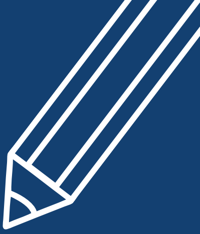
University of Cambridge

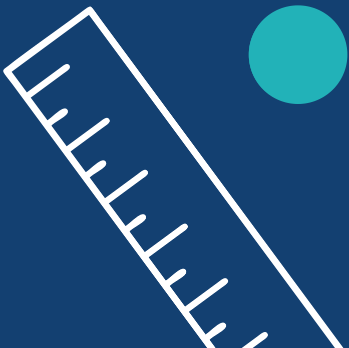


Biochemistry & Molecular Biology 2020

Course Handbook

General Information,
Lecture Overviews &
Practical Information

Department of Biochemistry



Biochemistry & Molecular Biology Course Handbook 2020-21

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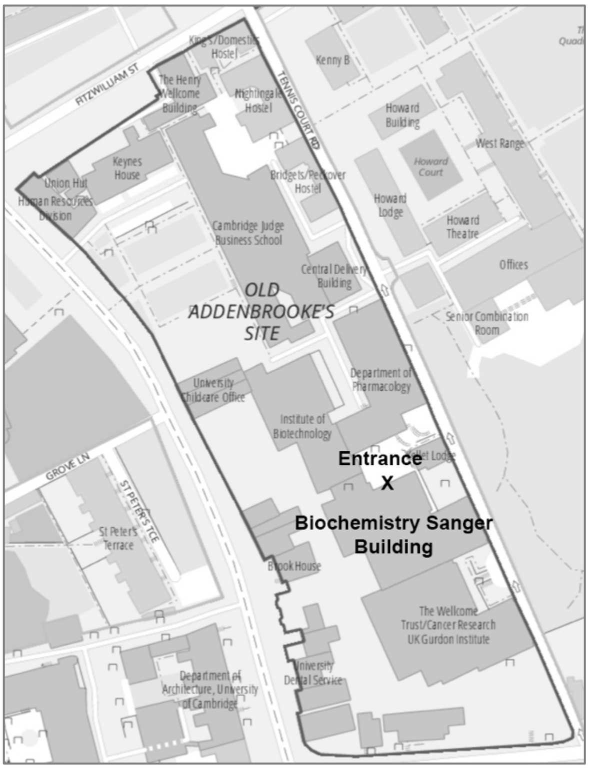
Department of Biochemistry

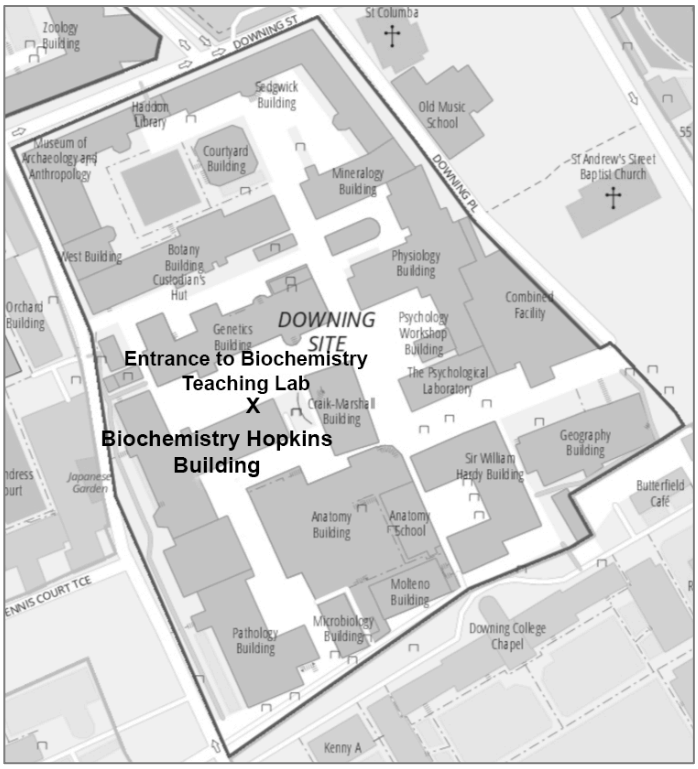
NATURAL SCIENCES TRIPOS PART IB

Biochemistry & Molecular Biology (BMB) Course Handbook

Course Organiser: Dr Dee Scadden (Email: adjs100@cam.ac.uk)


FITZWILLIAM ST
King's/Domestic Hostel
The Henry Wellcome Building
Nightingale Hostel
TENNIS COURT RD
Kenny B
Keynes House
Union Hut
Human Resources Division
Bridgets/Pettover Hostel
Howard Building
Howard Court
West Range
Howard Lodge
Howard Theatre
Cambridge Judge Business School
Central Delivery Building
Offices
OLD ADDENBROOKE'S SITE
Senior Combination Room
University Childcare Office
Department of Pharmacology
Institute of Biotechnology
Entrance
X
Biochemistry Sanger Building
GROVE LN
ST PETER'S TCE
St Peter's Terrace
Brook House
The Wellcome Trust/Cancer Research UK Gurdon Institute
University Dental Service
Department of Architecture, University of Cambridge

Biochemistry Sanger Building: Lectures


Zoology Building
DOWNING ST
St Columba
Sedgwick Building
Haddon Library
Old Music School
Museum of Archaeology and Anthropology
Courtyard Building
Mineralogy Building
DOWNING PL
St Andrew's Street Baptist Church
West Building
Botany Building
Custodian's Hut
Physiology Building
Orchard Building
Combined Facility
Genetics Building
DOWNING SITE
Psychology Workshop Building
Entrance to Biochemistry Teaching Lab
X
The Psychological Laboratory
Craik-Marshall Building
Biochemistry Hopkins Building
Geography Building
Japanese Garden
Sir William Hardy Building
Butterfield Café
Anatomy Building
Anatomy School
TENNIS COURT TCE
Molteno Building
Pathology Building
Microbiology Building
Downing College Chapel
Kenny A

Biochemistry Sanger Building: Practicals

The Department of Biochemistry recognises that each student has their own optimal way of studying and learning.

Please take time to read this course handbook to see what different resources are available to you, and to try them out to see what works best for you.

Specific information for BMB in 2020-21 during Covid19 pandemic

Dear BMB student,

I am pleased to welcome you to the BMB course, in this most unusual year where things are far from normal. I wanted to take this opportunity to reassure you that while things may be different than we had hoped, we have worked hard to ensure that we can deliver a rich and exciting course that will build on the material you bring from Part IA. It's especially important to note that while changes have been made to how we deliver the course, the aims and objectives remain constant.

This handbook is largely in keeping with the information we have previously included in the handbook provided in a more conventional year. However, the handbook has been updated, where relevant, to be consistent with the structure of the course for the 2020-21 academic year. Please note that there are some exceptions, as follows:

- Within the introductory sections there is some text in blue – this indicates information that has been retained in the handbook for your information, but not directly applicable this year.
- The information pertaining to lab safety is for your information only. Whilst the practical classes still form a key part of the curriculum, this year they will largely be online where safety considerations are very different!
- While the material describing the detailed experimental procedures in the wet practical labs will not be applied directly, it is essential that you are familiar with the introductory material for each practical, as this will still provide the background for the online practical sessions. Similarly, many of the experimental details will underpin the online practicals.

In addition to this handbook, do make sure you're very familiar with the BMB Moodle site – the resources you'll need for the 2020-21 academic year will be added to the site. Please note that any last-minute changes to the course will be communicated via Moodle – so it is essential that you check your @cam email daily for any updates.

We understand that you will be anxious to know how examinations will be affected by the changes in place this year. Whilst the details of this are still in discussion, please be assured that we will let you know more as soon as the details are finalised; I'm sure you will appreciate that careful consideration of this is essential. Regardless, as usual the exams will draw on all content delivered within the course, including lectures, practicals, journal clubs etc. Further information regarding the format of the exams will be shared as soon as possible.

We look forward to meeting you soon and hope that you enjoy the year.

All the best,

Dee Scadden

BMB Course Organiser
Director of Teaching (Biochemistry)

Aims and Objectives for Biochemistry & Molecular Biology

Aims

The course aims to build on the NST IA Biology of Cells course, and to provide an advanced foundation for specialist further study of Biochemistry or other molecular biosciences, by giving students an understanding of:

A. the structural organisation of genes and the control of gene expression in prokaryotes and eukaryotes;
B. protein structure, enzyme catalysis and protein engineering;
C. the control of metabolic pathways, energy transduction and cell growth;
D. the methods used to analyse biochemical structures and processes, including the use of molecular genetic tools.

Objectives

By the end of the course, the students should be able to demonstrate knowledge and understanding of:

A. The principles and exploitation of methods of recombinant DNA technology
B. Chromatin structure, RNA synthesis, processing and translation
C. Protein domain structure and folding, conformational mobility and stability, enzyme kinetics, enzyme mechanisms, allostery, and antibody recognition and protein design
D. The structural basis and mechanism of energy transduction processes in mitochondria, photosynthetic bacteria and chloroplasts, and of the control of metabolic flux
E. Signal transduction across membranes and within and between cells
F. Basic Immunology
G. Bacterial chemotaxis
H. The control of eukaryotic cell cycle
I. What happens when biology is not what you expect

and be skilled in:

J. The design and execution of simple experimental protocols, the interpretation of data, and to be able to use computer applications to analyse gene and protein sequences and structures
K. Analysis and critical interpretation of the results of biochemical experiments, using examples from their own practical exercises, journal clubs and lectures

and continue their learning by:

L. Building on their knowledge, understanding and skills by further study of specialised biomolecular courses within the Natural Sciences Tripos.

Introduction to Biochemistry & Molecular Biology

BMB Moodle Site

We provide a website for the Biochemistry & Molecular Biology (BMB) course using the University's Virtual Learning Environment (VLE), Moodle, accessed from www.vle.cam.ac.uk. You will be registered for the BMB site once we have collected your User IDs at the practical registration. There is a lot of important information on Moodle:

- Look out for course announcements
- Information about the course is provided on Moodle
- Moodle provides on-line access to course materials and resources:

  e.g. lecture handouts, films of lectures (via Panopto – see below), pre-practical quizzes, interactive practical materials, exam quizzes, background information (e.g. techniques posters and films), information for the practical classes, tutorial films, etc.
- Additional material to support the BMB course will be provided on Moodle throughout the academic year – do keep an eye out for new additions or developments.

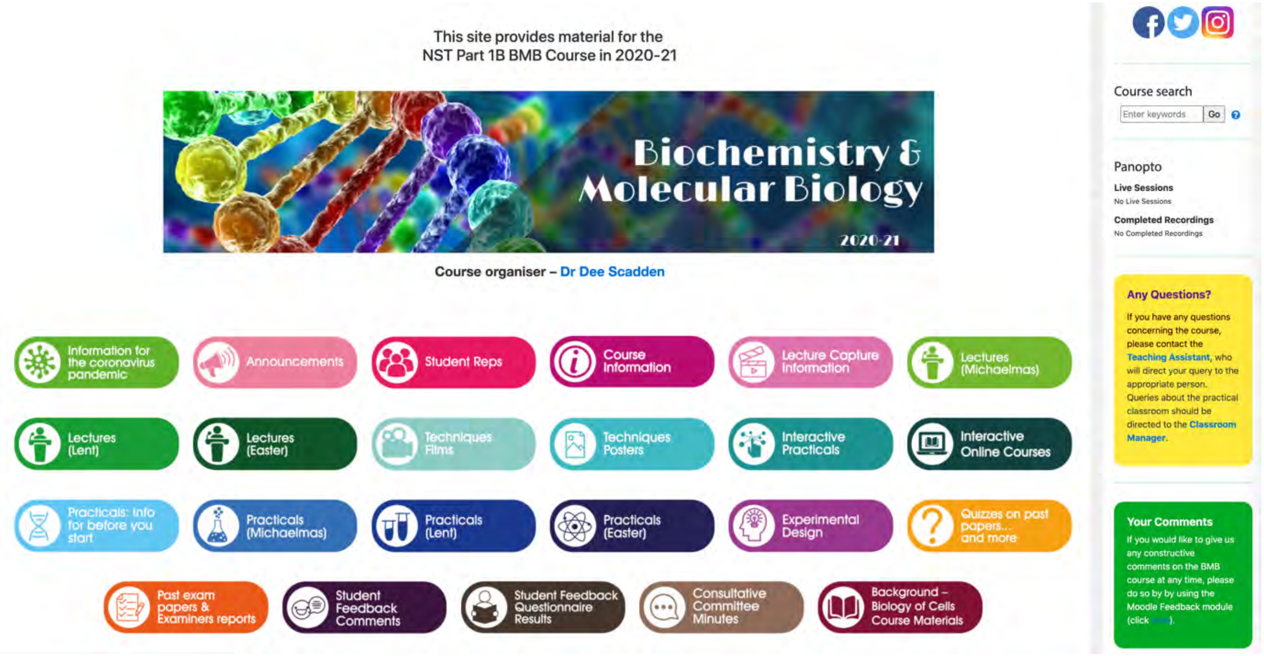
Do take the time to explore the BMB Moodle site, as there are many resources that will support your learning, both for lectures and practical classes.

Please note that there are resources on Moodle that are absolutely essential for your course work. In addition, there are also a large number of additional resources that have been provided to support your learning (e.g. quizzes, interactive practical materials, extra experimental design exercises, information about biochemical techniques etc.), and **it is not essential** that you complete or engage with **all** of the material provided.

The virtual learning environment should support the way YOU learn best, so feel free to pick and choose from the resources provided to use those best suited to your individual learning style.

A few practical details about using Moodle for BMB

The format of the BMB Moodle site may be arranged slightly different to what you've used previously. Here are a few helpful tips to get you started: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Information for the coronavirus pandemic
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Lecture Capture Information
Lectures (Michaelmas)
Lectures (Lent)
Lectures (Easter)
Techniques Films
Techniques Posters
Interactive Practicals
Interactive Online Courses
Practicals: Info for before you start
Practicals (Michaelmas)
Practicals (Lent)
Practicals (Easter)
Experimental Design
Quizzes on past papers... and more
Past exam papers & Examiners reports
Student Feedback Comments
Student Feedback Questionnaire Results
Consultative Committee Minutes
Background – Biology of Cells Course Materials
Course search
Enter keywords
Go
Panopto

This site provides material for the NST Part 1B BMB Course in 2020-21

Course organiser – Dr Dee Scadden

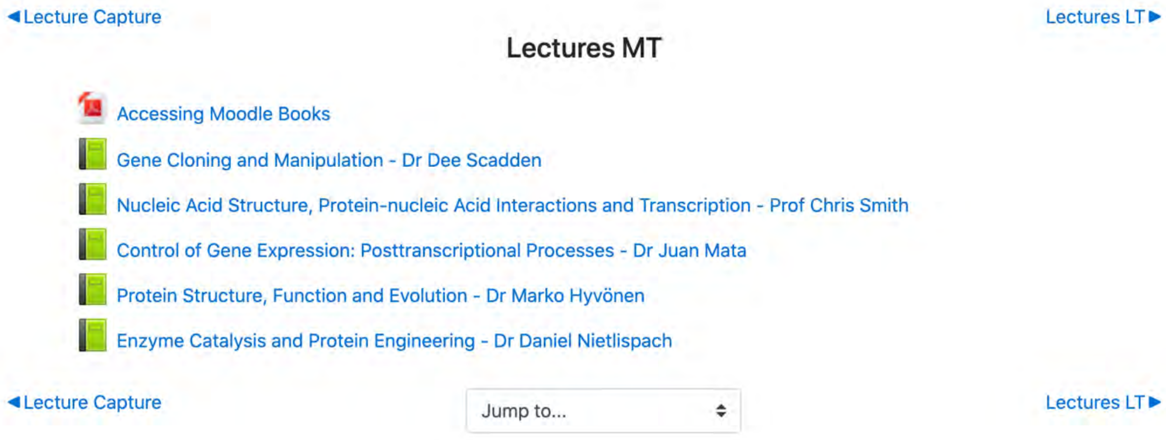
Live Sessions
No Live Sessions

Completed Recordings
No Completed Recordings

Any Questions?
If you have any questions concerning the course, please contact the **Teaching Assistant**, who will direct your query to the appropriate person. Queries about the practical classroom should be directed to the **Classroom Manager**.

Your Comments
If you would like to give us any constructive comments on the BMB course at any time, please do so by by using the Moodle Feedback module (click here).

- Helpful information can be found in the blocks on the right-hand side of Moodle
- Click on each coloured box on the front page to access the various course materials or information
- Lecture materials etc. in each block are generally organised into '**Moodle Books**', where there are separate Moodle Books for each lecture set.
  e.g. Within the block 'Lectures (Michaelmas)':


◀Lecture Capture
Lectures LT▶
Jump to...

Lectures MT

Accessing Moodle Books

Gene Cloning and Manipulation - Dr Dee Scadden

Nucleic Acid Structure, Protein-nucleic Acid Interactions and Transcription - Prof Chris Smith

Control of Gene Expression: Posttranscriptional Processes - Dr Juan Mata

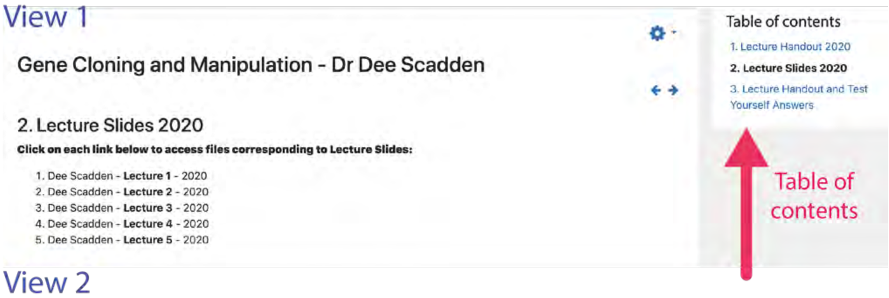
Protein Structure, Function and Evolution - Dr Marko Hyvönen

Enzyme Catalysis and Protein Engineering - Dr Daniel Nietlispach

- To access the contents of Moodle Books, click on the book and the chapters will appear in the top right-hand side of the screen or below the main middle screen (depending on screen size), and the contents of each chapter will be in the middle of the screen:

  e.g. Chapter 1: Lecture handout 2020

View 1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1. Lecture Handout 2020
2. Lecture Slides 2020
3. Lecture Handout and Test Yourself Answers
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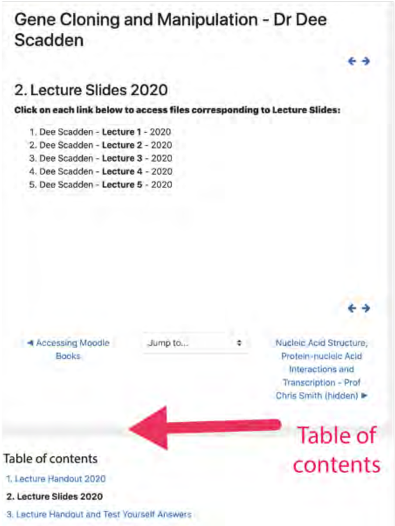
Gene Cloning and Manipulation - Dr Dee Scadden

2. Lecture Slides 2020

Click on each link below to access files corresponding to Lecture Slides:

1. Dee Scadden - **Lecture 1** - 2020
2. Dee Scadden - **Lecture 2** - 2020
3. Dee Scadden - **Lecture 3** - 2020
4. Dee Scadden - **Lecture 4** - 2020
5. Dee Scadden - **Lecture 5** - 2020

View 2


◄ Accessing Moodle Books
Jump to...
Nucleic Acid Structure, Protein-nucleic Acid Interactions and Transcription - Prof Chris Smith (hidden) ►
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2. Lecture Slides 2020
3. Lecture Handout and Test Yourself Answers

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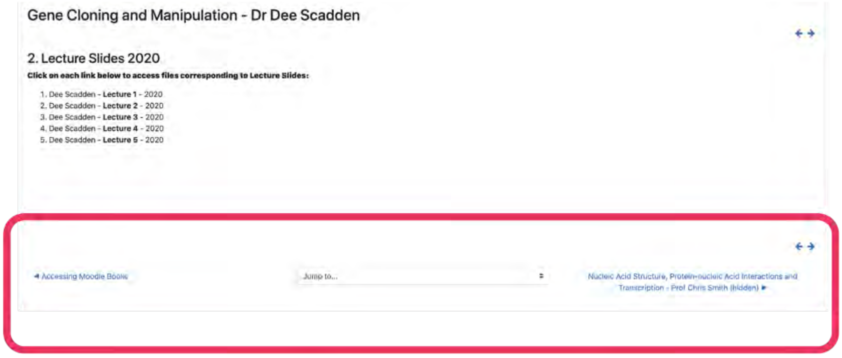
1. Dee Scadden - **Lecture 1** - 2020
2. Dee Scadden - **Lecture 2** - 2020
3. Dee Scadden - **Lecture 3** - 2020
4. Dee Scadden - **Lecture 4** - 2020
5. Dee Scadden - **Lecture 5** - 2020

- All the materials on the BMB Moodle site can be accessed by
  - Navigating from the front page (click on the top image (collage) at any time to take you back to the BMB front page)
  - Using the drop-down menu in each section 'Jump to…'
  - Using the navigation arrows/text at the sides of each section

  e.g.


◄ Accessing Moodle Books
Jump to...
Nucleic Acid Structure, Protein-nucleic Acid Interactions and Transcription - Prof Chris Smith (hidden) ►

Gene Cloning and Manipulation - Dr Dee Scadden

2. Lecture Slides 2020

Click on each link below to access files corresponding to Lecture Slides:

1. Dee Scadden - **Lecture 1** - 2020
2. Dee Scadden - **Lecture 2** - 2020
3. Dee Scadden - **Lecture 3** - 2020
4. Dee Scadden - **Lecture 4** - 2020
5. Dee Scadden - **Lecture 5** - 2020

Some course information will also be available via the Biochemistry Department at http://www.bioc.cam.ac.uk/teaching/second-year/bmb

Lectures

Lecture handouts are provided in advance on Moodle. To aid discussion in supervisions, lecturers will provide some questions related to the lecture material, if you and your supervisors wish to use them. Your supervisors will be provided with guides to answering these questions.

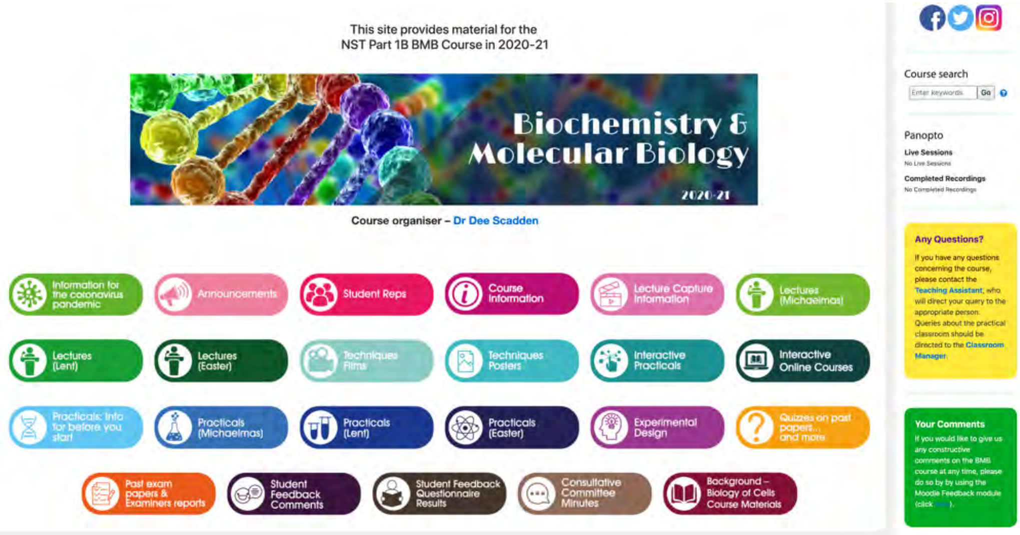
Various resources to support the lectures are included on Moodle. These are as follows:

- Lecture handouts
- Lecture slides
- Questions based on the lectures as provided by lecturers
- Literature sources, reading lists etc.
- Lecture recordings – via Panopto software (see below)

Lecture Recordings – Panopto

All lectures in BMB will be recorded using ‘Panopto’ and will be accessible via the BMB Moodle site. Here are a few tips for using Panopto for BMB.

- Lecture recordings for BMB can be accessed in two ways:
  - via the Panopto block (top right-hand side of BMB Moodle page) where the recordings will be listed by date



This site provides material for the NST Part 1B BMB Course in 2020-21

Course organiser – Dr Dee Scadden

Any Questions?

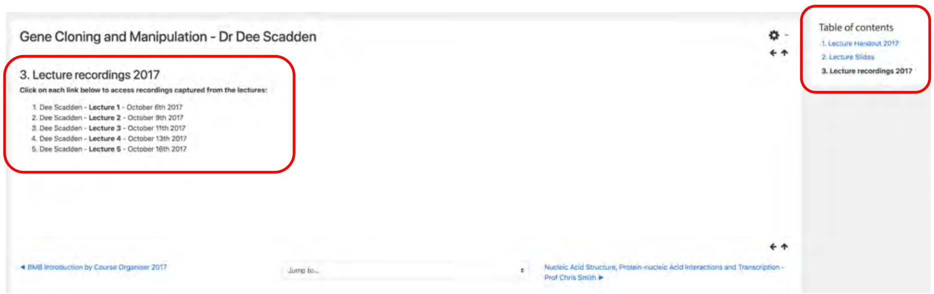
If you have any questions concerning the course, please contact the Teaching Assistant, who will direct your query to the appropriate person. Queries about the practical classroom should be directed to the Classroom Manager.

Your Comments

If you would like to give us any constructive comments on the BMB course at any time, please do so by by using the Moodle Feedback module (click here).

Biochemistry & Molecular Biology
2020-21
Course search
Enter keywords
Go
Panopto
Live Sessions
No Live Sessions
Completed Recordings
No Completed Recordings
Information for the coronavirus pandemic
Announcements
Student Reps
Course Information
Lecture Capture Information
Lectures (Michaelmas)
Lectures (Lent)
Lectures (Easter)
Techniques Films
Techniques Posters
Interactive Practicals
Interactive Online Courses
Practicals: Info for before you start
Practicals (Michaelmas)
Practicals (Lent)
Practicals (Easter)
Experimental Design
Quizzes on past papers... and more
Past exam papers & Examiners reports
Student Feedback Comments
Student Feedback Questionnaire Results
Consultative Committee Minutes
Background – Biology of Cells Course Materials

  - via links in the Moodle book for each set of lectures



Gene Cloning and Manipulation - Dr Dee Scadden

3. Lecture recordings 2017

Click on each link below to access recordings captured from the lectures:

1. Dee Scadden - **Lecture 1** - October 6th 2017
2. Dee Scadden - **Lecture 2** - October 9th 2017
3. Dee Scadden - **Lecture 3** - October 11th 2017
4. Dee Scadden - **Lecture 4** - October 13th 2017
5. Dee Scadden - **Lecture 5** - October 16th 2017

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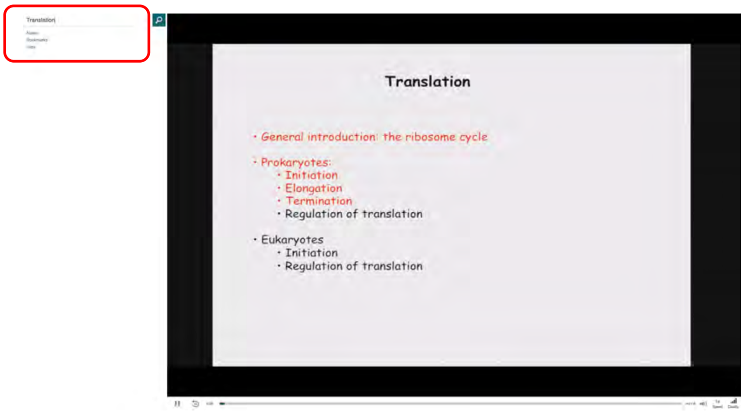
1. Lecture Handout 2017
2. Lecture Slides
3. Lecture recordings 2017

◄ BMB introduction by Course Organiser 2017
Jump to...
Nucleic Acid Structure, Protein-nucleic Acid Interactions and Transcription - Prof Chris Smith ►

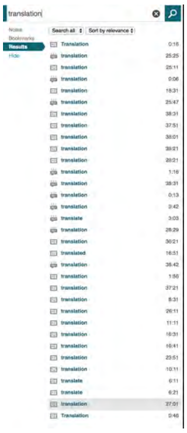
- You can search for key words in recordings – these will be identified by voice recognition and from text on slides:

  e.g. if you type ‘translation’ in search box (top left-hand side of Panopto screen), you will get a list of places where that word occurs, and at what time. You can then skip forward to the part you are looking for.

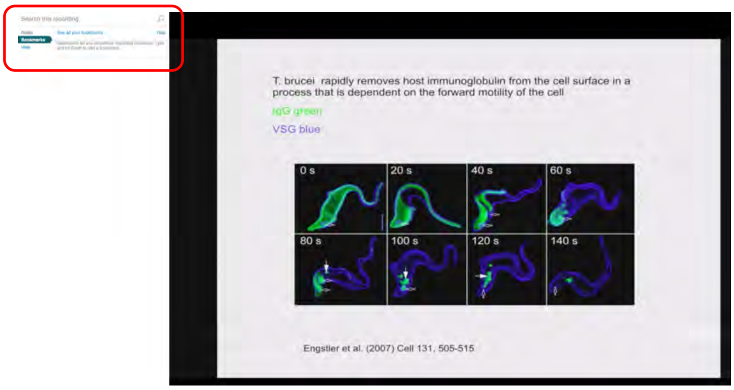


Translation

- General introduction: the ribosome cycle
- Prokaryotes:
  - Initiation
  - Elongation
  - Termination
  - Regulation of translation
- Eukaryotes
  - Initiation
  - Regulation of translation



- You can also use bookmarks to mark bits that you might want to go back to later:



T. brucei rapidly removes host immunoglobulin from the cell surface in a process that is dependent on the forward motility of the cell

IgG green

VSG blue

Engstler et al. (2007) Cell 131, 505-515

Practical Classes

Aim of the practical classes

Progress in science is achieved through observation and experiment. Biochemistry (and its close cousin, molecular biology) is an experimental science that advances from well-thought out investigations in the laboratory. No serious student should neglect the opportunities that this course provides to appreciate this fact. Your course includes experiments devised for you to gain some insight into how laboratory investigations are carried out and how data are processed and interpreted. To obtain useful results an experiment should be designed to answer a definite question and the detailed planning should be sufficiently rigorous to exclude adventitious errors. The course gives you the opportunity to plan some experiments for yourselves. You should benefit from the practicals in three ways:

(i) You will learn a variety of experimental techniques, all of which are currently used in biochemical research. The practicals have been designed to complement the lectures and fit in with their sequence as far as possible. The hands-on experience should link to the mental framework provided by the lectures and give you a deeper understanding and more realistic perspective of the topics discussed.

(ii) You will learn to handle experimental data effectively, and to extract the maximum information content without falling into the trap of over-interpretation.

(iii) You will be helped when it comes to the data handling questions in the Tripos examination. A collection of question papers from previous years is available on the course website. Quizzes based on the previous papers can also be found on Moodle.

The Demonstrators

Demonstrators are there to help you. The senior demonstrator will normally be a member of the Staff of the Biochemistry Department – often a lecturer in your course. The assistant demonstrators will be graduate students working for a PhD research degree, or post-doctoral research workers who have recent and sympathetic memories of the difficulties felt by studying the subject. Rely on them not only to sort out practical difficulties but also to help you make sure you understand what the experiments are about and what your results mean. They may well also be able to help you understand theoretical or lecture material.

Discussions

As the class size is relatively small, discussion of the practical work will be informal and will normally take place in the laboratory at the end of the experimental work. In 2020-21, these discussions will take place on Zoom (link on Moodle). Demonstrators will be on hand at these occasions and you are urged to make the most of these discussions and take an active part in them.

Safety and care in the laboratory

PLEASE be careful in the laboratory. Attend to your own safety and that of others around you: this is a statutory obligation – a matter of law.

PLEASE also extend your consideration to the equipment in the laboratory. Most of it is costly to repair or replace.

Policy, rules, and guidance on safety are given on the pages in this handbook headed 'Safety in the Biochemistry Part I Teaching Laboratory'. You **must** read this before coming to the first practical. In addition, you **must** also watch the short film about lab safety before coming to the first practical, which can be found on Moodle.

At the end of the safety pages, there is a declaration form to acknowledge that you have read the document and will abide by the rules set out. Copies of the form can be downloaded from Moodle and will also be available at the first practical class. This form **must** be signed and will be collected during the first practical.

Practical sheets

You are given comprehensive notes for each practical. The **practical notes must be read before you begin the practical.** In addition, there will be a '**pre-practical' quiz available on Moodle that can be completed prior to the practical class, which will help you to complete your practical most efficiently.**

a) The Background

For each practical class, there are pages at the beginning that set the context and state the learning objectives for each practical. If appropriate, they will indicate the lecture handout material that you might want to review before reading the practical notes.

b) The experimental plan and instructions.

These are NOT a recipe to be read for the first time when you are faced with the experiment. Make a practice of scanning the experimental information in advance, a day or two before the practical. Not to take in every detail, which will only make sense when you have the apparatus in front of you, but to get an overview of the planned experiment. Once in the laboratory, the senior demonstrator will give a brief introduction and you should then read and follow the instructions carefully. **Take a few minutes to read through the instructions again at the beginning of the practical** – don't just dive in, no matter how busy everyone else looks. Always try to think out the principles of what you are doing as you go along, and to understand what is going on in the procedures you carry out. Ask the demonstrators – or they may well ask you first!

Probably the most important contribution you can make to the success of your experiments is in making up reaction mixtures. **Think** about what you are adding, so you add the **correct** components. **Measure** and **dispense** the volume of component reagents **accurately** using the semi-automatic Gilson pipettes provided. If in doubt as to how to use a Gilson properly, watch the video on the course Moodle site.

c) Data processing and Questions

There are sheets following the experimental pages for processing the data and also contain questions about the practical to help you understand what you have done, and why. They are a form of self-assessment – we don't take them in and give marks, but demonstrators can advise and comment on your efforts. You will find them helpful when preparing for the end of year exam, so it is important to get them filled out properly. Going from raw numbers that come straight off an instrument to a meaningful calculated result is found quite difficult by most students and may be tested in the examination. So take advantage of the help available.

You should complete the question sheets during the practical if that is feasible, or as soon after as possible after each practical. Bring your calculators to each session. Graph paper is available if you don't have your own. Compare your results against the yellow sheets (see below) or discuss with a demonstrator.

d) Appendices to the Practicals

There are appendices in the handbook immediately before practical notes that contain important background material related to several practicals and practical aspects of biochemistry. Note that the appendices are now also available on Moodle as an interactive resource.

The appendices include an introduction to units, to pH and buffers, and to the principles of spectrophotometry. Try and have a brief look at them before your first practical. Raise any difficulties with your supervisor or a demonstrator.

Yellow sheets

The YELLOW SHEETS contain specimen results and, where relevant, examples of how to analyse them. They will generally be handed out either at the end of the practical or at the appropriate discussion period.

Recording your results

You should ensure you have a notebook or loose-leaf book to use for the practical classes. Whether this is an online or in person practical, for each experiment plan what observations you will make. Record your results and interpretations directly into the notebook as you go along rather than on scraps of paper that will surely get lost. Also record any arguments and conclusions from your data if there is not enough space for these on the summary sheets.

You should endeavour to complete this writing up of your argument and conclusions during the experiment itself. There is no need to write out again what is already in your practical sheets. Concentrate on ensuring that you get down the key arguments and conclusions. **Any writing up after the experiment is not expected to take more than about an hour for a day's practical, but it is important to do it at once while the experiment is still fresh in your mind.**

The results of an experiment are often best recorded graphically as well as numerically. So far as possible the graph is best drawn while you are doing the experiment as you go along, so that in doubtful regions points may be repeated or additional ones obtained. The conventional way of plotting a graph is to plot the dependent variable (the thing you measure) on the ordinate (vertical axis) against the independent variable (the thing you fix) on the abscissa (horizontal axis). Remember to label the axes with the quantity being plotted and units involved. Remember, too, that the way you plot a graph shows your interpretation of the experiment. Consult Appendix 1 on "Units" for further guidance.

Your notebooks are not "marked" as part of a "summative assessment" or examination. Rather they are to help you get to grips with each practical as you do it and write it up so that you still understand it when it comes to revision later in the year. To help with this, we ask you to complete the question sheets (see above). If you feel all at sea, or if your answers are full of blank spaces make a point of asking the demonstrator for help (e.g. this can be in the Zoom sessions if online).

Moodle and the Practicals

In the 2020-21 academic year, the majority of practicals will be online. The information and materials for the practical class will be circulated ahead of each session via Moodle.

In addition to the structured online practicals, there is a significant amount of information on Moodle to support the practical classes, and it is important that you access this prior to beginning your first practical. The information you will find is as follows:

- A film that provides **essential** safety information for the classroom – while you may not be in the lab initially this year, it is still worth a watch, and must be watched **prior to in person practical classes**
- Films that provide information about practical lab techniques (e.g. use of Gilson micropipettes)
- Pre-practical quizzes that are available before the start of each practical class.
- Interactive practicals materials (Learning Science) – great for checking you understand the techniques used in the practical classes
- An interactive session outlining the information contained in the practical appendices mentioned above.
- Technique posters that provide a ‘snapshot’ of information that describes commonly used biochemical techniques. This knowledge is typically assumed in lectures and practical classes, so use these resources to remind yourself of necessary techniques.
- Tutorial videos to explain biochemical methods (e.g. primer design)
- Materials required for the Journal Clubs (Michaelmas and Lent Terms) and the Experimental Design Exercise (Lent Term).

Journal Clubs

There are two Journal Clubs, one on a molecular topic and the other more cell biological. You will be given a published paper, with some guidance notes and questions, to analyse during the week before the practical session in which you critically evaluate its merits in a small group directed by one of the Biochemistry staff. Most students find this a challenging but worthwhile exercise, since it gives exposure to the raw material of the scientific literature.

Experimental Design Exercise

There will be a teaching session on experimental design in Lent Term. You will be briefed in advance of the session. You will be allocated to groups and given an outline of a problem to address. You will be required to think about an experimental strategy to investigate the problem and will discuss this as a group, led by a member of staff.

Examinations

General advice on examination skills and on the criteria used for marking and classing have been drawn up by the Faculty of Biology and the Management Committee for the Natural Sciences Tripos. They can be found by following links from these web sites

http://www.biology.cam.ac.uk/exams/skills

http://www.natsci.tripos.cam.ac.uk/exams

https://www.biology.cam.ac.uk/exams/raven/marking-tripos

Some more specific information relating to the Biochemistry & Molecular Biology examination is given below. Copies of recent past papers are provided on Moodle. Answers to the data-handling questions (previously Paper 3) have been made available to your supervisors; it's better for you to try the questions before looking at the answers! Quizzes based on the data-handling questions in Paper 3 from previous exams can also be found on Moodle.

General University guidance can be found at:

http://www.admin.cam.ac.uk/students/studentregistry/examinations/

Reports from the Senior Examiners are also available on Moodle.

NST Part IB Biochemistry & Molecular Biology

Beginning in the 2020-21 academic year, the format of the BMB exams has been changed to include only two papers, each of three hours' duration, where each paper carries equal marks. The exact structure of these papers will be confirmed as soon as possible, but the format will be based on the following:

Paper 1 consists of five sections, and students should answer one essay question from each section. Each section will carry equal marks.

Paper 2 will comprise two sections:

Section A will be made up of short-answer factual questions based on the whole lecture course, and carrying equal marks. Students should attempt all questions in Section A.

Section B will be concerned with practical techniques covered in the practical classes and journal clubs, as well as other material in the practical section of the course handbook and experimental techniques covered in the lecture course. All questions in Section B should be attempted.

When answering essay questions, take particular care that you have absorbed what the question is specifically asking for. It is a common fault for candidates to react unthinkingly to a "trigger word" and simply write all they know in response. Take a little time to reflect, rather than leaping straight in.

The examiners will have regard to the style and methods of candidates' answers. Where relevant, you should write legibly and not adopt note form unless specifically requested to do so. Helpful diagrams are welcome as part of an essay. You are perfectly free to use abbreviations that are standard scientific vocabulary without definition (e.g. G6P, ATP, DNA, RNA).

BMB Lecture Timetable 2020-21

Course Organiser: Dr Dee Scadden (adjs100@cam.ac.uk)

All lectures start at **10am** online via the Panopto on the BMB Moodle site.
There will be an opportunity to ask the lecturer questions immediately after each lecture via Zoom (https://zoom.us/my/**bmb20**) – changes to this link will be available on Moodle.
Please note that the Q&A will begin at 10:50am for 10-15 min.

MICHAELMAS TERM: Genes and proteins; macromolecules in action

First Lecture on Friday, October 9th 2020; Last Lecture on Wednesday, December 2nd 2020

Date	No.	Title	Lecturer	Most relevant Techniques Posters
Oct 9		Introduction to the course (in lecture 1)	Dr Dee Scadden	
Oct 9, 12, 14, 16, 19	5	Gene cloning and manipulation	Dr Dee Scadden	1, 2, 4, 9, 13, 15, 16, 17, 19, 21, 20, 22, 26, 28, 29, 30, 31, 33
Oct 21, 23, 26, 28, 30	5	Nucleic acid structure, protein-nucleic acid interactions and transcription	Prof Chris Smith	5, 7, 17, 22, 25, 26, 27
Nov 2, 4, 6, 9,11	5	Post transcriptional control of gene expression	Dr Juan Mata	
Nov 13, 16, 18, 20, 23	5	Protein structure, function and evolution	Dr Marko Hyvönen	
Nov 25, 27, Dec 30, 2	4	Enzyme catalysis and protein Engineering	Dr Daniel Nietlispach	

LENT TERM: Energy transduction, cell signalling and cell proliferation

First Lecture on Wednesday, January 20th; Last Lecture on Monday, March 15th 2021

Date	**No.**	**Title**	**Lecturer**	**Most relevant Techniques Posters**
Jan 20, 22, 25, 27, 29	5	Control of metabolism	Dr Bill Broadhurst	
Feb 1, 3, 5, 8, 10, 12	6	Energy transduction in bacteria, mitochondria and chloroplasts	Prof Chris Howe	
Feb 15, 17, 19, 22, 24	5	Transmembrane signalling; molecules and mechanisms	Prof Sarah Lummis	
Feb 26	1	Immunology: the basics	Dr Marc de la Roche	
Mar 1, 3, 5	3	Bacterial chemotaxis	Dr Martin Welch	
March 8, 10, 12, 15	4	Control of eukaryotic cell growth	Prof Mark Carrington	

EASTER TERM: When biology is not what you expect *First Lecture on Wednesday, April 28th 2021, last lecture on May 10th 2021*				
Date	**No.**	**Title**	**Lecturer**	**Most relevant Techniques Posters**
April 28, 30	2	How the other three quarters lives - how protists break the rules of biochemistry	Prof Chris Howe	
May 3, 5, 7, 10	4	Oncogenes, tumour suppressor genes and cancer	Prof Gerard Evan & Dr Trevor Littlewood	

BMB Practical Timetable 2020-21

- While practicals will largely be online during the 2020-21 academic year, the format of individual practicals will be posted on Moodle. Some online sessions will be face-to-face via Zoom, while others will be carried out remotely, followed by a face-to-face Zoom discussion session.
- Practicals commence at **11am**, unless stated otherwise. **Monday sessions commence at 12 noon**.
- **Where indicated (*), a follow up discussion session will be held via Zoom each day. Details for Zoom sessions will be posted on Moodle.**
- **General zoom address (unless told otherwise): https://zoom.us/my/bmb20**
- We hope to carry out 'in person' Practical Skills sessions during Easter Term in the Part IB Teaching Laboratory in Biochemistry – details will be communicated via Moodle.

MICHAELMAS TERM 2020			
Dates	**Practical**	**Senior Demonstrator**	**Most relevant techniques posters**
Week 1 October 8 – 14 ***Zoom session**	Safety Cloning of Oct1 POU domain gene into *E. coli* plasmid. Restriction mapping of plasmid.	Dr Dee Scadden	2, 15, 26
Week 2 October 15 – 21 ***Zoom session**	Expression of POU domain in *E. coli*. Melting properties of DNA.	Prof Ben Luisi	
Week 3 October 22 – 28 ***Zoom session**	Purification of POU domain	Prof Nick Gay	13, 1, 3, 15
Week 4 October 29 – Nov 4 ***Zoom session**	Electrophoresis mobility shift assay	Dr Mairi Kilkenny	5, 7, 13, 15, 16, 18, 20, 22, 28
Week 5 November 5 – 11 ***Zoom session**	Using Online Databases as Tools	Dr Dee Scadden	13, 26
Week 6 November 12 –18	Discussion in lab of weeks 1-5: 11.00 am (Monday 12 noon). Venue: **see Moodle** Reactivity and enzyme catalysis (2-4pm, except 3-5pm Monday) Venue: **see Moodle**	Dr Darerca Owen Dr Katherine Stott	
Week 7 November 19 – 25	Journal Club (2 pm, except Monday 3 pm) Venue: **see Moodle**	Staff	22, 34
Week 8 November 26 – Dec 2 ***Zoom session**	Protein structure by molecular graphics	Dr Bill Broadhurst	

LENT TERM 2019			
Dates	**Practical**	**Senior Demonstrator**	**Most relevant techniques posters**
Week 1 January 21 – 27 ***Zoom session**	Kinetic analysis of catalysis by chymotrypsin	Dr Daniel Nietlispach	15
Week 2 January 28 – Feb 3 ***Zoom session**	Subcellular fractionation	Dr Marc de la Roche	
Week 3 February 4 – 10	Experimental design: 11-1 pm (Monday 3pm start) Venue: **via Zoom** Metabolic Control *in silico*: 2.00-4.30pm (Monday 12 noon start) ***Zoom session**	Dr Dee Scadden Dr Bill Broadhurst	
Week 4 February 11 – 17	Mitochondrial oxidative phosphorylation	Dr Helen Mott	
Week 5 February 18 – 24 ***Zoom session**	Cell signalling (1)	Prof Sarah Lummis	15, 33
Week 6 February 25 – March 3 ***Zoom session**	Cell signalling (2)	Dr Marko Hyvönen	15, 33
Week 7 March 4 – 10 ***Zoom session**	Cell signalling (3)	Dr Marko Hyvönen	10, 37, 38, 1, 19, 35, 28, 33
Week 8 March 11 – 17	Journal Club (2pm, except Monday 3pm) Venue: **via Zoom**	Staff	

EASTER TERM 2019			
Dates	**Practical**	**Senior Demonstrator**	**Most relevant techniques posters**
Week 1 April 29 – May 5	**Practical skills sessions** Venue: **Part IB teaching lab**	TBC	
Week 2 May 6 – May 12	**Practical skills sessions** Venue: **Part IB teaching lab**	TBC	

Reading List

For NST Part IB Biochemistry & Molecular Biology, NST 2020-21

Most of these books should be available in your College library, but to give as many students as possible an opportunity to use them on a regular basis the Department of Biochemistry also keeps copies in the Part I Section of the Colman Library (in the Hopkins Biochemistry building, facing Tennis Court Road on the Downing Site). Selected books may be borrowed overnight from this collection. You are also welcome to make use of the main collection of books and journals, but borrowing these is not permitted. Part II and Part III Biochemistry students have priority for seating in the library. The Assistant Librarian is available to help locating books. Part IB BMB students who wish to use the library outside the hours of 8.30am – 5pm, Monday-Thursday, or 8:30am - 4pm, Friday, should ask the Hopkins building receptionist to programme their University proximity card for access.

There are a number of very good biochemistry text books available – some of which are quite general and cover similar topics, while others are more specialized. It's probably a good idea to primarily use the 'General Biochemistry Texts', then use the others to dip in and out of as required. The use of relevant textbooks is **essential** for underpinning the material given to you in lectures – do make the most of the resources available to you.

Recommended books

If you wish to buy, we suggest that you browse first in libraries, book shops or third-years' book collections.

a. General Biochemistry Texts

There are good companion web sites that give further information about these books, and contain useful onward links.

- **Biochemistry**, Berg, J. M., Tymoczko, J. L., Gatto, G.J., and Stryer, L. (Freeman, 9th edition, 2019).

  For access to Student Resources see:

  https://www.macmillanlearning.com/college/us/search/?text=stryer

- **Molecular Biology of the Gene**, Watson, J. D. *et al.* (Pearson Education, 7th edition, 2017).

- **Lehninger Principles of Biochemistry**, Nelson, D. L. & Cox, M. M. (Freeman, 7th edition, 2017).

  Particularly useful for bioenergetics and metabolism. Good for signalling. For access to Student Resources see:

https://www.macmillanlearning.com/college/us/product/Lehninger-Principles-of-Biochemistry/p/1464126119?searchText=lehninger

- **Molecular Biology of the Cell**, Alberts, B. *et al.* (Garland Science, 6th edition 2015).

  The largest and most comprehensive of the cell and molecular biology textbooks, now with a CD-ROM as well as a revised Problems Book. Will be useful for Part II courses as well as IB.

www.garlandscience.com/product/isbn/9780815345244

- **Molecular Biology of the Cell – The Problems Book**. Wilson, J. & Hunt, T. (Garland Science, 6th edition 2014).

b. Books on specific topics

Protein structure, function and evolution

- Protein Structure and Function, Petsko, G. & Dagmar, R., 2008

Enzyme catalysis and protein engineering:

- Introduction to Protein Structure, Branden, C. & Tooze, J. 2nd edition, 1999.
- Biochemistry, Abeles, R.H., Frey, P.A. & Jencks, W.P., 1992.
- Proteins, Creighton, T.E. 2nd edition, 1995.
- From Enzyme Models to Model Enzymes, Kirby, A.J. & Hollfelder, F., 2009.
- Structure and Mechanism in Protein Science – A guide to Enzyme Catalysis and Protein Folding, Fersht, A., 1999.
- Enzymatic Reaction Mechanisms, Frey, P.A. and Hegemann, A.D., 2007.

Energy transduction

- Bioenergetics, Nicholls D.G. & Ferguson S.J. 4th edition, 2013.
- Molecular Mechanisms of Photosynthesis, Blankenship R.E. 2nd edition, 2014.

Oncogenes, tumour suppressor genes and cancer:

- The Biology of Cancer, Weinberg, R. Garland Science, 2nd edition, 2014.

  The most comprehensive cancer biology textbook complete with a CD-ROM. Will be useful for Part II courses as well as IB.

Other sources of information

a. Journals – well worth consulting

Research-oriented summaries (2-4 pages) of important topics in biochemistry and molecular biology are contained in the monthly journal *Trends in Biochemical Sciences* (usually known as TIBS). A similar journal is *Bioessays.* The *Scientific American* usually contains at least one biochemical article per issue and is well worth a glance. Copies of these journals are found near the Part I reading area.

b. Web Sites

Ever more useful source of information. We shall just give here two of our favourite general addresses, which have many forward links.

- Online Mendelian Inheritance in Man (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM)
- Swiss Institute of Bioinformatics (www.expasy.ch/)

c. Links to literature sources on Moodle

The Reading list and links to Textbooks and Journals can be found on Moodle in the book entitled “Course Information”.

- Textbooks via pubmed
- Links to Electronic Journals via the University
- Trends in Biochemical Sciences (TiBS)
- Current Opinions in Chemical Biology
- Current Opinions in Structural Biology

Plagiarism

With effect from 1 October 2019, the University has outlined Rules of Behaviour for both current and former registered students (Statutes and Ordinances 2019, Chapter II, Section 19; p.191). All registered students and formerly registered students are responsible for following the Rules of Behaviour. Not knowing or forgetting about the rules or their consequences is not a justification for not following them. These Rules include a definition of academic misconduct, which is replicated below:

'**Academic misconduct'** is gaining or attempting to gain, or helping others to gain or attempt to gain, an unfair academic advantage in formal University assessment, or any activity likely to undermine the integrity essential to scholarship and research. It includes being in possession of unauthorised materials or electronic devices during an examination, including recording or communication devices or devices that can store data, even where Registered Students are unaware that such materials or devices are unauthorised, have no intention of using them, or are unaware that they have them in their possession. Academic misconduct also includes:

- Plagiarism, Self-plagiarism,
- Contract cheating,
- Collusion,
- Impersonating someone or being impersonated in an examination or arranging for someone to impersonate someone else by sitting their examination,
- Fabrication, falsification or misrepresentation of data, results or other outputs or aspects of research,
- Failure to meet legal, ethical and professional obligations in carrying out research.

https://www.plagiarism.admin.cam.ac.uk/definition

https://www.biology.cam.ac.uk/exams/AllExams/plagiarism (raven log in required) states that:

In the context of an examination, this amounts to:
Passing off the work of others as your own to gain unfair advantage, or for self-plagiarism examples see below

Breaches of academic misconduct will be taken forward under the Student Discipline Procedure. Full information on the Student Discipline Procedure is available from the Office of Student Conduct, Complaints, and Appeals (OSCCA):

- visit the OSCCA website
- review OSCCA information on student discipline

Students are responsible for ensuring they have read and understood both the University's Statement on Plagiarism and the Faculty of Biology guidance.

General University of Cambridge guidelines can be found at:

https://www.plagiarism.admin.cam.ac.uk/definition with further details at
https://www.plagiarism.admin.cam.ac.uk/

The following guidance has been issued by the Faculty Board of Biology:

In general, plagiarism can be defined as:

"The unacknowledged use of the work of others as if this were your own original work"

In the context of an examination, this amounts to:

Passing off the work of others as your own to gain unfair advantage.

Such use of unfair means will not be tolerated by the University; if detected, the penalty may be severe and may lead to disciplinary proceedings being taken against you.

1. The scope of plagiarism

Plagiarism is defined as submitting as one's own work, irrespective of intent to deceive, that which derives in part or in its entirety from the work of others without due acknowledgement. It is both poor scholarship and a breach of academic integrity.

Examples of plagiarism include **copying** (using another person's language and/or ideas as if they are a candidate's own), by:

- **quoting verbatim** another person's work without due acknowledgement of the source;
- **paraphrasing** another person's work by changing some of the words, or the order of the words, without due acknowledgement of the source;
- **using ideas** taken from someone else without reference to the originator;
- **cutting and pasting** from the Internet to make a pastiche of online sources;
- **submitting someone else's work** as part of a candidate's own without identifying clearly who did the work. For example, buying or commissioning work via professional agencies such as 'essay banks' or 'paper mills', or not attributing research contributed by others to a joint project.

Plagiarism might also arise from **colluding** with another person, including another candidate, other than as permitted for joint project work (i.e. where collaboration is concealed or has been forbidden). A candidate should include a general acknowledgement where he or she has received substantial help, for example with the language and style of a piece of written work.

Plagiarism can occur in respect to all types of sources and media:

- text, illustrations, musical quotations, mathematical derivations, computer code, etc.
- material downloaded from websites or drawn from manuscripts or other media
- published and unpublished material, including lecture handouts and other students' work

Acceptable means of acknowledging the work of others (by referencing, in footnotes, or otherwise) vary according to the subject matter and mode of assessment. Faculties or Departments should issue written guidance on the relevant scholarly conventions for submitted work, and also make it clear to candidates what level of acknowledgement might be expected in written examinations. Candidates are required to familiarize themselves with this guidance, to follow it in all work submitted for assessment, and may be required to sign a declaration to that effect. If a candidate has any outstanding queries, clarification should be sought from her or his Director of Studies, Course Director or Supervisor as appropriate.

Failure to conform to the expected standards of scholarship (e.g. by not referencing sources) in examinations may affect the mark given to the candidate's work. In addition, suspected cases of the use of unfair means (of which plagiarism is one form) will be investigated and may be brought to one of the University's Courts. The Courts have wide powers to discipline those found guilty of using unfair means in an examination, including depriving such persons of membership of the University, and deprivation of a degree.

2. How to avoid plagiarism

The stylistic conventions for different subjects vary and you should consult your Course Organiser or project supervisor about the conventions pertaining in your particular subject area. Most courses will issue written guidance on the relevant scholarly conventions and you are expected to have read and to follow this advice. However, the main points that apply to submitted work (e.g. dissertations, project reports) are:

- when presenting the views and work of others, include in the text an indication of the source of the material, e.g. 'as Sharpe (1993) has shown,' and give the full details of the work quoted in your bibliography
- if you quote text verbatim, place the sentence in inverted commas and give the appropriate reference, e.g. 'The elk is of necessity less graceful than the gazelle' (Thompson, 1942, p 46) and give the full details in your bibliography as above
- if you wish to set out the work of another at length so that you can produce a counter-argument, set the quoted text apart from your own text (e.g. by indenting a paragraph) and identify it by using inverted commas and adding a reference as above. NB long quotations may infringe copyright, which exists for the life of the author plus 70 years
- if you are copying text, keep a note of the author and the reference as you go along, with the copied text, so that you will not mistakenly think the material to be your own work when you come back to it in a few weeks' time
- if you reproduce an illustration or include someone else's data in a graph include the reference to the original work in the legend, e.g. (figure redrawn from Webb, 1976) or (triangles = data from Webb, 1976)
- if you wish to **collaborate** with another person on your project, you should check with the Course Organiser to see whether this might be allowed and then seek their permission
- if you have been **authorised to work together** with another candidate or other researchers, you must acknowledge their contribution fully in your introductory section. If there is likely to be any doubt as to who contributed which parts of the work, you should make this clear in the text wherever necessary, e.g. 'I am grateful to A. Smith for analysing the sodium content of these samples'
- be especially careful if **cutting and pasting** work from electronic media; do not fail to attribute the work to its source. If authorship of the electronic source is not given, ask yourself whether it is worth copying

Please note that during written answers for unseen examination papers, you will not be penalised for failures to reference information in this manner.

3. The Golden Rule:

The examiners must be in no doubt as to which parts of your work are your own original work and which are the rightful property of someone else.

For the University-wide statement on plagiarism, and further information on the topic, please see:

http://www.admin.cam.ac.uk/univ/plagiarism/

Synopsis of the BMB lecture course

***Note**: This information is provided at the beginning of the year for your guidance and that of your supervisors. It is not intended to be a comprehensive list of contents. Lecturers will all issue their own handouts and may vary the topics and the order in which they are presented.*

Michaelmas Term: Genes and proteins – macromolecules in action

In Michaelmas, the course examines the molecular biology of DNA and protein structure. How is DNA packaged in cells? How does chromatin structure affect gene expression? How is genetic engineering actually carried out? How are transcription and translation regulated? What are the principles of protein design and how can we exploit them through protein engineering?

***Dr Dee Scadden**: Gene cloning and Manipulation*

These lectures introduce the techniques of gene cloning and manipulation that underpin much of the work described in the rest of the course. Building on material covered in the Part IA Biology of Cells lectures, we look at the use of various techniques to ask specific experimental questions. Examples of topics covered include:

- The polymerase chain reaction and its various applications
- Vectors and hosts that are used in conventional gene cloning
- Investigating how clones may be used experimentally. e.g. for preparing recombinant fusion proteins, making RNA for *in vitro* studies, etc.
- Methods for reducing gene expression (e.g. RNAi, CRISPR/Cas9), and for creating transgenic mice.

***Prof. Chris Smith:** Nucleic acid structure, Protein-Nucleic acid Interactions and Transcription*

These lectures cover the first step in gene expression – transcription of RNA using genomic DNA as template. How do RNA polymerases recognise the correct locations at which to initiate transcription, and how can this be regulated? Six main topics will be covered:

- DNA and RNA structure
- Prokaryotic transcription mechanisms
- Prokaryotic transcriptional regulation
- Packaging of eukaryotic DNA into chromatin
- Eukaryotic transcription – core promoter and general transcription factors (GTFs)
- Eukaryotic transcription – activating transcription factors and enhancers

The overarching theme of DNA-protein interactions – both sequence-specific and non-specific – runs through all of these topics. At appropriate points, relevant experimental approaches and techniques will be highlighted.

***Dr Juan Mata:** Post-Transcriptional Control of Gene Expression*

The production of functional proteins involves multiple processes in addition to transcription. Although these steps are usually referred to as post-transcriptional, many of them occur concurrently with transcription. These lectures will introduce the processes required for the formation of a mature RNA in eukaryotic cells (capping, splicing and 3' end processing), translation (in both prokaryotes and eukaryotes) and RNA decay. The basic machinery that carries out these processes, as well as the mechanisms by which this machinery is modulated in a gene-specific manner, will be addressed.

Dr Marko Hyvönen: *Protein Structure, Function and Evolution*

Proteins play most of the effector roles in living organisms. They maintain the structures of cells, of the extracellular matrix and tissues; they catalyze most reactions in cells and generate mechanical force in the muscles; they are involved in information transfer through recognition of other molecules and can act as ligands, as receptors, as messengers, and as transcription factors; they act as receptors, gates and channels in membranes. The aim of these lectures is to understand the unique principles of protein structure from primary structure to formation of large oligomeric complexes and molecular machines and to introduce the methods that are used to study protein structures from optical spectroscopies through X-ray crystallography and NMR to cryo electron microscopy. We will also discuss how proteins have evolved and how analysis of protein structure can help us to understand the evolutionary relationships between different proteins and their function.

Dr Daniel Nietlispach: *Enzyme Catalysis and Protein Engineering*

This lecture series focuses on how the peptide and protein structures discussed in the preceding module can assume functions – and on experiments that delineate the mechanisms involved. First, we develop ideas about enzyme catalysis, mechanism and kinetics. We look in detail at the co- operative (allosteric) molecular basis of metabolic regulation. Other protein structures that are discussed include immunoglobulins and their binding to specific antigens, and the principles of protein folding and stability. Finally, we look at the 'holy grail' of protein engineering and mechanistic enzymology – how to create novel, functional proteins, by rational design, semi-rational approaches, and by directed evolution.

Lent Term: Energy transduction, cell signalling and cell proliferation

The course now builds on the molecular foundations laid in the Michaelmas Term to develop an integrated view of cellular processes. How do cells make a continuous supply of energy available for transcription, translation, ion pumping, biosynthesis and a host of other processes? How is metabolism regulated according to the varying needs of the cell? What are the mechanisms by which hormones regulate intracellular processes?

Dr Bill Broadhurst: *Control of Metabolism*

The aims of these lectures are:

- To examine the different ways in which enzyme activity may be controlled.
- To consider the benefits these different modes of control offer for the regulation of flux in metabolic pathways.

This discussion takes place in a wider context, as these various modes of control are employed throughout biological systems. Textbook descriptions of control in the metabolic pathways tend to assume that the enzymes involved are 'soluble' and homogeneously distributed in the cell cytoplasm. We will see how this is not the case: rather, a high degree of spatial organisation is critical to the control of these pathways.

Various experimental approaches are described for studying how metabolism is controlled, with particular emphasis on methods that may be used to study intact systems. These include:

- Metabolic control analysis, which allows for quantitative determination of the importance of any enzyme for flux control *in vivo*.
- Two key non-invasive spectroscopic techniques – fluorescence and NMR – that permit the study of metabolic events in intact cells and tissues.

Prof. Chris Howe: *Energy Transduction in Bacteria, Mitochondria and Chloroplasts*

Bioenergetics is the study of how energy is acquired and used in living systems. Recent discoveries of key structures and mechanisms have greatly enhanced our understanding of this process. This knowledge is being applied to medicine, nanotechnology, and the energy industries, informing our attempts to develop renewable biological energy sources. The six lectures explore how bacteria, plants and animals use light, electrons, protons and ATP to transduce energy from the sub-molecular to the cellular level. The lectures use an evolutionary emphasis to make it easier to understand the diversity of bioenergetics systems in nature.

Prof. Sarah Lummis: *Transmembrane Signalling: Molecules and Mechanisms*

Cells are continuously bombarded by many different types of signal; the ability of these cells to respond appropriately to such signals is critical for cell survival, adaptation, and specification of function, whether they are individual amoebae or components of a large, complex organism such as a human. This lecture course explores how cells monitor the presence of specific extra-cellular signalling molecules and how these signals then instigate and drive complex and interwoven intracellular responses. The course will focus on:

- The diversity of signals carrying information to cells; these range from single photons and small molecules to complex proteins.
- The relatively few mechanisms, usually involving plasma membrane receptors, by which the cell perceives the signal.

- The means by which the cell decodes 'the message', a process which may be very rapid, as in neurotransmission, or much slower, as in the signals that regulate gene expression and control growth.

The lectures will, for example, examine the roles of the ‘second messengers’ that often mediate part of cell signalling cascades, and will explore how these cascades allow very low concentrations of initiating signals to generate large responses in their target cells. Special attention is paid to G-protein coupled responses, and to the multiple roles played by protein phosphorylation in relaying intracellular signals.

This lecture course will be complemented by two successive practical classes in which students gain hands-on experience of the techniques used to probe the roles of proteins in three different cell signalling pathways.

Dr Marc de la Roche: *Immunology: the basics*

The aim of “Immunology, The Basics” is to introduce the immune system as a society of professional cells with myriad functions in our bodies – preventing the spread of infection, eliminating pathogens, wound healing, even guiding neural synapse development. The lecture will take a bottom-up approach by considering the evolution of the immune system, its cell types and their functions, and ending with a discussing of immune responses to infection and cancer.

Topics covered will include:

- Evolution and development of the immune system
- The innate immune system
- The adaptive immune system
- Infection by pathogens and how we deal with it
- Immunology’s evil, pro-cancer twin

Dr Martin Welch: *Bacterial Chemotaxis and Signal Transduction*

The field of bacterial chemotaxis and motility encompasses perhaps the best-understood prokaryotic signalling pathway. We start by using video footage of motile *E. coli* cells to define the basic swimming behaviour of bacteria in the unstimulated state. We then look at how this behaviour is altered when the cells are challenged with chemostimuli and demonstrate that the observed changes correlate with the sense of flagellar motor rotation. The altered bias in flagellar motor rotation brought about by exposure to chemostimuli causes structural changes in the architecture of the flagellar filaments, and we examine how these subtle molecular alterations can give rise to substantial changes in the behaviour of the whole cell.

We also look at how the molecular components of the chemotaxis and motility apparatus of the cell were discovered, and at the techniques that have been used to piece together the complex signal transduction pathway that is involved in integrating the multiple chemosensory inputs received by the cell at any given time into a single output. This signal transduction pathway involves multiple protein components, transient protein-protein interactions, phospho-transfer events and other chemical modifications, and its workings are now beginning to be understood at the atomic level.

We look at how the signalling pathway is assembled, how it works, and how its output influences the rotational bias of the flagellar motor (and therefore, ultimately, the swimming behaviour of the cell). Finally, we look at what is known about the flagellar motor itself – the world's smallest multi-speed motor, incorporating both forward and reverse gears. The ingenious methods that have been developed to study this remarkable device are discussed, including some video footage of the motor in action. Moreover, the study of chemotaxis and motility is not simply an esoteric branch of microbiology. With the recent completion of many eukaryotic genome sequences (including the human genome), it has become clear that homologues of the chemotaxis proteins are widespread in "higher" organisms, so these findings are likely to yield valuable insights into the function of many other organisms.

Prof. Mark Carrington: *Control of Eukaryotic Cell Growth*

The cell cycle is the term used to describe the succession of events that occur to produce two cells from one. An understanding of the molecular events involved in progression through the cell cycle is central to solving the larger problems of how the tightly controlled expansion of cell populations during the development and growth of any organism occurs and how the loss of regulation of the cycle results in disease – not just cancer but also the inappropriate growth of normal cells.

The aims of the lectures are:

- To give an understanding of the experimental approaches that can be taken to investigate the molecular machinery of a complex biological process.
- To explain how the molecular components that regulate cell cycle progression were identified and how their function was determined.
- To discuss a model of how the ordering of transitions that ultimately lead to cell division is regulated.

Easter Term: When biology is not what you expect

How is normal eukaryotic cell growth controlled, and what goes wrong when such control is pathologically disturbed in cancer?

Prof Chris Howe: *How the other three quarters lives - how protists break the rules of biochemistry*

Most of what you have heard so far in the course about the biochemistry and molecular biology of eukaryotes is based on a limited number of model organisms, such as *Saccharomyces*, *Drosophila*, humans, and *Arabidopsis*.

However, there is an enormous range of eukaryotic diversity outside those model lineages. This range includes organisms that are tremendously important as pathogens (such as trypanosomes) or ecologically (such as dinoflagellates that underpin coral reefs). Many of these organisms – most of them single celled protists - are strikingly different from the model ones in their basic biochemistry.

These lectures will start with a broad survey of eukaryotic diversity, to see what is out there, and then consider some examples of how some protists break the ‘rules’ of biochemistry, for example in doing away with histones for DNA packaging, or how they process RNA. At the same time, this will provide an opportunity for revision of earlier parts of the course.

Prof. G Evan & Dr T Littlewood: *Oncogenes, Tumour Suppressor Genes and Cancer*

The next four lectures build on the story of the cell cycle in eggs and yeasts by describing how normal mammalian cell proliferation is controlled. The focus is on the mechanisms of normal signalling pathways – growth factors and mitogens, their receptors and the mitogenic signals they generate inside the cell, and the pathways that then transduce such mitogenic signals to the various intracellular effectors that precipitate cell growth and replication. The principal effector responses to mitogenic signalling are transcriptional activation of proliferation-associated and cell survival genes and repression of growth suppressing genes, activation of RNA and protein synthesis, and an abrupt shift of metabolism to biosynthesis and aerobic glycolysis.

These lectures address the question of what happens in diseases, such as cancer, where control of cell growth, proliferation, survival and migration is lost through activating mutations in proto-oncogenes, and inactivating mutations in tumour suppressor genes. This introduction to molecular oncogenesis sets the scene for a more comprehensive analysis of cancer biology in one of the Part II Biochemistry courses.

Course Management and Student Feedback

The Biochemistry & Molecular Biology Course is run by a Management Committee, consisting of the Course Organiser and lecturers from the course. The Management Committee decides broadly on the content of the lectures and practicals and has the responsibility for organising and delivering these.

Decisions made by the Management Committee are influenced by the Consultative Committee, which comprises **student representatives** (chosen by you) and lecturers from the course. The Consultative Committee meets at the end of each term (dates below) and looks at the results of student feedback questionnaires as well as hearing your views about the course.

The course is revised on a yearly basis in the light of comments made by you in student feedback questionnaires and by your representatives on the Consultative Committee.

Dates for the Consultative Committee Meetings for BMB:

- Wednesday December 2th 2020, 11.15am
- Monday March 15th 2021, 11.15am
- Wednesday June 9th 2021, 10.00am

Minutes of the Consultative Committees and Analysis of Student Feedback Questionnaires

The minutes of the Consultative Committee meetings and the results of the student feedback questionnaires can be found on Moodle.

Contact Information:

Contact	Name	Email	Role
Chair of the Management Committee	Dr Dee Scadden	adjs100@cam.ac.uk	Academic issues relating to the course
Assistant Director of Teaching	Dr Mairi Kilkenny	mlk26@cam.ac.uk	Academic issues relating to the course
Teaching Administrator	Mrs Wendy Bundy	tchasst@bioc.cam.ac.uk	Organisation of meetings of the Consultative Committee, and Moodle enquiries
Classroom Manager	Dr Siolian Ball	slrb2@cam.ac.uk	Organisation of the practical laboratory

Safety Information

Part I Practical Classroom

University of Cambridge
Department of Biochemistry

Safety in the Biochemistry Part I Practical Teaching Laboratory

These pages ***MUST be read*** *before your first practical class.*

The 'Safety Acknowledgement and Compliance Form' and the 'Health Record Form' **MUST be read, then completed and signed. Please note that the forms in this handbook are for INFORMATION ONLY**, and forms for completion will be provided in your first practical class.

Alternatively, copies of these forms can be downloaded from Moodle in the file "Experimental Practicals – information for before you begin the course".

1. Departmental policy

1.1 It is the overriding policy of the University and Department of Biochemistry that experimental work, whether associated with teaching or research, be done efficiently and safely. All employees, students and research workers in the Department are required to observe the provisions of the Health and Safety at Work etc. Act 1974, the Radioactive Substances Act 1993, the Ionising Radiations Regulations 1999, and the Control of Substances Hazardous to Health (COSHH) Regulations 1988, as and when appropriate. Copies of the Acts and Regulations are available in the University Safety Office; copies of the University and Departmental Safety Regulations are available in the Teaching Laboratories. Copies of the risk assessments are available at the end of the Safety Instructions. Copies of the COSHH assessments will be put up on the notice board in the practical laboratory.

1.2 Safety in the Department begins with the individual's personal responsibility. However, in addition, each member of the academic staff, each research worker, and each technician in charge of a section or a laboratory has a statutory duty to take reasonable care for persons under his/her supervision or visitors to his/her area.

1.3 It is advisable to inform the Classroom Supervisor if you suffer from any medical problem (e.g. allergies, asthma, serious hearing or sight impairment etc.) that might be aggravated by an experiment or might affect your ability to carry out an experiment.

1.4 The staff involved with practical teaching in the Part I Laboratory have ensured, so far as is reasonably practical, that students will not be exposed to risks to their health and safety. Students have a statutory obligation to protect themselves and others from hazards resulting from their acts or omissions in the laboratories.

2. General

2.1 Smoking, eating and drinking are NOT permitted in the laboratory.

2.2 Outdoor clothing, umbrellas and bags must be placed in the under-bench cupboards provided for the purpose and not allowed to obstruct gangways or bench tops.

2.3 Suitable laboratory coats must be worn (fastened up) in the laboratory and removed before leaving. Safety spectacles must be worn when carrying out all procedures, and gloves of the appropriate type must be worn when necessary. Long hair must be restrained e.g. by means of a cap, ribbon, headband or net.

2.4 The notes for each practical should be read before coming to the practical and note taken of any safety matters. Students are NOT permitted to do any experimental work unless a supervisor (demonstrator or member of staff) is present.

2.5 The use of unfamiliar equipment (particularly centrifuges, vortex mixers, chart recorders, spectrophotometers, and oxygen electrodes) and the handling of potentially hazardous materials will be explained to students. If a student, for some reason, misses the appropriate explanation, then it is the student's responsibility to bring the lack of knowledge to the attention of the Classroom Supervisor, so that appropriate arrangements can be made to remedy the situation. See 3.2 below.

2.6 Glassware and plastic ware that is being used, for example to make up mixtures, should be labelled with marker pen; this avoids confusion and will help the laboratory staff.

2.7 Hazard and other labels that have been fixed to solution containers must NOT be tampered with or removed.

2.8 Hand-washing facilities are available in the laboratory. Hands should be washed before leaving.

2.9 Lavatory facilities are available just outside the main entrance to the laboratory.

3. Substances and procedures hazardous to health

3.1 Where a potential hazard (e.g. certain chemicals, biological materials and procedures) exists in a particular practical, this will be discussed in the talk before the practical and details of safe working methods will be highlighted in the practical notes.

3.2 Students must NOT use unfamiliar equipment or procedures without them having been given instruction. All safety instructions given in the preliminary talk and practical notes must be adhered to.

3.3 A hazard assessment (COSHH assessment) has been prepared for each practical; these are available for reference on the notice board situated to the right of the doorway to the NST IB classroom and exit. Extra copies of the COSHH assessments for all practicals will be made available either prior or during each practical session.

4. Waste

4.1 Broken glassware must be placed in one of the special bins provided in the laboratories where the sign 'BROKEN GLASS DISPOSAL POINT' is displayed. It must NOT be put into any other waste container.

4.2 Ordinary waste (e.g. tissues, sealing film and its backing paper, gloves) should be placed in one of the waste buckets provided at the ends of the benches. It must NOT be put into the bins for broken glass (referred to in 4.1 above).

4.3 Used plastic pipette tips must be placed in the appropriate labelled container on the bench.

4.4 Certain waste materials (e.g. used lancets, syringe needles, particular chemicals) that need specific disposal methods must be placed in the appropriate containers provided during that practical. They must NOT be placed in any other waste container.

4.5 No liquids should be poured down the sinks. Liquids should be disposed of in the disposal vessels supplied.

4.6 Benches should be left as waste-free and tidy as possible at the end of each practical – this reduces potential for accidents and spillages and is of considerable help to the laboratory staff.

5. Accidents

5.1 All accidents and spillages, including any personal injuries and damage caused to equipment, must be reported as soon as practicable to the Senior Demonstrator and Laboratory Manager or any of the other technicians. They will organise first aid if required and attend to the required written report.

5.2 Spillages must be cleared up immediately and the area decontaminated; they must NOT be left as a hazard to others.

5.3 A first aid box is located in the Classroom Supervisor's office area; see the classroom plan in the practical manual. Consult a Demonstrator or member of the Technical Staff if you need items from the First Aid Box.

6. Evacuation procedures

6.1 Any fire **must** be reported immediately to a technician or demonstrator.

6.2 A two-tone siren means evacuate the building immediately, by the designated routes (see plan).

6.3 In the event of fire do not panic, hesitate, or run; follow any oral instructions immediately and leave in an orderly manner. Do NOT use the lift.

6.4 The designated fire exits at both ends of the laboratory have clear signs and are shown on the plan of the laboratory. Students must identify those exits on their first visit to the laboratory.

7. Summary

Safe working is an attitude of mind, and the result of the sensible application of acquired knowledge. Always plan experimental work with safety in mind. Anticipate possible hazards and seek to minimise them, but be alert for the unexpected. Students should learn to carry out their work safely and maintain their working area in a safe condition, for the benefit of themselves and others working in the area.

If in Doubt – ASK!!

Please note that the 'Safety Acknowledgement and Compliance form' and the 'Health Record form' in this handbook are for INFORMATION ONLY – forms for completion will be provided in the first practical class.

Safety Acknowledgement and Compliance Form

NAME ____________________________ COLLEGE ________________
(please print)

BENCH/GROUP No __________________
(This is the number on your side of the blue sticker at the centre/back of the bench).

PRACTICAL DAY _______________________

I have read the document entitled *Safety in the Biochemistry Part I Practical Teaching Laboratory*. I agree to abide by the rules contained therein.

Signed ________________________________ Date __________________

This form to be handed to: **Laboratory Manager or her deputy**

Form received by______________________ Date _______

Health Record Form – Record of Hazardous Substance Usage

The COSHH Regulations require all individuals working with substances that can cause certain identifiable diseases or adverse health effects to be kept under health surveillance. For most workers this is confined to maintaining a record of a person's involvement in such work. Individuals who work with respiratory sensitizers, mercury, latex, arsenic and skin sensitizers will have additional health surveillance arranged by the Occupational Health Service. As a precautionary measure the University also requires health surveillance for all individuals working with Nanoparticles. Therefore, all persons working with nanoparticles should use this health record form and register with the University's Occupational Health Service. For further information on the criteria for health surveillance see the University's Hazardous Substances Policy: Policy and Guidance.

Personal Details for Workers in the Department of Biochemistry

Surname:	**Forenames**:	Male/Female/Prefer not to say: (circle)	Date of birth:
N.I. Number:	Date commenced present job:	Permanent address:	
Status: Undergraduate student (NST 1B BMB)			
Supervisor's name: Siolian LR Ball	Supervisor's phone no: 333344		
Your signature:	Date:	Postcode:	Dept. Tel No:

SUBSTANCE DETAILS

Name of substance	**Nature of hazard**[1]	**Physical state**[2]	**Quantity, amount**[3]	**Frequency of use**[4]	**Control measures**[5]
Ampicillin (sodium Salt)	Respiratory sensitiser	Solid	100 μg/ml in agar plates	**Rarely**	PPE – gloves, lab coat, safety specs
Bis/Acrylamide	Substance toxic to reproduction	Liquid (37:5:1)	10 ml	**Rarely**	PPE – gloves, lab coat, safety specs
Boric Acid	Impair fertility and toxic to unborn child	Liquid	8.9 mM in 400 ml	**Rarely**	PPE – gloves, lab coat, safety specs
Carbonyle cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)	Skin sensitiser	Liquid	20 μM in 200 μl	**Rarely**	PPE – gloves, lab coat, safety specs
α-Chymotrypsin	Respiratory sensitiser	Liquid	4 mg/ml (0.5 ml)	**Rarely**	PPE – gloves, lab coat, safety specs
Gel Red	Unknown	Solid In Polymerised Gel (<0.01%v/v)	Up to 45 ml	**Rarely**	PPE – gloves, lab coat, safety specs
Nitrilotriacetic Acid Disodium Salt	Cat 2 Carcinogen	Liquid	5 mM (30 ml)	**Rarely**	PPE – gloves, lab coat, safety specs
2-Mercaptoethanol	Skin Sensitiser	Liquid (5% v/v)	10 μl per sample	**Rarely**	PPE – gloves, lab coat, safety specs
SYBR Gold™	Unknown	1:10,000 dilution from stock	20 ml	**Rarely**	PPE – gloves, lab coat, safety specs
Virkon	Respiratory sensitiser. Irritating to skin. Can cause irreparable damage to eyes.	Liquid (1-10%)	200 ml	**Rarely**	PPE – gloves, lab coat, safety specs

(1) Carcinogen, mutagen, substance toxic to reproduction, respiratory sensitiser (i.e. asthmagen), skin sensitiser, (Relevant Risk Phrases/Hazard Statements: R42/H334, R43/H317, R45/H350, R46/H340, R49/H350i, R60/H360f, R61/H360d, R64/H362 where listed) **(2)** Liquid, solid, dust, vapour, gas or nanoparticle (particles of approximately 100 nm or less in at least one dimension) **(3)** Include amount and units **(4)** Daily, weekly, monthly, rarely **(5)** Fume cupboard, laminar flow bench, local exhaust ventilation (LEV), glove box or other form of containment, personal protective equipment (PPE; specify).

The completed form should be given to the Safety Secretary (Biochemistry), who will contact the Occupational Health Service. **This record must be kept for 40 years**.

Risk Assessments

Level of risk = [severity of the event] x [likelihood of the event happening]

Severity descriptors ↓ Likelihood →	1 very low <2% likely	2 low 2-5% likely	3 medium 5-10% likely	4 high 10-20% likely	5 very high >20%
1 (Insignificant) None	1	2	3	4	5
2 (Minor) Minor reversible injury	2	4	6	8	10
3 (Moderate) Major reversible injury	3	6	9	12	15
4 (Severe) Irreversible injury or death	4	8	12	16	20
5 (Very Severe) Irreversible injury or death to multiple people	5	10	15	20	25

Overall Risk Levels	**Rating**	**Monitoring and Reviewing Guidelines**
1-6	Low	Should be reviewed annually
8-12	Medium	Should be monitored and reviewed 6 monthly
14-20	High	Should be constantly monitored and reviewed quarterly
Above 20	Very high	Should be constantly monitored and reviewed monthly

This is a guide to how risk is assessed. The control measures we employ (which includes how we behave around dangerous activities and chemicals) can modify these risks. As you read the risk assessments you will notice that the safety instructions given to you in your protocols and your training (control measures) can reduce the risk by affecting the **likelihood** that something bad will happen, rather than the **severity** of the result. You ***must*** read and ensure you understand the risk assessments and the COSHH (Chemical Hazard) forms for each experiment. The COSHH forms will be supplied during each experimental session.

UNIVERSITY OF CAMBRIDGE
DEPARTMENT OF BIOCHEMISTRY

CLASSROOM RISK ASSESSMENT FOR STUDENTS

Assessors: Dr Siolian Ball

Date: 01/09/19

Aseptic Technique: Culturing Microorganisms

Staff Affected: Staff, Students and academic visitors

Activity / Hazard		Risk level Severity x Likelihood = (Total risk)	Control Measures Implemented	Revised Risk Level
1	Use of Bunsen burner/Burns	4x5 **(20)**	Equipment supplied is of correct type and in good working condition Safe operating procedures are described in practical protocol User to use PPE as described in practical protocol User will be adequately trained and supervised	4x1 **(4)**
2	Handling microbes/Pathogenic Microbial contamination	4x5 **(20)**	Equipment supplied is of correct type and in good working condition Non-Pathogenic strains used *(Escherichia coli K12 derivatives, Serratia sp ATCC39006, Chromobacterium violaceum)* Safe operating procedures are described in practical protocol User to use PPE as described in practical protocol User will be adequately trained and supervised	4x1 **(4)**
3	Handling microbes/Puncture injuries from use of cocktail sticks causing pathogenic microbial infection	4x5 **(20)**	Equipment supplied is of correct type and in good working condition Safe operating procedures are described in practical protocol User to use PPE as described in practical protocol User will be adequately trained and supervised	4x1 **(4)**

Further Information:

Review Date: July 2020

Overall Risk Levels	Rating	Monitoring and Reviewing Guidelines
1-6	Low	Should be reviewed annually
8-12	Medium	Should be monitored and reviewed 6 monthly
14-20	High	Should be constantly monitored and reviewed quarterly
Above 20	Very high	Should be constantly monitored and reviewed monthly

UNIVERSITY OF CAMBRIDGE DEPARTMENT OF BIOCHEMISTRY	**CLASSROOM RISK ASSESSMENT FOR STUDENTS**		
	Assessors: Dr Siolian Ball	**Date: 01/09/19**	**Liquid Handling and mixing**
Staff Affected:	Staff, Students and academic visitors		

Activity / Hazard		**Risk level** Severity x Likelihood = **(Total risk)**	**Control Measures Implemented**	**Revised Risk Level**
1	Vortex mixer use/Possible electrocution	4x2 (8)	Equipment supplied is of correct type and in good working condition Safe operating procedures are described in practical protocol User will be adequately trained and supervised	4x1 (4)
2	Vortex mixer use/Vibration injury	3x2 (6)	Safe operating procedures are described in practical protocol User will be adequately trained and supervised	3x1 (3)
3	Exposure to substances hazardous to health	4x5 (20)	Concentrations and volumes reduced to minimal values Non-Pathogenic strains used *(Escherichia coli K12 derivatives, Serratia sp ATCC39006, Chromobacterium violaceum)* Safe handling procedures described in practical protocol User to use PPE as described in practical protocol and by demonstrators User will be adequately trained and supervised Refer to relevant COSHH forms	3x1 (3)
4	Liquid Handling/Possible RSI	2x2 (4)	Equipment supplied is of correct type and in good working condition Safe operating procedures are described in practical protocol User will be trained and adequately supervised	2x1 (2)
Further Information:			**Review Date: July 2020**	

Overall Risk Levels	**Rating**	**Monitoring and Reviewing Guidelines**
1-6	Low	Should be reviewed annually
8-12	Medium	Should be monitored and reviewed 6 monthly
14-20	High	Should be constantly monitored and reviewed quarterly
Above 20	Very high	Should be constantly monitored and reviewed monthly

UNIVERSITY OF CAMBRIDGE **DEPARTMENT OF BIOCHEMISTRY**	**CLASSROOM RISK ASSESSMENT FOR STUDENTS**		Page 46 of 274
	Assessors: Dr Siolian Ball	**Date: 01/09/19**	**Electrophoresis**
Staff Affected:	Staff, Students and academic visitors		

Activity / Hazard		**Risk level** Severity x Likelihood = **(Total risk)**	**Control Measures Implemented**	**Revised Risk Level**
1	Gel electrophoresis/Possible electrocution from gel rig	4x2 **(8)**	Equipment supplied is of correct type and in good working condition Safe operating procedures are described in practical protocol User to use PPE as described in practical protocol User will be adequately trained and supervised	4x1 **(4)**
2	Gel electrophoresis/Exposure to substances hazardous to health	4x5 **(20)**	Concentrations and volumes reduced to minimal values Safe handling procedures described in practical protocol User to use PPE as described in practical protocol User will be adequately trained and supervised Refer to relevant practical COSHH forms	4x1 **(4)**
3	Gel electrophoresis/Risk of injury from breaking glass plates	3x3 **(9)**	Safe handling procedures described in practical protocol User to use PPE as described in practical protocol User will be adequately trained and supervised	3x1 **(3)**
4	Liquid Handling/Possible contamination with substances hazardous to health	4x5 **(20)**	Equipment supplied is of correct type and in good working condition Safe operating procedures are described in practical protocol User to use PPE as described in practical protocol User will be adequately trained and supervised Refer to relevant practical COSHH forms	4x1 **(4)**
Further Information:				**Review Date: July 2020**

Overall Risk Levels	**Rating**	**Monitoring and Reviewing Guidelines**
1-6	Low	Should be reviewed annually
8-12	Medium	Should be monitored and reviewed 6 monthly
14-20	High	Should be constantly monitored and reviewed quarterly
Above 20	Very high	Should be constantly monitored and reviewed monthly

	Activity / Hazard	Risk level Severity x Likelihood = (Total risk)	Control Measures Implemented	Revised Risk Level
1	Use of Glass Homogenisers/Exposure to substances hazardous to health	4x5 (20)	Concentrations and volumes reduced to minimal values Safe handling procedures described in practical protocol User to use PPE as described in practical protocol User will be adequately trained and supervised Refer to relevant practical COSHH forms	4x1 (4)
2	Use of Glass Homogenisers/Risk of injury from breaking glass	3x3 (9)	Safe handling procedures described in practical protocol User to use PPE as described in practical protocol User will be adequately trained and supervised	3x1 (3)
Further Information:				**Review Date: July 2020**

Overall Risk Levels	Rating	Monitoring and Reviewing Guidelines
1-6	Low	Should be reviewed annually
8-12	Medium	Should be monitored and reviewed 6 monthly
14-20	High	Should be constantly monitored and reviewed quarterly
Above 20	Very high	Should be constantly monitored and reviewed monthly

UNIVERSITY OF CAMBRIDGE **DEPARTMENT OF BIOCHEMISTRY**	**CLASSROOM RISK ASSESSMENT FOR STUDENTS**		Page 48 of 274
	Assessor: Dr Siolian Ball	**Date: 01/09/19**	**Centrifuges**
Staff Affected:	Staff, Students and academic visitors		

Activity / Hazard		**Risk level** Severity x Likelihood = **(Total risk)**	**Control Measures Implemented**	**Revised Risk Level**
1	Use of centrifuge/Electrocution	4x2 **(8)**	Equipment supplied is of correct type and in good working condition Safe operating procedures are described in practical protocol User to use PPE as described in practical protocol User will be trained and adequately supervised	4x1 **(4)**
2	Use of centrifuge/Exposure to substances hazardous to health	4x5 (**20**)	Concentrations and volumes reduced to minimal values Safe handling procedures described in practical protocol User to use PPE as described in practical protocol User will be trained and adequately supervised Refer to relevant practical COSHH forms	4x1 **(4)**
3	Use of centrifuge/Physical injury	3x2 **(6)**	Equipment supplied is of correct type and in good working condition Safe operating procedures are described in practical protocol User to use PPE as described in practical protocol User will be trained and adequately supervised	4x1 **(4)**
Further Information:			**Review Date: July 2020**	

Overall Risk Levels	**Rating**	**Monitoring and Reviewing Guidelines**
1-6	Low	Should be reviewed annually
8-12	Medium	Should be monitored and reviewed 6 monthly
14-20	High	Should be constantly monitored and reviewed quarterly
Above 20	Very high	Should be constantly monitored and reviewed monthly

UNIVERSITY OF CAMBRIDGE

DEPARTMENT OF BIOCHEMISTRY

CLASSROOM RISK ASSESSMENT FOR STUDENTS

Assessor: Dr Siolian Ball

Date: 01/09/19

Oxygen Electrodes

Staff Affected: Staff, Students and academic visitors

	Activity / Hazard	**Risk level** Severity x Likelihood = **(Total risk)**	**Control Measures Implemented**	**Revised Risk Level**
1	Use of oxygen electrode stirrer/Electrocution	4x2 (8)	Equipment supplied is of correct type and in good working condition Safe operating procedures are described in practical protocol User to use PPE as described in practical protocol User will be trained and adequately supervised	4x1 (4)
2	Use of oxygen electrode/Exposure to substances hazardous to health	4x5 (20)	Concentrations and volumes reduced to minimal values Safe handling procedures described in practical protocol User to use PPE as described in practical protocol User will be trained and adequately supervised Refer to relevant practical COSHH forms	4x1 (4)
3	Use of chart recorders/Electrocution	4x2 (8)	Equipment supplied is of correct type and in good working condition Safe operating procedures are described in practical protocol User to use PPE as described in practical protocol User will be trained and adequately supervised	4x1 (4)
4	Liquid Handling/Possible RSI	2x2 (4)	Equipment supplied is of correct type and in good working condition Safe operating procedures are described in practical protocol User will be trained and adequately supervised	2x1 (2)

Further Information:

Review Date: July 2020

Overall Risk Levels	**Rating**	**Monitoring and Reviewing Guidelines**
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UNIVERSITY OF CAMBRIDGE **DEPARTMENT OF BIOCHEMISTRY**	**CLASSROOM RISK ASSESSMENT FOR STUDENTS**		Page 50 of 274
	Assessor: Dr Siolian Ball	**Date: 01/09/19**	**Heat Blocks**
Staff Affected:	Staff, Students and academic visitors		

Activity / Hazard		**Risk level** Severity x Likelihood = **(Total risk)**	**Control Measures Implemented**	**Revised Risk Level**
1	Use of heat blocks/Burns	4x2 **(8)**	Equipment supplied is of correct type and in good working condition Safe operating procedures are described in practical protocol User to use PPE as described in practical protocol User will be adequately trained and supervised	4x1 **(4)**
2	Use of heat blocks/Electrocution	4x2 **(8)**	Equipment supplied is of correct type and in good working condition Safe operating procedures are described in practical protocol User to use PPE as described in practical protocol User will be adequately trained and supervised	4x1 **(4)**
Further Information:				**Review Date: July 2020**

Overall Risk Levels	**Rating**	**Monitoring and Reviewing Guidelines**
1-6	Low	Should be reviewed annually
8-12	Medium	Should be monitored and reviewed 6 monthly
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Appendix 1: Units – prefixes, amount and concentration

Units

Quantitative results MUST be given in the appropriate units. Biochemical calculations often involve going from raw data to units that enable you to compare sets of measurements. When doing calculations, being crystal clear about the units at each stage will help you to keep track of what you are doing and where you are going. If you make a mistake in a calculation - perhaps dividing by a volume instead of multiplying – the units can alert you to the error.

Some simple but important points are noted below.

Prefixes

You will be familiar with milligrams (mg; milli = 10^{-3}) and kilograms (kg; kilo = 10^{3}) and cubic decimetres (dm^3; deci = 10^{-1}). The full range of prefixes is as follows:

atto	(a)	= 10^{-18}
femto	(f)	= 10^{-15}
pico	(p)	= 10^{-12}
nano	(n)	= 10^{-9}
micro	(μ)	= 10^{-6}
milli	(m)	= 10^{-3}
kilo	(k)	= 10^{3}
mega	(M)	= 10^{6}
giga	(G)	= 10^{9}
tera	(T)	= 10^{12}
peta	(P)	= 10^{15}
eta	(E)	= 10^{18}

The distinction between amount and concentration

Somewhat surprisingly, students often confuse amount with concentration.

Amount

Quantities (amounts) of substances are usually best expressed in moles. A mole is defined as that amount of a substance that contains as many elementary units (i.e. atoms, molecules or whatever) as there are carbon atoms in exactly 12 g of the isotope carbon-12. The symbol for mole or moles is mol, with a prefix if appropriate (e.g. mmol, μmol). Thus, 1 mol of glucose ($C_6H_{12}O_6$) would weigh (6x12) + (12x1) + (6x16) = 180 g.

Moles are used to enable quantities easily to be compared – e.g. to find how many molecules of a product are formed per second by a molecule of enzyme. Of course, if the molecular weight is unknown, units of mass (grams etc) must be used. (It would be neither helpful nor feasible to consider the molecular weight of a sausage or a banana. However, the molecular weight of a defined complex of components such as a ribosome is available and useful).

Biochemists often express the mass of a molecule in daltons (which is just another name for unified atomic mass units). So a protein with a molecular mass of 50, 000 atomic mass units can be described as a 50, 000 dalton protein. Usually, this would be shortened to 50 kDa (kilodaltons). Another term that can be used is relative molecular mass (M_r), which is also called the molecular weight. This has no units, because it is relative to 1/12 of a C atom – check back to 'A' level Chemistry. The M_r of our example would be 50, 000.

Concentration

This is amount per unit volume. Biochemists (and chemists of course) often work in molar concentrations. A solution that contains 1 mol of a substance in a volume of 1 dm^3 has a concentration of 1 molar, symbolised 1 M. Biochemists usually refer to the cubic decimetre (dm^3) as a litre (l), and a cubic centimetre as a millilitre (ml, pronounced "mill").

Thus, you need to become fluent in the types of quantities shown below

1 litre of a 1 M solution contains 1 mol of solute
1 ml of a 1 M solution contains 1 mmol of solute
1 ml of a 1 mM solution contains 1 µmol of solute
1 ml of a 1 µM solution contains 1 nmol of solute
1 µl of a 1 µM solution contains 1 pmol of solute etc.

Never use M to mean number of moles: M means molar, a unit of concentration equal to moles per litre.

Dilutions

It is frequently necessary to dilute stock solutions when working in a biochemical laboratory.
Some sample calculations will be made available on the course Moodle site. You will get practice at this throughout the year and should feel comfortable with this by the time you sit the examination in June. If you are having problems you will not be alone in this. Please consult with your supervisor early in the year for help.

Appendix 2: Acids, Bases, pH and Buffers

Why are acid-base concepts important in Biochemistry?

Very simply, cells are largely composed of molecules with functional groups that can alter their electric charge by loss of protons (acidic groups) or gain of protons (basic groups). Such molecules include proteins, nucleic acids and phospholipids (components of cell membranes) as well as metabolites such as the intermediates of glycolysis and the citric acid cycle. The properties of molecules with ionisable groups therefore can alter with the acidity of the medium they are exposed to, whether within living cells or in solutions prepared in the laboratory.

Important points that affect ionisation and hence molecular properties are:

1. The strength of acids and bases – how easily are protons lost or gained?
2. How the electric charge of ionisable groups is affected by the proton concentration of the ambient solution.
3. How the proton concentration of the ambient solution can be regulated by buffers.

What are protons, acids and bases?

Strictly speaking, a proton is the nucleus of a hydrogen atom that has lost its single electron – an H^+ ion. However, in aqueous solution protons are not naked (or "dry") but become hydrated by reacting with H_2O to form H_3O^+ ions called hydroxonium ions. For convenience, biochemists call these hydrated ions simply "protons" (or sometimes "hydrons") unless the context makes it important to be more precise.

Acids are donors of protons, and bases are acceptors. For example, in proteins -COOH groups are acids, and $-NH_2$ groups are bases. A -COOH or other acidic group is said to dissociate when it donates its proton to water to form the anion, $-COO^-$, which is the conjugate base of the -COOH acidic group.

$$R{\cdot}COOH + H_2O = R{\cdot}COO^- + H_3O^+$$

This is shown simply as

$$R{\cdot}COOH = R{\cdot}COO^- + H^+$$

Similarly, for $R{\cdot}NH_2$

$$R{\cdot}NH_2 + H^+ = R{\cdot}NH_3^+$$

Here, $R{\cdot}NH_3^+$ is the conjugate acid of the $R{\cdot}NH_2$ basic group.

Any acid has a dissociation constant, K, which is the equilibrium constant for the dissociation. Sometimes, a subscript "a" is added, to show that the constant refers to an acid, K_a: For our carboxyl group example,

$$K = \frac{[H^+][R{\cdot}COO^-]}{[R{\cdot}COOH]}$$

and for the amino group

$$K = \frac{[H^+][R{\cdot}NH_2]}{[R{\cdot}NH_3^+]}$$

Water itself dissociates, and has a dissociation constant called K_w.

$$H_2O = H^+ + OH^-$$

Because the concentration of H_2O is effectively constant in ordinary aqueous solution, it is generally given an arbitrary value of 1 and omitted from equilibrium constants. Hence

$$K_w = [H^+][OH^-]$$

The value of K_w is close to 10^{-14} M^2. (Note that the unit is M^2 because of the convention adopted for water concentration).

Logarithms: definition of pH

Very wide ranges of proton concentration need to be considered in a laboratory context: 1 M HCl contains 1 M H^+ and 1 M NaOH contains 10^{-14} M H^+ (see below). This is one reason why a more convenient and compressed scale of acidity than simple molar concentration of H^+ is used. Of course, this is the pH scale and pH is defined as the negative logarithm to base 10 of the H^+ ion concentration[1].

$$pH = -\log_{10}[H^+]$$

This can also be written as the logarithm of the reciprocal of H+ concentration. (Note that $1/[H^+] = [H^+]^{-1}$).

$$pH = \log_{10}\left(\frac{1}{[H^+]}\right)$$

So, a solution at pH 5 would have $[H^+] = 10^{-5}$ M. The higher the pH, the lower the value of $[H^+]$. A solution of 1 M HCl would have 1 M H^+ concentration and pH = 0 (because $\log_{10}1 = 0$). In the case of 1M NaOH, the concentration of OH^- is 1 M. As explained above, K_w is 10^{-14} M^2 so that $[H^+]$ is 10^{-14} M, corresponding to a pH of 14.

The Henderson-Hasselbalch equation and its uses

The expression for the dissociation constant of acids can be manipulated into a more convenient form, replacing $[H^+]$ by pH, and K by pK. For a general acid, HA

$$K = \frac{[H^+][A^-]}{[HA]} \quad \text{Eqn (1)}$$

Rearrange

---

[1] Logarithms: if $y = 10^x$, then $\log_{10}y = x$. For example, if $y = 10^{-5}$ then $\log_{10}y = -5$, and $-\log_{10}y = 5$.

$$\frac{1}{[H^+]} = \left(\frac{1}{K}\right)\left(\frac{[A^-]}{[HA]}\right)$$

Now take the logarithm of each side. [Note that log (A ·B) = log A + log B]

$$pH = \log\left(\frac{1}{K}\right) + \log\left(\frac{[A^-]}{[HA]}\right)$$

We give the symbol p*K* to log(1/*K*), to get the **Henderson-Hasselbalch equation**[2]**.**

$$pH = pK + \log\left(\frac{[A^-]}{[HA]}\right) \quad \text{Eqn (2)}$$

The Henderson-Hasselbalch equation

For carboxyl groups, $\left(\frac{[A^-]}{[HA]}\right) = \left(\frac{[RCOO^-]}{[RCOOH]}\right)$ and for amino groups the ratio is $\left(\frac{[RNH_2]}{[RNH_3^+]}\right)$.

In general, the less-protonated form (base: proton acceptor) is the numerator of the ratio, and the more protonated form (acid: proton donor) is the denominator.

The characteristics of the p*K*

Notice from the definition of *K*, in equation (1) that the stronger the acid the lower is its p*K* value, and *vice versa* the weaker the acid the higher the p*K*. For example, acetic acid (ethanoic acid) with p*K* = 4.7 (so $K = 10^{-4.7}$) is a much stronger acid than phenol (p*K* = 10.0). For bases, the same applies: the higher the p*K* the weaker is the conjugate acid form, and the stronger is the proton-accepting conjugate base. For example, the side chain of the amino acid arginine has a p*K* = 12.5 and is a hundred-times stronger base than the side chain of lysine (p*K* = 10.5).

Notice from equation (2), the Henderson-Hasselbalch equation, that when the pH is equal to the p*K*, the concentrations of A^- and HA are equal because[3] (pH - p*K*) = $\log\left(\frac{[A^-]}{[HA]}\right) = 0$. So the p*K* is the pH at which there are equal concentrations of the acid and its conjugate base, in other words the acid is 50% dissociated.

Curves of pH-dependence

You can see from the Henderson-Hasselbalch equation how the extent of dissociation of an acid will vary with pH above and below the p*K*. We can calculate the ratio of $\left(\frac{[A^-]}{[HA]}\right)$ at any pH we like, expressed as a difference from the p*K*.

At pH = p*K* - 1, $\log\left(\frac{[A^-]}{[HA]}\right) = -1$ so that $\left(\frac{[A^-]}{[HA]}\right) = 1/10$

---

[2] This equation dates from 1908/1916 and was originally given for the carbon dioxide/carbonic acid system that is so important in the acid-base balance of blood. See Physiology.

[3] If you don't see that: the ratio of $[A^-]/[HA] = 1$, and the log of 1 = 0 because $1 = 10^0$

At pH = pK , $\log\left(\frac{[A^-]}{[HA]}\right)$ = 0 so that $\left(\frac{[A^-]}{[HA]}\right)$ = 1/1

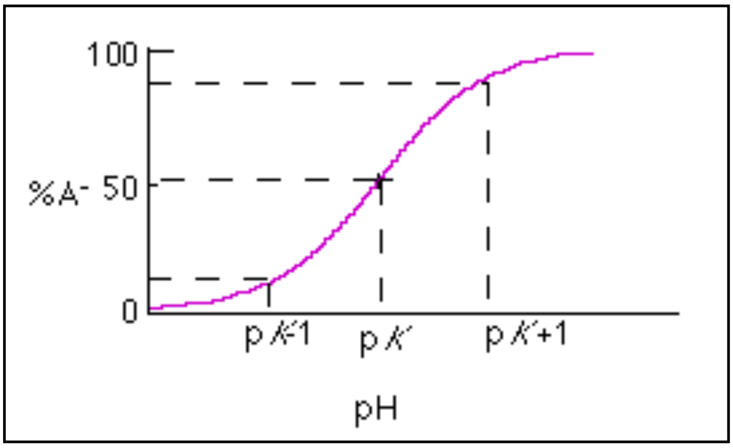
At pH = pK + 1, $\log\left(\frac{[A^-]}{[HA]}\right)$ = 1, so that $\left(\frac{[A^-]}{[HA]}\right)$ = 10/1

In order to calculate the fraction (or percentage) of the total acid species that is dissociated, it is necessary to appreciate the difference between the RATIO of [A$^-$] to [HA] and the FRACTION that A$^-$ forms of the total species present (A$^-$ and HA).

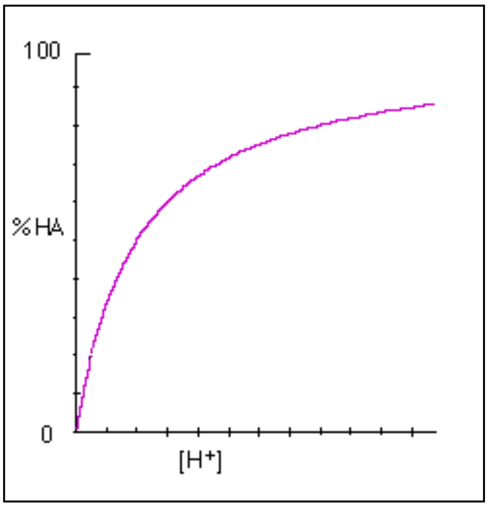
This simple point is a common source of confusion for students. The total number of "parts" into which the “whole” is divided is numerator + denominator. So if the ratio is 1/10, there are 1+10 = 11 parts in all, and the fraction that A$^-$ contributes to the total is 1/11. Look at the table below to see how it is done. We can build up a curve showing the pH dependence of the extent of dissociation of an ionising group. Why not try this for yourself?

pH - p*K*	**RATIO** $\left(\frac{[A^-]}{[HA]}\right)$	**FRACTION DISSOCIATED** $\frac{[A^-]}{[HA]+[A^-]}$	**% DISSOCIATED**
-1	1/10	1/(10 + 1) = 1/11 = 0.091	9.1%
-0.5	0.32/1	0.32/(0.32 + 1) = 0.32/1.32 = 0.24	24%
0	1/1	1/(1 + 1) = 1/2 = 0.5	50%
+0.5	3.2/1	3.2/(3.2 + 1) = 3.2/4.2 = 0.76	76%
+1	10/1	10/(10 + 1) = 10/11 = 0.91	91%

The general shape of a pH-dependence curve is shown below.



Notice that if we didn't want to bother with pH and pK, and just plotted the percent of total species present as HA as a direct function of H$^+$ concentration, we would have the rectangular hyperbola that describes any simple type of equilibrium where two molecules combine reversibly – in this case H$^+$ and A$^-$ associating to form HA.



Properties of buffers

A buffer resists pH changes: if H^+ or OH^- are added to a buffered solution, its pH changes less than would the pH of water alone. Any mixture of an acid and its conjugate base can act as a buffer - for example a mixture of acetic acid and sodium acetate. This is because if H^+ is added, some ions will be removed by protonating the conjugate base (acetate anions); similarly, H^+ removed by combining with added OH^- to form water will be replaced by dissociation of the acid.

$$R{\cdot}COOH \;=\; R{\cdot}COO^- + H^+$$

Buffers are only effective near their p*K* values

When H^+ is added to a buffer, some A^- will be converted to HA so that the ratio of $\left(\frac{[A^-]}{[HA]}\right)$ will fall. Provided that the ratio is quite close to 1 in the first place, then there will be no large proportional change in either and the pH will not change much. But if A^- (or HA) is the predominant species, the buffering power will be lower because the proportional change will be larger. In practice, a good buffer requires reasonably large concentrations of both buffering species, and so its pH must not be too far from its p*K*.

The following worked example illustrates this principle in action.

Consider a series of buffer solutions, each of volume 1 litre and containing 1 M total concentration of A^- (salt) and HA (acid), but in different proportions. To each buffer, add 100 ml of 1 M HCl (i.e. 100 mmol). Suppose that the p*K* of the acid is 5.0. Now apply the Henderson-Hasselbalch equation to see how the composition of the buffer affects the pH change after addition of HCl.

BEFORE HCl			AFTER 100 mmol HCl			Change in pH
$[A^-]$ mM	[HA] mM	old pH	$[A^-]$ mM	[HA] mM	new pH	
200	800	4.40	100	900	4.06	0.34
500	500	5.00	400	600	4.82	0.18
900	100	5.95	800	200	5.60	0.35
Just pure water		7.00	0.09 M HCl		1.05	5.95

Buffers are effective if they are concentrated

The ratio of $[A^-]/[HA]$ will alter less for a given quantity of one species changed into the other as their concentrations increase. Hence, buffers become more effective at resisting pH changes the higher their concentrations.

The pH of a buffer is independent of its concentration

Provided that [HA] and $[A^-]$ remain large compared with $[H^+]$ and $[OH^-]$, dilution of the buffer to lower its concentration will not alter the ratio $\left(\frac{[A^-]}{[HA]}\right)$. Hence, dilution will not affect the pH of a buffer solution, provided it behaves “ideally”; it will only make it a less powerful buffer. In practice, concentrated solutions are not “ideal” because solute molecules no longer behave independently. This means that dissociation constants can change with the total ionic strength of the solution, so small changes in buffer pH do occur on dilution.

Types of buffer useful for experimental purposes

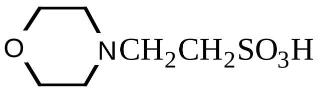
What criteria influence the choice of a buffer for experimental purposes?

Obviously the buffering capacity must be sufficient to stabilise the pH within an acceptable range. The p*K* of the buffer is central to this, but buffer concentration can also be raised although this may be undesirable because it will increase total ionic strength and osmolarity. Ionic strength effects will be diminished if the buffer anion or cation is univalent. Other practical criteria are that the buffer substance should be easily available in pure form, not be too expensive, and not interfere with the assay method. Thus, it should be optically transparent to the wavelength used in a spectrophotometric assay, and not interfere with other substances in assay mixtures by forming strong complexes or insoluble salts with metal ions.

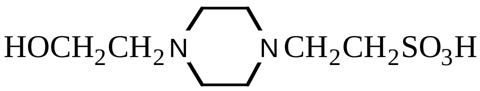
Buffers should not interfere with reactions being studied. Thus in enzyme assays, buffers should not contain substances that are substrates, products, inhibitors or activators of the enzyme. For example, phosphate buffer ($H_2PO_4^-/HPO_4^{2-}$; $pK = 7.2$) would not be suitable for assaying glucose 6-phosphatase (phosphate is a product) or glycogen phosphorylase (phosphate is a substrate). You will meet these enzymes in the lectures on carbohydrate metabolism. When experiments concern membrane-bound organelles, or intact cells it is important that the buffer should not cross the membranes to cause osmotic problems or unwanted changes in the pH of compartments. For example, that would rule out acetate buffers for many purposes because the CH_3COOH species is membrane permeant.

Until the mid1960s, biochemists were rather restricted in the types of buffer for use at "biological" pH values (roughly pH 5.5 - 8.5). Norman Good led a search to identify a range of suitable buffers with p*K* values between 6 and 8, with high water solubility but poor solubility in "lipid solvents" so they would not readily penetrate biological membranes, and with poor chelating ability for metal ions.

Typical "Good" (capital "G") buffers are HEPES (p*K* 6.15) and MES (p*K* 7.55).

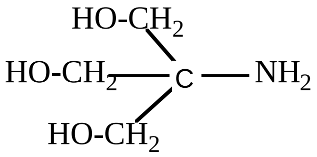


Structure of MES



Structure of HEPES

Another commonly used buffer, which you will encounter in several practicals is Tris (p*K* 8.2). The name is an abbreviation of tris(hydroxymethyl)aminomethane.



Structure of Tris

Some Exercises (answers below)

Working through these should help you get a feel for buffers. The answers are given below.

Q1. What fraction of lactic acid will be present as the free acid at pH 4.5, given that its p*K* is 3.9?

Q2 (a). What is the pH of a solution of 0.1 M $NaHCO_3$ and 0.08 M Na_2CO_3, given that the p*K* for dissociation of bicarbonate to carbonate is 10.0?

Q2 (b). What will its pH be when 1 ml of 0.5 M NaOH is added to 10 ml of this solution?

Q3. What concentration of HCl must be added to a solution of 0.6 M sodium acetate to achieve a pH of 5.0? The p*K* of acetic acid is 4.7.

Answers to exercises

Answer 1: Applying the Henderson-Hasselbalch equation,

$$4.5 = 3.9 + \log\frac{[\text{lactate anion}]}{[\text{lactic acid}]}$$

$$0.6 = \log\frac{[\text{lactate anion}]}{[\text{lactic acid}]}$$

$$4 \quad = \frac{[\text{lactate anion}]}{[\text{lactic acid}]}$$

So $\frac{[\text{lactic acid}]}{[\text{lactic acid}]+[\text{lactate anion}]} = \frac{1}{(4+1)} = 0.2$ (20%).

Answer 2(a): Applying the Henderson-Hasselbalch equation to the dissociation

$$HCO_3^- = CO_3^{2-} + H^+$$

$$\text{pH} = 10 + \log\left(\frac{[CO_3^{2-}]}{[HCO_3^-]}\right) = 10 + \log\frac{0.08}{0.1} = 10 + \log 0.8 = 10 - 0.1 = 9.9$$

Answer 2(b): Proceed by converting concentrations into amounts.

10 ml of the buffer contains 1 mmol of HCO_3^- and 0.8 mmol of CO_3^{2-}.
1 ml of 0.5 M NaOH contains 0.5 mmol of NaOH.

The 0.5 mmol of NaOH reacts with 0.5 mmol of HCO_3^- and converts it to 0.5 mmol of CO_3^{2-} according to the equation

$$HCO_3^- + OH^- = CO_3^{2-} + H_2O$$

The result of this reaction is that the amount of CO_3^{2-} increases from 0.8 to 1.3 mmol, and the amount of HCO_3^- decreases from 1 to 0.5 mmol. Since these are both obviously dissolved in the same volume (11 ml), their concentrations will have the same ratio as their amounts, $\frac{1.3}{0.5}$.

Calculate the new pH from the Henderson-Hasselbalch equation

$$\text{pH} = 10 + \log\frac{1.3}{0.5} = 10 + \log 2.6 = 10 + 0.4 = 10.4$$

Answer 3: The pH wanted is 0.3 above the p*K*. From the Henderson-Hasselbalch equation,

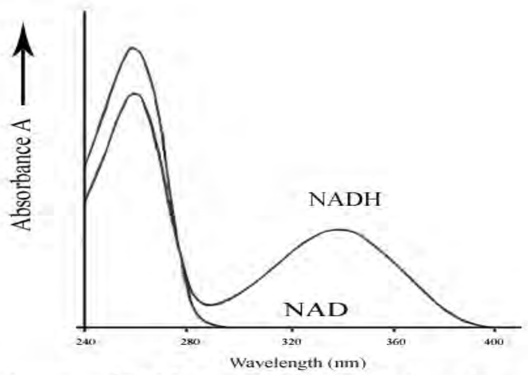
$\log\frac{[\text{acetate anion}]}{[\text{acetic acid}]}$ is 0.3. Hence, the ratio $\frac{[\text{acetate anion}]}{[\text{acetic acid}]}$ is 2/1. Therefore to achieve this ratio by adding HCl to the original 0.6 M sodium acetate (assumed to be fully dissociated), 1/3 of the initial concentration must be converted to acetic acid. This is done by 0.2 M HCl.

Appendix 3: Principles of Spectrophotometry

Absorption of light

Water is colourless; it does not absorb visible light, or light in the ultraviolet region of the spectrum. But many substances dissolve to give coloured solutions (e.g. haemoglobin, cytochrome *c*), because their molecules do absorb visible light. Others, like nucleic acids, proteins, and NAD(H) absorb in the ultraviolet. The higher the concentration of solute, the more light is absorbed. **Spectrophotometry** is a simple technique based on this quantitative relationship that allows concentrations of dissolved substances to be determined by measuring light absorption of solutions.

For example, reduced cytochrome *c* is yellow and has absorption peaks at around 550 nm and 420 nm. This is due to the haem prosthetic group, which is a **chromophore** (literally, colour carrier). NADH has peaks in the ultraviolet at 260 and 340 nm, while NAD absorbs at 260 nm only. Thus the activity of NAD-linked dehydrogenases can be measured by changes in light absorption at 340 nm.

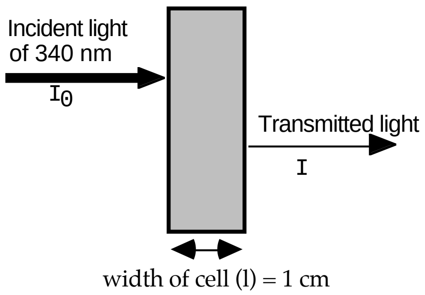


Absorbance of NAD and NADH as a function of wavelength

Quantitative Applications: The Beer-Lambert Law

Imagine a solution of NADH in a spectrophotometer cell (width 1 cm) and that monochromatic ultraviolet light at 340 nm is shone through it.

Let the intensity of the incident light = I_O and of the transmitted light = I



The most intuitive measure of how much light has been absorbed is the **transmission**, I/I_O. This can be expressed as a percentage.

However, it is more useful to use the **absorbance**, A. This is defined as the logarithm (to base 10) of I_O/I. Note that I_O/I is the *reciprocal* of the transmission.

$$A = \log I_O/I$$

Incident Intensity (I_O) Arbitrary units	Transmitted Intensity (I)	Percent transmission $I/I_O \times 100$	Absorbance $\log I_O/I$
100	100	100%	0
100	50	50%	0.3
100	10	10%	1.0
100	1	1%	2.0

Relationship between transmission and absorbance

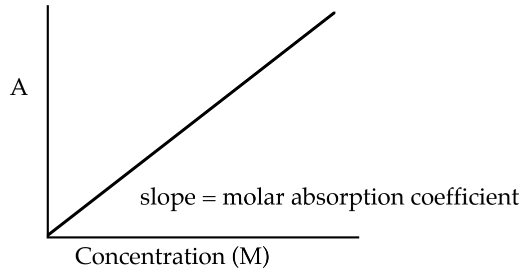
The reason why absorbance is useful is simply that it is directly proportional to the concentration of the substance that absorbs the light (NADH in our example). The absorbance is also directly proportional to the distance travelled by light through the sample, the path length – in practice the width of the spectrophotometer cell (cuvette)[4]. Absorbances much higher than 2 are difficult to measure, since less than 1% of the incident light is available to be detected by the instrument's photomultiplier. (Note that some instruments claim linearity to absorbances of 3 and beyond, but such values should be treated with caution.)

The constant that relates absorbance to concentration and path length is called the **absorption coefficient**, or sometimes the **extinction coefficient**. When concentrations are in units of moles per litre (molarity), we have a **molar absorption coefficient** that is symbolised ε_M. (The "ε" is the Greek letter epsilon). We can express this relationship as an equation, where A = absorbance, c = concentration (mol l^{-1}) and l = width of cell (cm). This expresses the **Beer-Lambert law**.

$$A = \varepsilon_M \cdot c \cdot l$$

The units of ε_M are $M^{-1} \cdot cm^{-1}$ (that is, litre$\cdot$mol$^{-1} \cdot$cm^{-1}). For NADH, the value of ε_M is 6220 $M^{-1} \cdot cm^{-1}$.

The Beer-Lambert law can also be represented as a straight-line graph of A *vs* c. The slope is then ε_M.l. It is most common to use a 1-cm wide cell, and in that case the slope is numerically ε_M.



Graphical representation of the Beer-Lambert law

N.B. The absorption coefficient of any substance varies with wavelength, so the Beer-Lambert law only holds for monochromatic light.

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[4] The reason why absorbance (a logarithm) is linearly proportional to concentration and width of cell (path length) is that intensity of the transmitted light falls off exponentially as it passes through the absorbing solution.

Values of ε_M are known (and can be looked up) for many substances of biological interest, or they can be determined by making up accurately solutions of the pure substance. It is then a simple matter to determine the concentration of the substance in a solution from its absorbance – for example, if a solution of the amino acid tryptophan has an absorbance of 0.167 in a 1 cm cell at 280 nm, we may calculate the concentration from the Beer-Lambert law, given that ε_M at 280 nm for tryptophan = 5580 litre·mol^{-1}·cm^{-1}:

$$A = \varepsilon_M \cdot c \cdot l$$

Rearranging:

$$c = A/\varepsilon_M \cdot l$$

So

$$c = 0.167/5580 = 3.0 \times 10^{-5} \text{ mol } l^{-1}$$

Some practical points about measuring absorbance

1. Spectrophotometer cells can be made of glass or synthetic polymers (such as polystyrene) and will work down to about 260 nM (the wavelength at which nucleic acids have peak absorbance). Polystyrene can easily warp though and so it is conventional to use quartz cuvettes to measure absorbance of wavelengths in the UV (Ultra Violet) range. The surfaces must be flat and clean and be separated by an accurately known path length (often 1 cm). Quartz cells are both fragile and expensive, so take care if you should need to use them! Only handle cells by their opaque surfaces.
2. It is usual to set the spectrophotometer to read zero absorbance using a reference cuvette that contains solvent only. This is because light losses due to the cuvette material itself and to the solvent need to be allowed for in order to determine the true absorbance of a solute in the sample solution.
3. For any particular instrument, make sure that you understand the arrangement for “data output”. A spectrophotometer and linked recorder can usually be set at different sensitivities (as well as allowing either absorbance or transmission to be displayed). Typically, you will use a recorder set up so that the full width of the chart paper corresponds to 1 absorbance unit. The speed at which the recorder moves the chart paper can also be varied – you might well use a speed of 0.5 or 1 mm per second. Alternatively, the data may be displayed on a screen on the spectrophotometer. In this way, you can follow the rate of an enzyme-catalysed reaction that is consuming or producing NADH by measuring the change in A_{340} with time. You will encounter this application in the practical course.
4. It is common to measure absorbance using 96-well format instruments, that may operate at pre-set wavelengths determined by optical filters in the light path. On more sophisticated instruments, wavelength can be varied using an inbuilt diffraction grating monochromator. In the Lent term, you will use ELISA methodology that uses a simple 96-well plate reader and provides absorbance values for each well. Normally, blank wells or wells containing controls will provide a background that can be subtracted from other sample readings. The multi-well format has the great advantage that it allows a range of standards to be measured alongside the test samples.

Weeks 1-4 (Michaelmas) – Basic Techniques in Molecular Biology

These first four practicals of the course occupy weeks 1 to 4.

Objectives

We intend to give you some "hands on" experience of some of the simpler procedures that are central to modern molecular biology and are covered in the lectures by Drs Scadden and Mata, and Professor Smith this term. You will apply these to the study of the DNA binding domain (the POU domain, NB rhymes with cow not moo!) from a transcription factor called Oct-1. You will use PCR to amplify the DNA corresponding to the POU domain from a longer template and incorporate a different restriction site at each end to allow directional cloning. You will then cut the PCR product with suitable restriction enzymes and ligate it into a vector for blue/white screening. The following week you will express the POU domain in *Escherichia coli*. The expression plasmid is called pMAT8 and contains the DNA encoding the POU domain as a fusion protein with maltose binding protein (MBP) to aid solubility and a hexahistidine tag $(His)_6$ for purification. The fusion protein is under the control of a promoter recognized by the RNA polymerase from bacteriophage T7, whose expression is in turn switched on in the presence of lactose or its stable analogue isopropyl β-D-1-thiogalactopyranoside (IPTG). In week 3, you will purify the protein that you expressed the previous week, using an affinity column and in the final week you will use electrophoresis mobility shifts assays (EMSA) to characterize DNA binding by the POU domain.

In a different type of experiment, in week 2, while you are waiting for the protein to express, you will study the melting of eukaryotic DNA and factors that influence the stability of the double helix.

At the end of these practicals you should be able to perform the following tasks:

- Perform a PCR (polymerase chain reaction) to amplify a desired section of DNA.
- Digest the PCR product with restriction endonucleases and clone the resulting fragment into a vector.
- Express a recombinant protein in *E. coli* and purify it using a Ni^{2+}-affinity column.
- Use your purified protein to perform a gel shift assay to investigate its DNA-binding properties.
- Use spectrophotometry to investigate the melting of nucleic acid double helices by changes in light absorption at 260 nm.

Summary of Schedule

Week 1

- Use PCR to amplify OctI POU domain coding sequence cDNA
- Clone amplified fragment into pBluescript plasmid

Week 2

- Express His-MBP-POU fusion protein in *E. coli* cells
- Study DNA melting

Week 3

- Purify His-MBP-POU using immobilized metal affinity column
- Estimate the amount of protein produced using a Bradford Assay

Week 4

- Assess DNA binding by the Oct1 POU domain in an Electrophoresis Mobility Shift Assay (EMSA)

Week 1

There are several steps in the practical today:

1. PCR to amplify the POU domain from a template DNA molecule that encodes the domain.
2. Incubate the PCR product with restriction enzymes to generate sticky ends.
3. Separate and visualise the digested PCR products by agarose gel electrophoresis
4. Ligate the digested PCR product into a plasmid vector for analysis.

Week 1 – Introduction and background

PCR

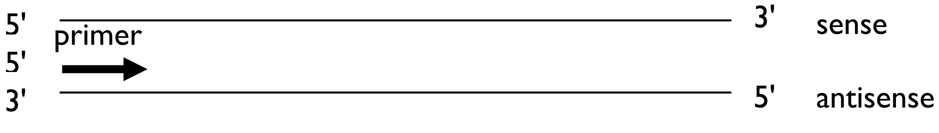
PCR is based on three simple steps: (1) ***denaturation*** of the template DNA into single strands; (2) ***annealing*** of oligonucleotide primers to each original strand for new strand synthesis; and (3) ***extension*** of the new DNA strands from the primers.

PCR achieves the repeated synthesis of new DNA strands, exponentially amplifying a defined region of the starting material, and allowing the birth of a new technology that has revolutionised daily life in labs and hospitals. To achieve multiple rounds of synthesis, the DNA templates must be repeatedly denatured, which requires temperatures well above those that inactivate most enzymes. A heat-stable DNA polymerase from a thermophilic bacterium, *Thermus aquaticus (Taq)*, is often used in PCR. The polymerase chain reaction is discussed in some detail in Dr Scadden's lectures and handout. Please consult that for further background information.

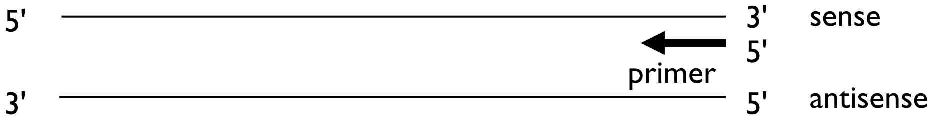
A reminder about primer design for PCR:

The success of any PCR reaction is dependent upon the specificity of primer hybridization to the targeted site with respect to non-specific regions of the input DNA. The most important aspect of primer design is to ensure that the sequence chosen for the primer is unique i.e. that the primer anneals to a single site in the DNA template. These days, one simply searches the DNA database for homologous sequences (as you will be doing in the Bioinformatics computer practical).

To amplify double stranded DNA it is necessary to have one primer for each strand. PCR primers are DNA (unlike in DNA replication in the cell, where RNA primers are used). The sense strand of a gene is identical to the mRNA sequence (with T in DNA corresponding to U in RNA of course) and is **by convention written 5'→3'** when describing a gene. The antisense strand is complementary to the sense strand and often is not written out at all in a gene sequence. To amplify the sense strand the primer needs to be **identical** to the 5′ end of the sense strand; the antisense strand is used as the template for the DNA polymerase, such as *Taq,* included in the reaction mixture: →



Remember, DNA polymerase works in the **5′ → 3′ direction** *i.e* nucleotides are added to the 3′ end of the primer. To amplify the antisense strand, the primer is designed in the same way, but is now **identical to the 5′ end of the antisense strand**, with the sense strand being used as the template for the polymerase:



Finally, the primers are designed so that they anneal to the template DNA at similar temperatures.

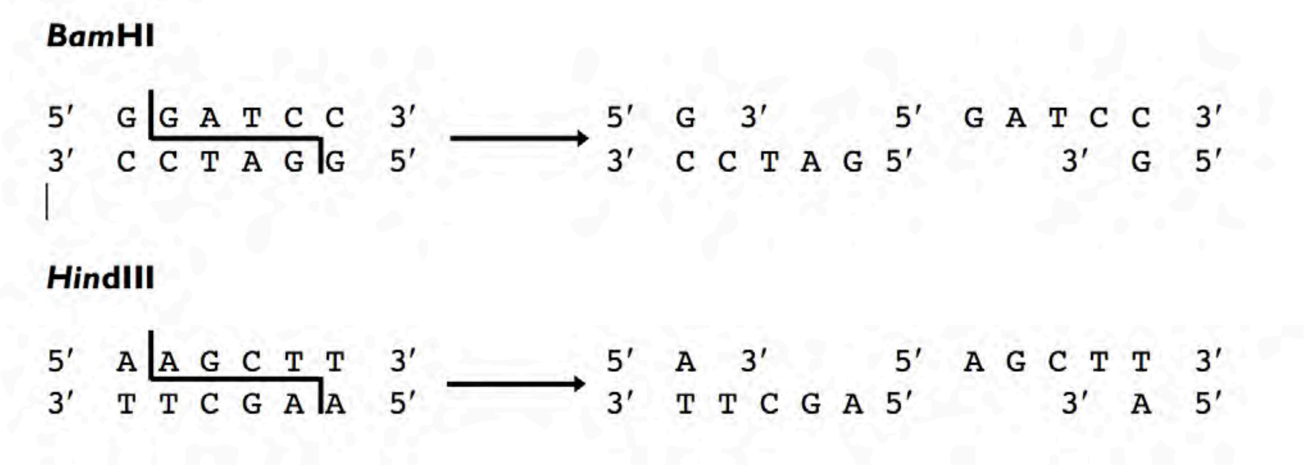
Restriction endonucleases

Restriction enzymes were so named because their presence in a number of bacteria restricts the ability of certain bacteriophages to grow in those bacteria. The enzymes do this by degrading the viral DNA before any harm can come to the cell.

You will be using two restriction enzymes today –

- *Bam*HI (isolated from *Bacillus amyloliquefaciens* strain H)
- *Hind*III (isolated from *Haemophilus influenzae* strain d)

The sequences they recognise and cut are:



Note that these recognition sites are palindromic, so that the resulting overhanging ends are identical when read from 5’ to 3’. Also notice that these recognition sites contain 6 specific bases, so they are expected to occur once in every 4^6 base pairs, or roughly 4 kbp.

Amplification of the POU domain

Today you are going to amplify the cDNA encoding the POU domain of Oct-1. cDNA is an abbreviation for complementary DNA, this means that the DNA is a double stranded DNA copy of the mRNA encoding the POU domain. Oct-1 is a ubiquitous transcription factor in mammalian cells that regulates transcription of some common genes. Oct-1 can also regulate the transcription of cell-specific genes, *e.g.* immunoglobulin genes in B cells, by recruiting co-activators (see Prof. Smith’s and Dr Mata’s lectures). Oct-1 is known to bind to DNA via a small domain of 162 amino acids called the POU domain.

The POU domain of Oct-1 encompasses amino acid residues 282 – 443. **Here is the cDNA sequence that encodes these amino acids:**

```
5′CCCAGTGACCTTGAGGAGCTTGAGCAGTTTGCCAAGACCTTCAAACAAAGACGAATCAAACTTGGA
TTCACTCAGGGTGATGTTGGGCTCGCTATGGGGAAACTATATGGAAATGACTTCAGCCAAACTACCAT
CTCTCGATTTGAAGCCTTGAACCTCAGCTTTAAGAACATGTGCAAGTTGAAGCCACTTTTAGAGAAGT
GGCTAAATGATGCAGAGAACCTCTCATCTGATTCGTCCCTCTCCAGCCCAAGTGCCCTGAATTCTCCA
GGAATTGAGGGCTTGAGCCGTAGGAGGAAGAAACGCACCAGCATAGAGACCAACATCCGTGTGGCCTT
AGAGAAGAGTTTCTTGGAGAATCAAAAGCCTACCTCGGAAGAGATCACTATGATTGCTGATCAGCTCA
ATATGGAAAAAGAGGTGATTCGTGTTTGGTTCTGTAACCGCCGCCAGAAAGAAAAAAGAATCAACCCA
CCAAGCAGTGGATA 3′
```

Often it is necessary to ensure that the PCR product contains DNA sequences at its ends that enable it to be cleaved by restriction endonucleases so that the amplified target sequence can be cloned into an appropriate vector. Any such extra sequences are added to the 5' ends of the primers so that they do not interfere with copying the target sequence (where nucleotides are added to the 3' end of the primer): the rest of the primer is designed as already described. As the extra sequences are included in the primers they will quickly be amplified even though they are not in the original target sequence template.

The primers that you will use for your PCR are thus (restriction enzyme recognition sites in bold):

POU Forward (5' primer)

5'- CGC**GGATCC***CCCAGTGACCTTGAGGAGCTTGAGC*-3'

POU Reverse (3' primer)

5'- CGC**AAGCTT***TATCCACTGCTTGGTGGGTTGATTC*-3'

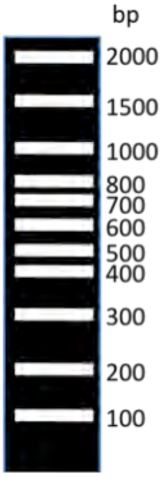
The region of the forward primer in italics is identical to the underlined sequence at the 5' end of the POU domain DNA sequence. The region of the reverse primer in italics is complementary to the underlined sequence at the 3' end of the POU domain sequence.

Measuring sizes of DNA molecules.

The most convenient way to measure the size of a DNA molecule is by gel electrophoresis. A DNA molecule is uniformly covered with negative charges and will move towards the positive electrode during electrophoresis. The small molecules thread their way through the gel matrix more easily than the large ones and so will move faster through the gel.

The gel matrix you will be using is agarose. This is a highly purified form of agar, with its physical and electrophoretic properties carefully defined. The DNA samples are loaded into wells at one end of the gel, which is then 'run' in an electric field to separate the fragments. Traditionally, a dye, ethidium bromide, is included in the gel. This is a flat, polycyclic molecule capable of fitting between the bases in the DNA (intercalation). Intercalated dye fluoresces much more strongly under UV light than free dye, so the position of DNA molecules in your gel can be easily determined. In this practical however, **GelRed** will be used to stain the nucleic acids in your gel. GelRed is structurally closely related to ethidium bromide, and consists of two ethidium subunits that are bridged by a linear spacer. Its fluorophore, and therefore its optical properties, are essentially identical to those of ethidium bromide. When exposed to ultraviolet light, it will fluoresce with an orange colour that strongly intensifies after binding to DNA. The substance is a **less toxic** and **more sensitive** alternative to ethidium bromide.

You will also include a sample of 'standard' sized DNA molecules to use as a size marker. In this case you will use a 100 bp ladder, which covers a convenient range of molecular weights.

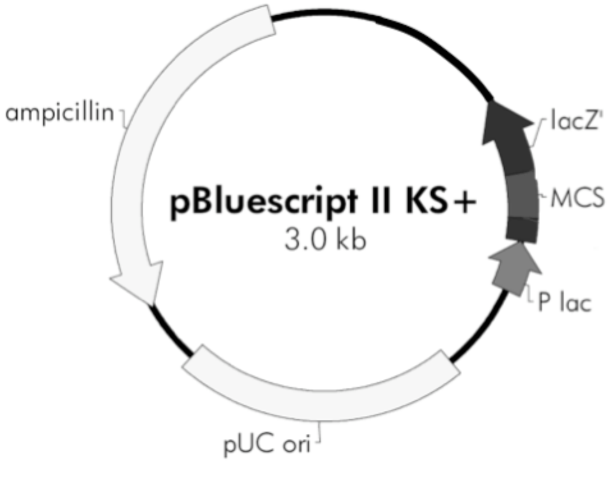


A graph of mobility against log of fragment size gives a straight line over a fairly wide range, so you can estimate the size(s) of fragments in your PCR products by comparison with the standards.

Note that this all relates to linear molecules. Circular molecules migrate rather anomalously in agarose gels, **so that you cannot use the mobility of an intact plasmid as a quantitative measure of its size.**

Ligation of the PCR product into a plasmid vector

Plasmids are DNA molecules found in a wide range of prokaryotic and some eukaryotic cells. They are usually independent of the main chromosome, although some, like the F factor in *E. coli*, can become integrated into it. Almost all are circular. Sizes vary widely, from only 1 kbp up to hundreds of kbp. Most confer some useful phenotype, such as antibiotic production and/or resistance, the ability to kill other bacteria, resistance to heavy metal poisoning and the ability to break down unusual sugars or other organic compounds. Plasmids are also widely used in genetic engineering.
The plasmid vector into which you will clone your POU domain fragment is called pBluescript. Here is a vector map:



The important features are marked on the map:
MCS: a multiple cloning site (see below)
ampicillin: the gene for ampicillin resistance
pUC ori: an origin of replication that allows the plasmid to be maintained independently in bacteria
P lac: the promoter for the *lac* operon
lacZ': one of the *lac* operon genes that encodes an enzyme called β-galactosidase.

The **MCS** is a cluster of restriction enzyme recognition sites, which in this plasmid includes sites for *Bam*HI and *Hin*dIII. If the PCR product and the vector are both cut with these two enzymes, they will have complementary sticky ends, which should anneal to each other. When this happens there will be a gap left in the DNA backbone at the joining site and you will use another enzyme called DNA ligase in the reaction to seal that gap – hence the whole operation is called a ligation. When you have successfully ligated the POU domain fragment into the vector, the resultant plasmid will have the lacZ' gene interrupted by the extra piece of DNA. The success of your ligation can therefore be monitored by an assay for **lacZ'**. For this you will use a modified version of the sugar galactose called 5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside (abbreviated to X-gal), which, when cleaved by β-galactosidase gives rise to a blue product.

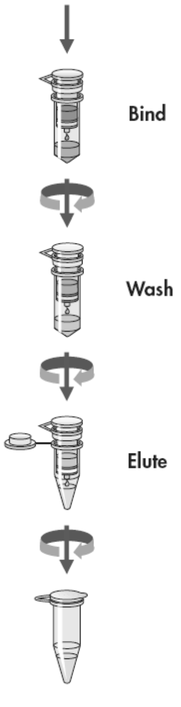
Purifying DNA after PCR and restriction enzyme digests

After the PCR you will purify the amplified DNA fragment to remove enzymes, primers up to 40 nucleotides long and dNTPs. For this you will use a QIAquick spin column®. This is a silica membrane assembly that efficiently binds single or double stranded DNA of more than 100 bp in a high salt buffer that contains a high concentration of chaotropic salts. Chaotropic agents disrupt the hydrogen-bonding network of water, destabilizing the DNA-water hydrogen bonds. The DNA can then establish binding interactions with the silica surface. Washing with a high percentage of ethanol removes unbound impurities, while enhancing the DNA binding. The DNA is then eluted with a low-salt buffer.

The same procedure is followed after the restriction enzyme digest, in this case to remove the small fragments that the restriction enzymes have removed from the PCR products as well as the enzymes themselves.

The QIAquick Spin Purification Procedure

(PCR reaction or restriction digest)



Buffer PB

Buffer PE

Buffer EB

Pure DNA fragment

Transformation of ligated DNA

After the ligation, it is necessary to amplify the successful plasmid products. The most efficient way to do this is to put the plasmid into bacteria by a process called *transformation*, and let them replicate the plasmid as they grow. *E. coli* do not readily take up plasmids unless they are made *competent* to do so. A common way of making *E. coli* competent is to treat them with $CaCl_2$, which works by an unknown mechanism to permit them to take up DNA when they are subjected to a heat shock. The bacteria can then be plated onto a medium containing the antibiotic present in the plasmid, to select only those that have taken up the plasmid.

Week 1: PCR and Cloning – Experimental

This part of the practical will run over two weeks: this week you will generate a fragment using PCR, which you will then ligate into the plasmid vector pBluescript. You will then plate out the ligation products on an agar plate that will be incubated at 37 °C overnight and then stored at 4 °C until next week, when you will be able to estimate the efficiency of the transformation (number of colonies) and the ligation (number of blue colonies).

It is critical that you label your plates clearly (with the day that you do the practical, your name, and your bench number) so they can easily be identified next week.

PCR

The stock reagents for the PCR reaction are as follows:

10x PCR buffer**:** 100 mM Tris-HCl, pH 9.0, 15 mM $MgCl_2$ and 500 mM KCl.
Primer 1 (POU Forward) – 10 μM
Primer 2 (POU Reverse) – 10 μM
DNA template – 0.2 mg/ml
dNTPs (mix of dATP, dGTP, dCTP, dTTP) – 2.5 mM
Taq DNA polymerase

Key to buffer and reagent solutions:

- **DNA** – DNA template for PCR
- **CT** – 10x PCR buffer, 2.5 mM dNTPs, ddH_2O
- **P** – POU F, POU R

***Remember**: the PCR primers have different restriction sites on the ends – so when the PCR products are cut, this will give a sticky ended product for directional cloning,*

Set up your PCR reaction in a **0.2 ml** centrifuge tube (**labelled with your bench number**), which contains the following:

1. **37 μl of CT, which is comprised of:**

   5 μl 10x PCR buffer
   4 μl dNTPs (200 μM final concentration)
   28 μl distilled, deionised (dd) H_2O
   Total 37 μl

2. **10 μl of the primer mix (P), which is comprised of:**

   5 μl POU F (1 μM final)
   5 μl POU R (1 μM final)

3. **2.5 μl plasmid DNA that contains the POU cDNA ("DNA")**

The final volume should be 49.5 μl.

A demonstrator will then add 0.5 µl of Taq DNA polymerase. Mix gently and **briefly** spin in the benchtop microcentrifuge (1-2 sec), using an empty 1.5 ml tube with the lid removed as a carrier for the PCR tube and a second empty tube as a balance.

When your PCR reaction is set up, put your tube in the thermocycler and run the PCR using the following program, which will already be set up for you:

95 °C 1 min, followed by:
95 °C 20 sec (to denature the DNA)
58 °C 20 sec (to anneal the primers to the template)
72 °C 20 sec (to allow the polymerase to copy the DNA)
for 15 cycles then
72 °C 3 min

The PCR cycles will take approximately 45 minutes to complete – this is a good time to go for lunch.

Purify the PCR product using a QIAquick spin column

Key to buffer and reagent solutions:

- **PB** – contains guanidine hydrochloride (a chaotropic agent) and propan-2-ol
- **PE** – wash buffer containing a high percentage of ethanol
- **EB** – 10 mM Tris-HCl, pH 8.5

Safety note: wear gloves and take care when handling buffer PB as guanidine hydrochloride is a severe irritant.

1. Transfer your PCR reaction to a 1.5 ml centrifuge tube, then add 250 µl PB Buffer and mix. Check that the colour of the mixture is yellow. PB contains a pH indicator, which will change colour to orange or violet if the pH is above 7.5. **If this happens, consult a demonstrator, as DNA will not stick to the column at high pH. A small amount (1-2 µl) of 3 M Sodium acetate, pH 5.2, will be added to reduce the pH to <7.5.**
2. Place the QIAquick spin column in a 2 ml collection tube
3. Apply your sample carefully to the QIAquick column. Put the column and the collection tube into the benchtop microcentrifuge, using a microfuge tube containing a similar volume of water as a balance, and centrifuge for 60 sec. The DNA should bind to the column.
4. Discard the flow-through (*i.e.* the contents of the 2 ml collection tube) into the waste pot provided. Place the QIAquick column back into the same tube.
5. Carefully add 0.75 ml PE Buffer to the QIAquick column. This will wash off any excess unbound material. Centrifuge for 60 seconds as in step 3.

Discard the flow-through and place the QIAquick column back in the same tube. Centrifuge the column for 60 seconds as in step 3. ***NB: residual ethanol from PE Buffer will not be completely removed unless the flow-through is discarded before this centrifugation.***

6. Place the QIAquick column into a new, clean 1.5 ml tube. Make sure that you cut the lid off before spinning in the microcentrifuge.
7. To elute the DNA, carefully add 50 µl EB Buffer to the centre of the QIAquick membrane, let the column stand for 1 minute (use a timer), and then centrifuge for 1 min as in step 3.

Digest the PCR product using restriction enzymes

Key to buffers:

- **CutSmart™** (supplied as 10x) 1x Buffer: 50 mM Potassium acetate, 20 mM Tris-acetate, 10 mM Magnesium acetate, 100 µg/ml BSA
- **LB** – 6x Loading Buffer: 60 mM Lithium acetate, 60 mM Boric acid (final concentration 10 mM Lithium acetate, 10 mM Boric acid, pH ~6.7), 60% (v/v) Glycerol, 0.03% (w/v) bromophenol blue

Add the following to a **1.5 ml** microfuge tube:

15 µl purified PCR product (from above)
5 µl of 10x restriction enzyme buffer (CutSmart™)
28 µl H_2O
Total 48 µl

A demonstrator will add 1 µl *Bam*HI and 1 µl *Hin*dIII to the tube. Mix gently and briefly spin in the benchtop microcentrifuge using an empty tube as a balance. Incubate the tube in the 37 °C heat block for 30 – 45 min.

Meanwhile, check the size of the PCR amplified DNA on a 0.8% agarose gel

- Add 5 µl of the purified PCR product to a new microfuge tube.
- Add 4 µl 6x Loading Buffer. This contains glycerol to make the sample dense and stop it diffusing out of the well into the buffer, and a blue dye (bromophenol blue) to help you judge how far the electrophoresis is going.
- Add 11 µl of H_2O to give a **final volume of 20 µl.**

You should also have a tube containing 20 µl of the 100 bp DNA ladder (labelled M1), which contains multiple DNA fragments of known size.

Loading the gel is the most difficult part of the whole practical – if in doubt about the loading, ask a demonstrator for help. It is best to use **2 adjacent tracks in the centre of the gel,** rather than at the side. Draw up the contents of one tube into a Gilson tip. Without pressing down on the plunger, poke the tip underwater into a well in the gel (over one of the red lines). **Do not jab the tip into the gel itself, keep it in the well.** Then gently depress the plunger to deliver the sample slowly into the well. Don't worry if it won't all go in.

Connect up the gel apparatus, with the negative terminal (black) at the end where you loaded the gel. The gel contains the same electrophoresis buffer that it is submerged in (10 mM Lithium acetate, 10 mM Boric acid, pH ~6.7). Switch on and run at ~150 V on constant voltage until the bromophenol blue dye has moved to the first coloured line in the bottom of the gel tray. This should take approximately 30 – 45 minutes.

Purify the restriction digested DNA using Qiaquick spin column (repeat of the procedure you performed on previous page)

1. Add 250 μl Buffer PB to the restriction reaction and mix. Check that the colour of the mixture is yellow.
2. Place a QIAquick spin column in a 2 ml collection tube.
3. Apply the sample to the QIAquick column and centrifuge for 60 sec (**balance!**).
4. Discard flow-through. Place the QIAquick column back into the same tube.
5. To wash, add 0.75 ml PE Buffer to QIAquick column and centrifuge for 60 sec.
6. Discard flow-through and place the QIAquick column back into the same tube. Centrifuge the column for an additional 1 min.
7. Place the QIAquick column in a clean 1.5 ml microfuge tube.
8. To elute DNA, add 50 μl EB Buffer to the centre of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.

Set up ligations using digested DNA

Key to buffers:

- **L** – 10x Ligation buffer. (**1x buffer composition**: 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT).
- **TG1** – TG1 *E. coli* competent cells
- **BS** – pBluescript plasmid
- **UC** – Uncut plasmid 20 ng/μl – **control**

The pBluescript plasmid has already been cut for you and the ends have been treated with alkaline phosphatase. This removes the 5' phosphate groups and reduces the chances that the plasmid will ligate to itself (*intramolecular ligation*). Even though this has been done, it is standard procedure to perform a control ligation with no insert DNA. Set up three 1.5 ml tubes (called Negative Control, Insert and Positive Control) and add the following to them:

Negative Control	**PCR Product Ligation (Insert)**	**Positive Control**
2 μl 10x ligation buffer (L) 2 μl pBluescript plasmid 5.5 μl H_2O	2 μl 10x ligation buffer (L) 2 μl pBluescript plasmid 2 μl digested DNA 3.5 μl H_2O	1 μl uncut plasmid (UC) 9.0 μl H_2O

The demonstrator will add 0.5 μl T4 DNA ligase to each of your tubes (except the positive control). Mix gently and spin down as before and leave at room temperature for at least 30 minutes. While the ligations are taking place, put the agar plates in the 37 °C oven to ensure that they are dry (hence spreadable).

Meanwhile, switch off the agarose gel when it has finished running. Remove gel (on plastic tray) from the gel tank and place it in a plastic tray provided to carry it to the UV-light box. Your gel will then be placed on the UV-light box to visualize the DNA. DNA is readily visualized as the gel contains GelRed.

Note: GelRed is more sensitive and less toxic than Ethidium Bromide. Nevertheless, do wear gloves when handling the gel, and avoid getting GelRed on your hands (or anywhere else). Note that any spills or drips from either your gel or running buffer should be cleaned up immediately with tissue.

A demonstrator will take a photograph of the gel. How big do you expect the PCR product to be? Is it the correct size? **Use the semi-log paper provided to determine the size of your PCR product.**

Transformation of *E. coli* TG1 cells

In experiments with bacteria you must avoid contamination from bacteria in the air. Aseptic techniques for the following procedures will be demonstrated to you. **In addition, ensure that you have your lab coat buttoned and that you wash your hands with soap before leaving the laboratory.**

The TG1 cells to be used for transformation are kept on dry ice and will be brought to the lab during the afternoon. Thaw in your ice bucket just before they are needed.

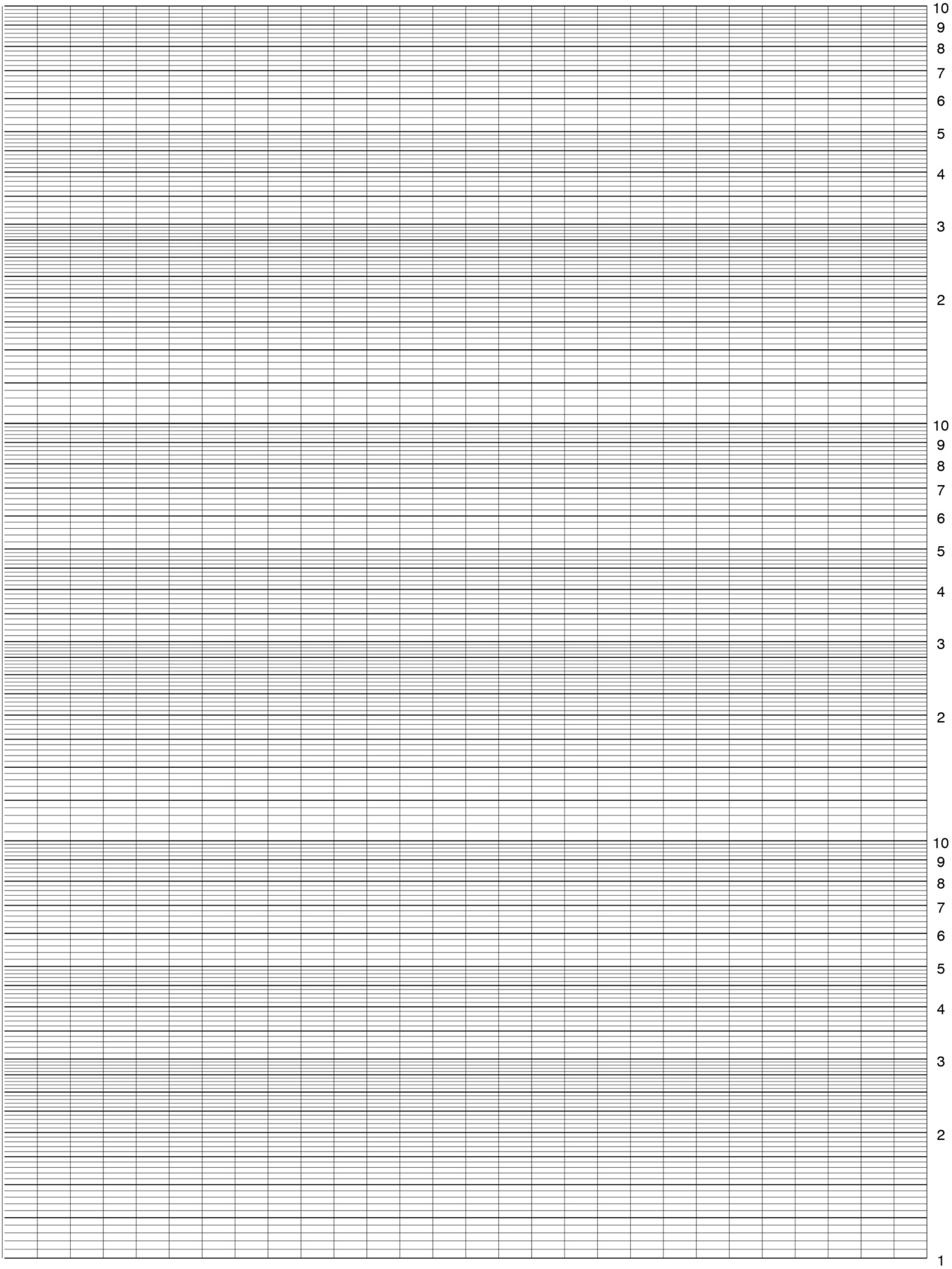
Take each of your samples (ligations and control) and put them into your ice bucket. Taking care to keep everything on ice, carefully add 100 μl of TG1 competent cells to each tube and mix **gently** by shaking the tube from side to side. **DO NOT VORTEX**.

Incubate the tubes on ice for at least 15 minutes.

Heat shock the cells by incubating them in the 42 °C heat block for **EXACTLY** 1 minute.

Using a Gilson pipette, transfer the transformed bacteria onto the surface of three agar plates (labelled + or – Control and Insert). Then, using a new sterile plastic spreader for each plate, gently distribute the cells across the whole of the plate. Be sure to keep the spreader flat, so that you do not damage the agar surface and try to keep the plate as sterile as possible. Ask the demonstrators to show you how to do this. The plate contains ampicillin, 0.1 mM IPTG to induce the *lac* promoter and 40 μg/ml X-gal for blue/white selection).

Once you have put the lids back on the plates, turn them over so that the agar is on top. This prevents condensation from the lid dripping onto the plate surface. Your plates will be incubated overnight at 37 °C and then stored at 4 °C for you to look at next week. **Make sure they are labelled with your (a) bench number, (b) your name and (c) the day that you do your practical**, as well as whether it is the positive control, the negative control (i.e. vector alone) or the real ligation (i.e. vector plus insert).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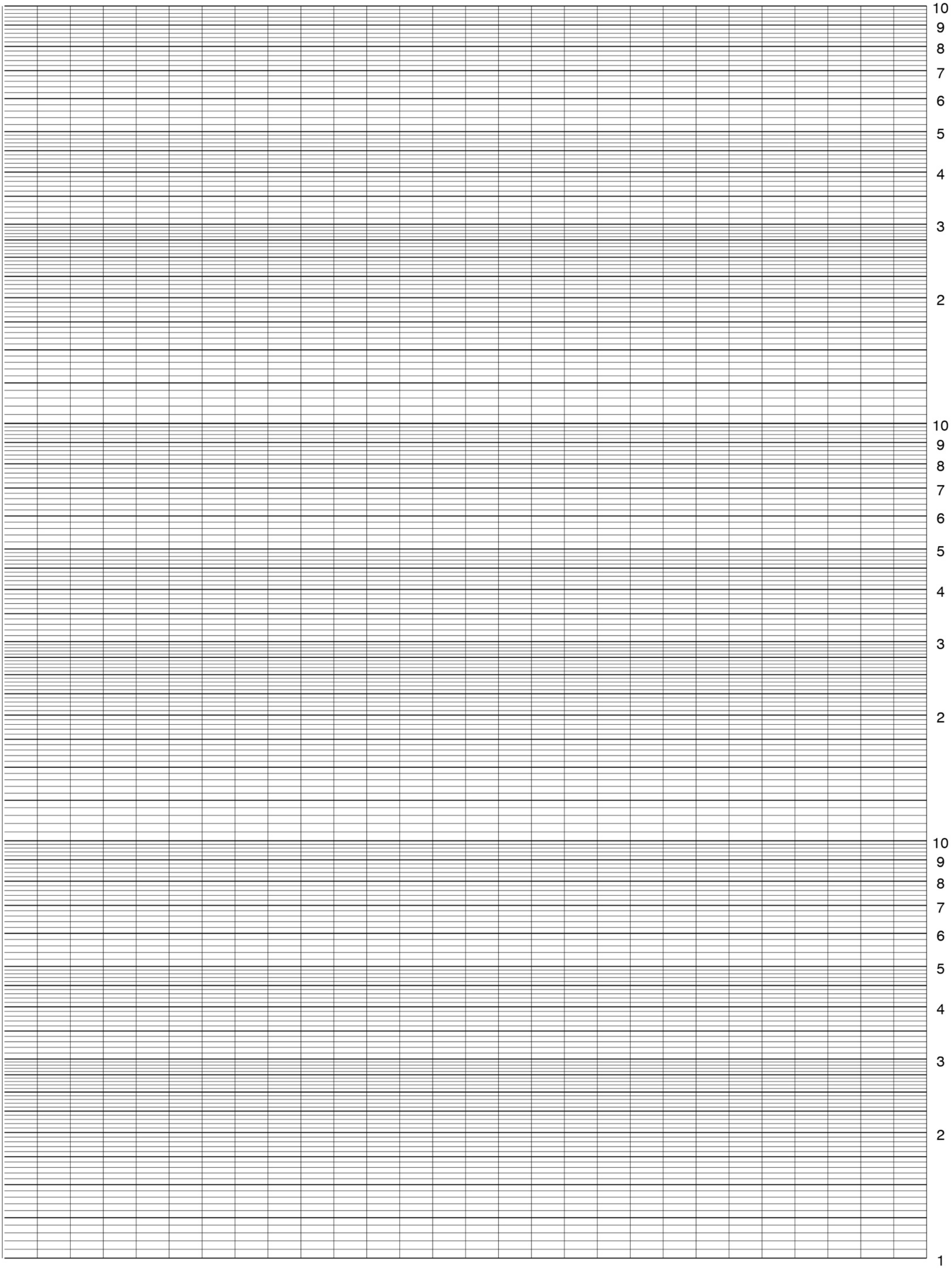
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Notes

Week 2

There are three parts to the practical today:

1. Expression of the POU domain in *E. coli*.
2. Assessment of the efficiency of the ligation that you performed last week.
3. Investigation of the melting of nucleic acids at high temperature.

Week 2 – Introduction and background

Protein Expression

The Oct-1 POU domain will be expressed in an *E. coli* strain called BL21(DE3). BL21 is often used for protein expression because it is deficient in two proteases (Lon and OmpT) that might otherwise degrade the recombinant protein, in this case to POU domain. The plasmid vector contains the POU domain coding sequence under the control of a promoter that is recognised by the RNA polymerase from the bacteriophage T7. This T7 promoter is used as it is small, the T7 RNA polymerase binds to and initiates within a 17 bp sequence, and has a high rate of initiation and processivity so it makes a lot of mRNA. T7 RNA polymerase is not normally present in *E. coli* and the (DE3) part of the name means that there is a DNA insert in the bacterial chromosome, which encodes the T7 polymerase under the control of a highly efficient promoter derived from the *lac* promoter so we can switch on its expression with IPTG, the lactose analogue. See Dr Scadden's lecture notes for a description of the other features of bacterial strains commonly used in molecular biology.

Expressing a foreign protein in large quantities is quite a strain for a bacterial cell and it is common for cell division to slow down once protein expression has been induced. For this reason, induction by IPTG is carefully timed, so that the bacterial cultures are in late log phase, that is, they are growing quickly but that they have not yet run out of nutrients in the medium. At this stage, there will be plenty of cells in the culture to produce protein. The density of bacteria in a culture can be estimated by measuring the apparent absorbance (technically turbidity due to the light scattering) at 600 nm in a spectrophotometer. Once they have been induced, the bacteria will be left to grow for three hours to express the protein. After this time, most of the nutrients in the medium will have been used up and there will be little extra protein produced.

Nucleic acid structures

All nucleic acids are composed of chains of nucleotides linked by phosphodiester bonds. The sugar-phosphate 'backbone' of the nucleic acid, from which the bases project, may be coiled in various configurations to give secondary structure to the molecules.

```
5'                               5'
deoxyribose 1'- adenine          ribose 1'- adenine
   3'                               3'
  /                                /
 P                                P
/                                /
5'                               5'
deoxyribose 1'- guanine          ribose 1'- guanine
   3'                               3'
  /                                /
 P                                P
/                                /
5'                               5'
deoxyribose 1'- thymine          ribose 1'- uracil
   3'                               3'
  /                                /
 P                                P
/                                /
5'                               5'
deoxyribose 1'- cytidine         ribose 1'- cytidine
   3'                               3'
  /                                /
 P                                P
/                                /


    single stranded DNA              single stranded RNA
```

In double stranded DNA, the form found in all prokaryotes and eukaryotes, two antiparallel strands are wound together to give a B-form helix, with the negatively charged sugar-phosphate backbone on the outside and the complementary base pairs (A–T, G–C) on the inside.

The base pairs are planar and roughly perpendicular to the axis of the helix. This double helical structure is stabilized by hydrogen-bonding between complementary bases and by hydrophobic interactions between adjacent base pairs (base stacking). In the absence of counterions, repulsion between negatively charged phosphate groups keeps the molecule in the extended form, so that DNA in solution behaves as an inflexible rod, hence the high viscosity of DNA solutions.

```
5'                                           3'
deoxyribose 1'- adenine  thymine - 1' deoxyribose
   3'                                           5'
  /                                            /
 P                                            P
/                                            /
5'                                           3'
deoxyribose 1'- guanine cytidine - 1'deoxyribose
   3'                                           5'
  /                                            /
 P                                            P
/                                            /
5'                                           3'
deoxyribose 1'- thymine  adenine - 1'deoxyribose
   3'                                           5'
  /                                            /
 P                                            P
/                                            /
5'                                           3'
deoxyribose 1'- cytidine guanine - 1'deoxyribose
   3'                                           5'
  /                                            /
 P                                            P
/                                            /

                 double stranded DNA
```

Most RNA molecules are single stranded. This does not mean that they lack secondary structure, but that secondary structure is a result of interactions within one molecule (i.e. intramolecular). The single stranded molecules fold up on themselves with limited base-pairing (A–U, G–C) occurring within a single molecule. The primary sequence of bases will determine the position and size of the stems and loops, with base-pairing occurring in the stem and non-base-paired regions at the ends as loops. In non-base-paired regions base stacking may occur which will stabilise the structure.

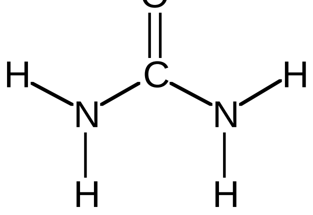
```
              U  G
             C    A
             U     U
              A - U
              U - A
              G - C
              G - C
              C - G
              U - A
              U - A
              A - U
......AGGCUGCAC - GGCGGAAAA......
```

All bases found in nucleic acids have a characteristic absorption of light in the ultraviolet region, and nucleic acids have a maximum absorbance at around 260 nm. Both hydrogen bonding and base-stacking interactions decrease this absorption by the bases. So the absorbance of intact, native, nucleic acid molecules is less than that of the nucleotides of which they are composed.

On disrupting the secondary structure of DNA either by heating or by exposing it to strongly alkaline pH, the absorption of light at 260 nm (A_{260}) rises to approximately 1.4 times its original value. This is known as the hyperchromic effect. The extra hydroxyl group on the 2' position in the backbone of RNA makes it sensitive to alkali hydrolysis, so changes in RNA structure are induced by heating.

The change in the structure of the DNA that produces this effect is a separation of the two strands (dsDNA to ssDNA). **No** hydrolysis of the backbone need take place in order to produce this increase in absorbance. The DNA is said to have **melted**; the transition occurs over a fairly narrow range of temperature due to the cooperative nature of the unwinding process (i. e. once a small region of dsDNA has separated to ssDNA the energy required for the next region to separate is reduced). The process is fully reversible under controlled conditions and the 're-annealed' DNA regains all physical and biological properties. The change in absorption at 260 nm of nucleic acids can be used to follow changes in the conformation of nucleic acids, for instance as a result of increasing temperature. Under standard conditions the temperature at which half the DNA is melted is known as the melting temperature (T_m). You might consider why the T_m can be used as an estimate of the percentage GC in any given DNA sample (this can vary between 30% and 70% depending on the organism of origin).

In the experiment today the effects of NaCl, $MgCl_2$ and urea upon the melting of dsDNA will be investigated. Also the melting of DNA and RNA will be compared. The structure of urea is shown below:



Week 2 – Basic Techniques in Biochemistry – Experimental

Check the bacterial cultures for the expression of the POU domain first and then investigate the DNA melting while you are waiting for the expression. You can count the colonies on your plates whenever you have a spare 10 minutes.

1. Expression of the Oct-1 POU domain in *E. coli*.

There is a 250 ml conical flask for each group in the orbital incubator. It contains 50 ml of LB (a rich medium for bacteria) and 100 µg/ml ampicillin. This flask was inoculated with 5 ml of an overnight culture of *E. coli* containing the plasmid that includes the DNA for the Oct-1 POU domain under the control of a T7 promoter.

1. When you are ready to start, check that the wavelength of the spectrophotometer is set to 600 nm. A demonstrator will show you how to use the spectrophotometer. Zero the spectrophotometer with a **plastic** cuvette containing 1 ml of LB (this is your blank).
2. Take the conical flask out of the incubator, remove 1 ml of culture and place it in a cuvette. Measure the absorbance of the culture at 600 nm. If the absorbance is between 0.6 and 0.8 go to step 3. If your absorbance has not yet reached the lower limit, put the flask back in the 37 °C incubator for a short time (consult a demonstrator to see how long) and then measure again. Discard the 1 ml of culture into Virkon disinfectant.
3. Once the absorbance of the cultures is 0.6-0.8, induce expression of the Oct-1 POU domain with 50 µl of 1M IPTG, under sterile conditions, and return to the 37 °C shaker for ~3 hours.
4. **After ~3 hours**, transfer ~35 ml of the culture into a centrifuge tube and pellet the cells in the benchtop centrifuge for 10 minutes at 4,000 rpm. Make sure you label the tube with your name, practical day and bench number. You will need another tube to balance the centrifuge, either by spinning at the same time as another group or by using a tube containing the same volume of water.
5. Carefully pour off the supernatant into a beaker containing Virkon. Make sure you do not disturb the pellet.
6. Transfer the remaining culture into the same tube and spin again for 10 minutes at 4,000 rpm, again ensuring that you balance the tube in the rotor appropriately. Pour off the supernatant into a beaker containing Virkon.
7. Place your tube on ice, making sure that it is clearly labelled with your name, practical day, and bench number. The pellet will be stored at -20 °C and returned to you next week.

In experiments with bacteria, ensure that you have your lab coat buttoned and that you wash your hands with soap before leaving the laboratory.

2. Assessing the efficiency of the ligation that you performed last week

You should have three plates (the positive and negative control plates and the ligation plate, which contains the products of the ligation containing insert) that you spread last week: they have been stored for you at 4 °C. Count the number of blue and white colonies on each of the plates (if you have too many to count, estimate the total by dividing the plate into 4 equal sections with a marker pen, then count the colonies in one quadrant and multiply by 4). Now calculate the percentage of blue and white colonies on each plate. How can white colonies arise on the control plate? How can blue colonies arise on the ligation plate?

3. Investigation of the unfolding of DNA and RNA at high temperature

Note the temperature of the heat block. Always use a thermometer; **do not rely on the thermostat setting**. If it is above 25 °C, turn off the heat block for at least 15 minutes, before turning it on again. The on/off button is at the rear of the machine.

You are provided with three of the following five buffers for the melting experiment:

1) TE
2) TE plus 4 mM sodium chloride.
3) TE plus 2 mM magnesium chloride.
4) TE plus 100 mM sodium chloride.
5) TE plus 2 M urea.

TE is 10 mM Tris-HCl, 1 mM EDTA, pH 7.5

Preparing the nucleic acid solutions

Dilute the stock DNA before making any measurement. You are given a stock of herring testis DNA solution of approximately 2 mg/ml in TE. Take 40 ml of each buffer and separately add 0.35 ml (350 µl) of stock DNA to each, mixing well. Take great care to mix well as the DNA solution is often viscous and **will not mix if you only give it a gentle swirl** – put the top of the tube on and invert a few times

You are also provided with a solution of yeast RNA at approximately 10 mg/ml in TE. Place 40 ml of TE buffer in a 50 ml tube, add your entire RNA stock solution (50–100 µl) to it and mix well.

Set up the spectrophotometer

Ask a demonstrator to show you how to use your spectrophotometer.

Using the plastic Pasteur pipette or 1 ml Gilson provided, transfer approximately 1 ml of TE buffer into a quartz cuvette to serve as a blank. Place the cuvette in the spectrophotometer. Ensure that the wavelength is set at 260 nm. This blank will usually be the same for all three buffers, but you should check that your other buffers have no absorbance at 260 nm – if either does, correct your later measurements accordingly.

Initial absorbance readings and denaturation of double-stranded DNA in alkali

Take a second quartz cuvette and add about 1 ml of DNA solution in TE. Place this cuvette into the spectrophotometer, and read the absorbance at A_{260}. It is also instructive to measure the absorbance of the DNA at 280 nm. **Do not forget to re-zero on the TE blank when you change the wavelength.** The ratio of A_{260}/A_{280} should be about 2. If the ratio is much lower than this, it indicates that the DNA is contaminated with protein. Nucleic acids have an absorption maximum at 260 nm, whereas proteins show an absorption peak at 280 nm, which is mainly due to the aromatic amino acids. Re-set the wavelength to 260 nm and re-zero with the TE blank.

Double-stranded DNA can be melted not only by heating, but also by alkali (at low temperature). To verify this, add 0.03 ml (30 µl) of 3 M NaOH, mix well, and repeat the measurement.

Remove the cuvette(s) from the spectrophotometer, and pour out the sample. **Take care to remove all the solution** including any large adhering droplets. Change solutions on the bench not in the spectrophotometer. This avoids the DNA solutions being splashed over the inside of the machine. Repeat the A_{260} measurement for the other nucleic acid solutions. The solutions should be in the range 0.3 to 0.6. If not, adjust the DNA concentrations accordingly. The A_{260} for RNA should be about 0.2.

Nucleic acid melting experiment

You are now ready to start the melting experiment. During the experiment **regularly check that the cuvette is clean**: the urea solution is concentrated and given the opportunity will crystallise out on the cuvette, so interfering with the readings. The procedure involves warming the solutions in a water bath, and taking A_{260} readings at noted temperatures. **Each reading takes 1 ml that you will throw away after the measurement, so plan how many readings to take – you don't want to run out of solution at 75 °C.**

Keep your cuvettes on top of the heat block so that they do not cool down and change the temperature of the solution. If you wash your cuvettes with H2O, you will cool them down so remember to reheat them by placing on top of the heat block, when they're dry, for at least 2 minutes.

Place each of your solutions into 2 ml microfuge tubes in the heatblock at the first temperature point (room temperature) and put a screw cap on to reduce evaporation. Adjust the heat block to the temperature you want by using the up/down keys at the front of the block. Start the block by pressing 'Start'. Measure the temperature by following temperature changes recorded by the thermometer placed in the heat block.

Record the temperature and immediately remove a sample and measure the new A_{260}. Discard this sample and repeat for each solution. Try to record the A_{260} at 10 °C intervals for each DNA sample and 5 °C intervals for RNA over the lower temperature range. Note that you cannot go down in temperature, so you must do the low temperature points first. When 60 °C has been reached continue to try to obtain readings at 5 °C intervals.

Always take care and try not to touch the heat blocks directly. Be very careful at higher temperatures as the gloves may melt onto your skin.

Plot your results as the experiment proceeds and estimate T_m from your graph for each of the solvents.

The most useful way is to use the starting A_{260} as A_0 and the A_{260} at temperature T as A_T then plot A_T/A_0 against temperature.

Linear graph paper

Linear graph paper

Notes

Notes

Week 3 – Introduction and background

Purification of His-tagged Proteins

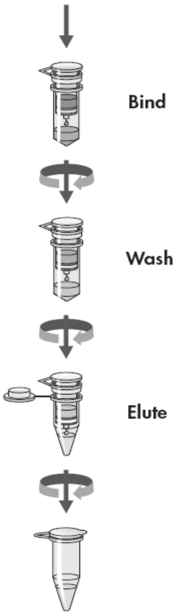
You have only one task in today's practical, to take the bacterial cell pellet from last week and extract your protein of interest in a relatively pure form.

The first task is to disrupt the bacterial cell walls to get at the protein inside. Many research labs use sonication (application of ultrasound) for this, but it is not suitable for a practical class. You will use a commercial solution called BugBuster® that is a mixture of detergents that perforate the cell wall without denaturing the proteins (which most detergents do). You will help the process along by adding the enzyme lysozyme, which as you know from IA Biology of Cells, breaks the peptidoglycan layer in bacterial cell walls. The final addition to the lysis buffer is the nuclease, DNase I. When the cell walls are disrupted and the contents of the cell are released the solution becomes very viscous due to the large amount of DNA present. DNase I cleaves the DNA into smaller pieces and prevents the high viscosity.

The protein that you expressed last week is a fusion protein that includes:

(His)$_6$ – maltose binding protein (MBP) – Oct1 POU domain

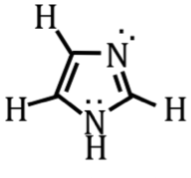
Both the His and MBP tags are present to aid protein purification but today you are going to utilise the His tag. At high pH, histidine sidechains chelate to metal ions, such as Ni^{2+} (why?). Ni^{2+} ions are immobilized on Sepharose, which is an inert, cross-linked polysaccharide polymer in the form of a small column. When the bacterial cell lysate is passed over the column, the His-tagged protein sticks to the Ni^{2+} and most other proteins, nucleic acids etc. will pass through. After washing to remove excess unbound material the His-tagged protein can be eluted from the Ni^{2+} by adding a high concentration of imidazole, which also binds tightly to Ni^{2+}. You are going to use a His SpinTrap® column today, which is suitable for small-scale protein production. It comprises 50 μl of Ni^{2+}-Sepharose in a small column that fits into a 1.5 ml microfuge tube and can be centrifuged.



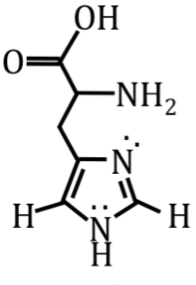
Wash buffer 1

Wash buffer 1 & 2

Elution buffer



Imidazole



Histidine

After the affinity purification, you will perform a Bradford assay to estimate the concentration of protein that you have produced. For this you will use the dye Coomassie

Brilliant Blue G250, which is **green-brown** when free in acid solution and becomes **blue** upon binding to protein. You will use the spectrophotometer to measure the intensity of the blue colour developed by a series of protein solutions of known concentration. This allows a standard curve to be constructed against which your samples can be compared and quantified.

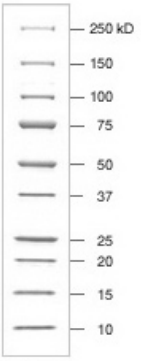
You will monitor the success of your protein purification using gel electrophoresis, where molecules are separated according to their size by applying a voltage across a fixed conducting medium. For large molecules like proteins (whose M_r values can lie between 1 kDa and 1 MDa), a porous medium of aqueous polyacrylamide is used. Polyacrylamide gels are prepared by dissolving acrylamide monomer, [CH_2=CH-CO-NH_2], and a cross linking agent called N,N'-methylenebis-(acrylamide), [(CH_2=CH-CO- NH-$)_2$ CH_2]. In the presence of a suitable catalyst, gels are formed, consisting of a three dimensional network of long chains of [-CH_2- CH_2 (-CO- NH_2)$]_n$- cross-linked at intervals by methylene groups.

The pore size of the gel can be modified by varying the acrylamide monomer concentration. For example, gels containing 5% acrylamide will separate polypeptides of 100-400 kDa M_r while gels containing 15% acrylamide separate polypeptides of 10-100 kDa M_r.

In the presence of the strong ionic detergent sodium dodecyl sulphate (SDS) CH_3-$(CH_2)_{11}$-O-SO_3- Na^+ proteins are completely unfolded. Most proteins bind equal amounts of anionic dodecyl sulphate per gram (1.4 g per g of protein) and so behave as polyanions with a constant ratio of negative charge to mass. Any inherent differences in charge due to the amino acid side chains are thus obscured and the proteins behave like rigid rods in solution. Provided that disulphide bonds have been reduced (by, for example, β-mercaptoethanol), multimeric proteins are also completely dissociated into their subunits by SDS.

During electrophoresis in a polyacrylamide gel that contains SDS, the proteins move at speeds determined **only by the size** of their SDS–protein complexes (i.e. length of polypeptide chain and therefore molecular mass). For a wide range of protein sizes, the speed of migration falls linearly **as the logarithm** of the M_r of the protein rises, because longer chains are more retarded by the gel medium.

You will run molecular weight markers at the same time as your unknown samples. Plotting the migration distance vs the log of M_r should yield a straight line that can be used to estimate the size of the proteins in the samples. Today, you will use the markers qualitatively, to help you figure out which band in the gel sample corresponds to your protein of interest.



Molecular weight markers used for the SDS-PAGE in today's practical.

Week 3 – Protein Purification – Experimental

Key to buffer and reagent solutions

BLD: BugBuster, containing 0.2 mg/ml lysozyme, 20 μg/ml DNase I, 20 mM imidazole

WB1: Wash Buffer 1: 20 mM sodium phosphate, pH 7.4, 500 mM NaCl

WB2: Wash Buffer 2: 20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 20 mM imidazole

EB3: Elution Buffer: 20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 500 mM imidazole

SB: 2x Sample Buffer: 0.26 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 40% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.05% bromophenol blue

PDB – Protein Dilution Buffer: 100 mM Tris-HCl, pH 7.5, 1.6 mM EDTA, 50 mM NaCl, 10% glycerol (N.B. MAY NOT BE NECESSARY).

Cell Lysis

Last week you made a pellet of cells that has been stored at -20 °C. These have been placed on ice on your bench.

1. Make sure that your pellet is **thoroughly** defrosted.
2. Add 1 ml of BLD to the centrifuge tube. Thoroughly resuspend the pellet by pipetting up and down using a Gilson pipette.
3. Using a Gilson pipette, divide the suspension equally into two microfuge tubes. Label them L1 and L2, there should be approximately 0.7 ml in each. Leave at room temperature for 30 minutes.

Preparation of His SpinTrap Column

Meanwhile, prepare the column for the purification of your protein:

1. The His SpinTrap column is in your microfuge rack.
2. Invert and shake the column to resuspend the beads in the storage buffer.
3. Loosen the cap 1/4 of a turn and break off the bottom closure. Cut off the lid from a 1.5 ml microfuge tube and place the column into the tube.
4. Place the column and microfuge tube into the microcentrifuge, using an empty tube as a balance tube. Press the pulse button on the microcentrifuge for 10-20 seconds to remove the storage buffer. Discard the flow-through in the microfuge tube into the beaker provided and not the sink! And replace the column.
5. Apply 600 μl of WB1 to wash the column.
6. Pulse for 30 seconds in the microcentrifuge and discard the flow-through in the tube. Your column is now ready for use.

Purification of the His-tagged protein

At various stages during the protocol you will take samples for analysis by SDS-PAGE. Label 1.5 ml microfuge tubes for the gel samples as follows: S1, S2, W1, W2, W3, W4, W5, W6, E1, E2.

1. Once the 30 minute lysis is complete, centrifuge your two lysate samples, L1 and L2, in a microcentrifuge for 10 minutes at high speed. **Make sure the His SpinTrap column is NOT in the microcentrifuge during this spin!** A pellet will form at the bottom of the L1 and L2 tubes.

2. Carefully remove the tubes from the microcentrifuge. Take 10 µl of the supernatant from L1 and add to the first gel sample tube, S1 and 10 µl from L2 and add to sample tube S2. Add 90 µl of 2x gel sample buffer (SB) to each of S1 and S2.

3. Carefully remove 600 µl of supernatant from L1 without disturbing the pellet and add it to the top of the His SpinTrap column. Put the column into the microcentrifuge, ensuring that it has an empty tube as a balance and is sitting in an empty tube to collect the flow-through. Pulse for 10-20 seconds as before. Discard the flow-through into a beaker containing a weak solution of Virkon. Remove 600 µl of supernatant from L2 without disturbing the pellet and add it to the His SpinTrap column. Pulse for 10-20 seconds and discard the flow-through into Virkon. If there is a small amount of supernatant left in L1 and L2, carefully remove it with a 200 µl Gilson pipette (yellow tip) taking care not to disturb the pellet, add it to the His SpinTrap column and pulse for 10-20 seconds, discarding the flow-through.

4. Wash the His SpinTrap column by applying 600 µl of WB1 to the top of the column and pulsing for 10-20 seconds in the microfuge. Take a 10 µl sample of the flow-through and add it to the gel sample tube 'W1'. Discard the remainder of the flow-through. Add 10 µl of SB to sample tube W1 and put the tube aside for analysis later. Repeat twice more, using sample tubes W2 and W3.

5. Wash the His SpinTrap column once more by applying 600 µl of WB2 to the top of the column and pulse for 10-20 seconds in the microfuge. Take a 10 µl sample of the flow-through and add it to the gel sample tube W4. Add 10 µl of SB to sample tube W4 and put the tube aside for analysis later. Discard the remainder of the flow-through. Repeat twice more, using tubes W5 and W6 for gel samples.

6. Place the His SpinTrap column into a clean 2 ml microfuge tube, discarding the previous flow-through and the tube into Virkon. Apply 200 µl of EB3 to the top of the column to elute the protein. Pulse in the microcentrifuge for 10-20 seconds. **Do not discard the flow-through (eluant).** Take the eluant and put it into a clean 1.5 ml tube (labelled Elution). Take a 10 µl sample of the eluant and add it to sample tube E1, adding 10 µl of SB. **Place the tube containing the eluant on ice.**

7. Place the HisSpinTrap column back into the tube. Apply another 200 µl of EB3 to the column and pulse in a microcentrifuge for 10-20 seconds. Take a 10 µl sample of the eluant and add it to sample tube E2 along with 10 µl of SB. Remove the remainder of the eluant and add it to the eluant from step 6.

8. **Place the pooled eluants on ice for the remainder of the practical.**

Analysis of the protein by SDS-Polyacrylamide Gel Electrophoresis

1. You should have 10 samples collected during the purification and you will have a microfuge tube marked M provided on your bench that contains protein molecular weight markers. Place all 11 samples into the 95 °C hot-block for 2 minutes making sure the safety caps are in place. Please leave the caps in the tray next to the heat block when you're done with your incubations.
2. Place the samples in the microcentrifuge and spin at high speed for 5 minutes.
3. Load 15 µl of each sample onto the gel into individual adjacent wells using a 20 µl Gilson pipette (yellow tip). Get a demonstrator to show you if you are unsure of how to do this. Load the samples in the order in which you collected them *i.e.* S1, S2, W1, W2, W3, W4, W5, W6, E1, E2 with M at end.
4. After making sure that the electrophoresis power supply is switched off, place the lid of the tank onto the base (taking care not to spill the buffer). Connect the power supply and the gel tank with the power leads. Switch on the power supply and adjust the voltage to 200 V and ensure that the switch is on constant voltage. Run the gel until the dye front just reaches the bottom of the gel (about 30 min).

Estimating the concentration of the protein produced

While your gel is running, you will measure the concentration of your protein using a Bradford assay. The change in colour is measured as Coomassie dye binds to protein and turns blue. A standard curve is constructed with known amounts of protein and the amounts of protein in the samples can then be estimated.

1. A stock solution of 20 mg/ml (=20 µg/µl) BSA (Bovine Serum Albumin) has been provided for you. First make two serial dilutions from this stock. Place 5 µl of 20 mg/ml BSA into a new 1.5 ml tube and add 95 µl of distilled water to make a 1 mg/ml solution. Now take 10 µl of the 1 mg/ml dilution into a new 1.5 ml tube and add 90 µl of distilled water to make a 0.1 mg/ml solution. Label the new tubes with their concentration.
2. Set up a series of 8 cuvettes, 6 of which will contain a different amount of BSA and the 7th and 8th to contain the sample you wish to test (this is the protein solution that you have stored on ice). Using the table below for quantities, add the water and the Bradford reagent to each cuvette first and finally the protein (BSA or test sample). Mix by covering the top of the cuvette with parafilm and inverting gently.

SAFETY NOTE: WEAR GLOVES WHILE HANDLING THE BRADFORD REAGENT AND MIXING THE TUBES

Final amount of BSA per tube (µg)	Volume of BSA needed	Volume of distilled water	Volume of Bradford reagent
0	0 µl	20 µl	980 µl
1	10 µl of 0.1 µg/µl	10 µl	980 µl
2	20 µl of 0.1 µg/µl	0 µl	980 µl
5	5 µl of 1 µg/µl	15 µl	980 µl
10	10 µl of 1 µg/µl	10 µl	980 µl
20	20 µl of 1 µg/µl	0 µl	980 µl
-	5 µl Sample	15 µl	980 µl
-	10 µl Sample	10 µl	980 µl

3. Leave the cuvettes to stand for 10 minutes.
4. Check that the spectrophotometer is set to 595 nm. Place 1 ml (1000 µl) sample of distilled water into a cuvette and zero the spectrophotometer. Now read the absorbance of your 7 samples, making a note of the readings in each case.
5. Plot a standard curve on the graph paper provided. Plot µg BSA on the x-axis and A_{595} on the y-axis. The plot should be linear within the range of BSA used. From the graph, read off the amount of protein present in the 5 µl sample in µg. Calculate the concentration of protein that you have purified in µg/ml.
6. **You will need 100 µl of an ~100 µg/ml protein sample for the band shift assay.**

   If your protein concentration is close to, or less than, 100 µg/ml (ask a demonstrator), you can use your solution directly in the assay. If your protein is too concentrated, you will need to make a dilution. For example, if your protein concentration is 500 µg/ml, you will need to add [(100/500) x 100] µl of protein solution (i.e. 20 µl) and then add PDB to make the volume up to 100 µl.

You must dilute your protein now. If it is frozen in too high a concentration it will precipitate out of solution once thawed and be ruined.

Staining and photographing the SDS-Polyacrylamide Gel

Once the blue dye has reached near the bottom of the gel **turn off** the power supply and then carefully remove the gel tank cover.

SAFETY NOTE: WEAR GLOVES WHEN HANDLING THE GELS. ACRYLAMIDE IS ABSORBED THROUGH THE SKIN AND IS NEUROTOXIC.

The gels are quite fragile so some care is required. Carefully remove the gel, sandwiched between glass plates, from the apparatus after removing the two clips which clamp it in place. Lay the gel sandwich on the bench (notched glass plate upwards) and gently prise the notched plate off by inserting a spatula between the glass plates, midway between the top and bottom of the plate and levering it up. The gel should stick to the lower plate with spacers.

Hold the glass plate with the gel upside down over the plastic tray and gradually peel off the gel with the spatula letting the gel fall into the plastic tray. Pour 10 ml of Instant Blue Stain into the plastic dish, then place the dish into the shaking waterbath. You should see the bands developing, stained a bright blue colour against a clear background.

Once the bands are sufficiently bright, discard the staining solution into the 'Waste Stain' pot. **Do not put it down the sink**.

A demonstrator will help you to photograph your gel.

Linear graph paper

Linear graph paper

Notes

Notes

Week 4 – Introduction and background

Electrophoresis mobility shift assay (EMSA)

This week you will take the protein that you purified last week and test its ability to bind to DNA. The Oct-1 POU domain binds to a specific 8 bp segment of DNA, ATGCAAAT, called the octamer, in certain promoters (see Dr Mata's and Prof. Smith's lectures). The POU domain itself is composed of two subdomains: the homeodomain (Pou_H) and the POU specific domain (Pou_S). Homeodomains use a helix-turn-helix motif to bind DNA and are found in several other proteins. The POU specific domain, as its name suggests, is only found in POU domain proteins. The structure of the complex of the Oct-1 POU domain with DNA has been solved by X-ray crystallography and it shows that both the subdomains bind to DNA. Today you will investigate the relative importance of the two binding sites for the affinity of the interaction.

Oct-1 POU domain binding to DNA. Pou_S is on the right and Pou_H is on the left. These subdomains bind on opposite faces of the DNA double helix and are connected by a flexible linker.

You will use the electrophoresis mobility shift assay (EMSA), also known as a gel shift or gel retardation assay, to investigate the binding of the POU domain to DNA. As you know already from the previous weeks, electrophoresis separates molecules on the basis of their size and charge. In the EMSA, you will look at the change in size when a small piece of DNA binds to the POU domain. The POU domain-DNA complex is larger than the DNA on its own and so the bound DNA is retarded in the gel. When a gel is run with free DNA and the complex DNA in separate lanes, it is possible to see the difference in the mobility of the bands. The migration of the DNA in the gel is detected by staining with Sybr Gold, a proprietary, asymmetric cyanine dye that exhibits >1000-fold fluorescence enhancement upon binding to nucleic acid – which is considerably (>x100) more sensitive than ethidium bromide (week 1).

It is important that the gel system used for EMSA is non-denaturing, otherwise the protein-DNA complex will not stay together while the gel is run. Thus SDS will not be included in the polyacrylamide gel or in the buffers this time.

The dissociation constant (K_d) is a useful measure to describe the strength of binding (or the affinity) between the protein (P) and DNA (D). The interaction between P and D can be described by the equilibrium:

$$P + D \rightleftharpoons PD$$

The balance between the concentration of the free protein ([P]) and the concentration of the bound complex ([PD]) depends on the strength of the interaction. At equilibrium, the dissociation constant is defined as:

$$K_d=[P][D]/[PD]$$

In practice, reactions are performed with an excess of protein, so the change in free protein concentration is negligible and the input protein concentration can be used as [P]. Then **the K_d equals the protein concentration when 50% of the DNA is bound.**

You will perform gel shift assays today with the following oligonucleotide sequences:

5′CCAGGGT**ATGCAAAT**TATTAAGGGC
3′GGTCCCA**TACGTTTA**ATAATTCCCG

The above is the so-called wild type (wt) sequence, with POU-binding nucleotides in bold.

You will compare POU binding to the wt sequence with one of three mutated dsDNA, altered as below:

Mutation 1 (Pou$_H$ mutation)

5′CCAGGGT**ATGCAA**GCTATTAAGGGC
3′GGTCCCA**TACGTT**CGATAATTCCCG

Mutation 2 (Pou$_S$ mutation)

5′CCAGGGTCG**GCAAAT**TATTAAGGGC
3′GGTCCCAGC**CGTTTA**ATAATTCCCG

Mutation 3 (1+2 combined)

5′CCAGGGTCG**GCAA**GCTATTAAGGGC
3′GGTCCCAGC**CGTT**CGATAATTCCCG

Or you will compare the wt with a random dsDNA of the same length.

These oligonucleotides were synthesized as single stranded DNA (just like the primers you used for PCR) and were annealed by heating to 85 °C and then slowly cooling.

Week 4 – Electrophoresis Mobility Shift Assay – Experimental

Key to buffer and reagent solutions

AB – 5x band shift assay buffer: 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5.

DNA – annealed oligonucleotides at a concentration of 0.58 μM.

LB – 10x gel loading buffer: 250 mM Tris-HCl, pH 7.5, 40% glycerol, 0.1% bromophenol blue.

Heparin – 1 mg/ml

You purified your POU protein last week and **should have** diluted it to a concentration of 100 μg/ml. This has been stored at -80 °C and is an ice bucket on your bench.

1. Label 6 microfuge tubes 1-6 and place on ice. Then, using the wt DNA, add the following from the table, adding the protein last:

Tube	**1** **7**	**2** **8**	**3** **9**	**4** **10**	**5** **11**	**6** **12**
DNA (µl)	2	2	2	2	2	2
AB Assay Buffer (µl)	3	3	3	3	3	3
Water (µl)	10	9	8	6	2	0
Protein (µl)	0	1	2	4	8	10

Do the same for the mutant/random DNA, this time with tubes labelled 7-12. We will allocate mutant or random DNA to each group. Some groups will add, to all of their 12 tubes, 1 µl of heparin, a highly-sulphated glycosaminoglycan.

2. Flick the tubes gently to mix, then briefly (1-2 sec) spin them in the microcentrifuge to make sure all reagents are at the bottom of the tube. Leave the tubes at room temperature for 30 minutes to allow the protein and DNA to bind.
3. Whilst your protein is binding, pre-run the gel. Mark the wells of the gel onto the glass plate, and remove the comb from the gel. Place the lid of the gel tank onto the base and connect the power supply to the gel tank. Switch on the power supply and adjust the voltage to 90 V, ensuring that the power supply is set to constant voltage. Run the gel for 10 minutes. This step removes all traces of ammonium persulphate (used to polymerize polyacrylamide gels) and ensures a constant gel temperature.
4. Once the 30 min protein-DNA incubation is complete, add 1 µl of LB to each tube, flick gently to mix then spin briefly in the microcentrifuge.
5. Make sure that the electrophoresis power supply is off, then remove the lid of the tank from the base, taking care not to spill the buffer. Load 10 µl of each sample onto the gel into individual adjacent wells using a clear tip. Get a demonstrator to show you if you are unsure of how to do this. Load the gel samples in order 1 to 12.

6. Place the lid of the tank onto the base (taking care not to spill the buffer). Connect the power supply and the gel tank with the power leads. Switch on the power supply, adjust the voltage to 90 V and ensure that the switch is on constant voltage. Run the gel until the dye front is about 3/4 of the way down the gel (about 45 min).

Safety note: wear gloves when handling the gels. Acrylamide is absorbed through the skin and is neurotoxic.

7. The gels are quite fragile so some care is required. Carefully remove the gel, sandwiched between glass plates, from the apparatus after removing the two clips which clamp it in place. Lay the gel sandwich on the bench (notched glass plate upwards) and gently prise the notched plate off by inserting a spatula between the glass plates midway between the top and bottom of the plate and levering it up. The gel should stick to the lower plate with spacers.
8. Pour 20 ml of SYBR Gold Stain into the plastic tray. Carefully hold the glass plate with the gel upside down over the plastic tray and gradually peel off the gel with the spatula letting the gel fall into the plastic tray. Place the dish in a shaking incubator. Make sure you cover your tray with foil as the stain is light sensitive. The bromophenol blue will lose its colour as the stain develops. Allow the gel to stain for 30 minutes.
9. After 30 minutes, discard the staining solution into the 'Waste Stain' pot. **Do not put it down the sink.**
10. A demonstrator will help you to photograph your gel.

Safety Note:

As SYBR Gold also binds to double stranded DNA we are instructed to treat it in the same manner as Ethidium Bromide.

Ethidium Bromide is a mutagen. Even though SYBR Gold is not yet proven to be one, wear two sets of gloves when handling the gel and avoid getting SYBR Gold on your hand, clothing, or anywhere else. Carry your gels on the plastic trays provided. There are very clear university guidelines on how to handle SYBR gold/Ethidium Bromide and these are posted on the classroom notice boards.

Notes

Notes

Question Sheets – Practicals 1 to 4

Week 1 – Questions

(Give one-line answers to the following questions)

Why do *Bacillus amyloliquefaciens* and *Haemophilus influenzae* not find their own DNA digested by the enzymes they contain?

How do you know that the Oct-1 POU domain was inserted in the correct orientation? How might you confirm this?

What potential problems might you encounter using Taq polymerase for PCR? How are these problems overcome? Is it likely to be a problem in the PCR in this practical?

Why is it important to treat the vector preparation with alkaline phosphatase prior to ligation? Where does alkaline phosphatase come from?

How are *E. coli* cells made 'competent' so they will take up DNA? What other methods might be used to transform *E. coli* with DNA?

You have just finished an arduous experiment using a new set of primers designed to amplify the β-globin gene but you have ended up with no PCR product. Suggest five reasons why your experiment might have failed (other than forgetting to add any of the components).

(i)

(ii)

(iii)

(iv)

(v)

You have performed another arduous experiment and now you obtain a myriad of PCR products where you expected only one band. Suggest a reason why this might have happened and how you would go about testing your hypothesis.

What is the theory that allows one to predict the annealing temperature of a primer? (Look it up).

The following problem, which was set in the 2002 NST IB Practical examination, illustrates several important aspects of PCR.

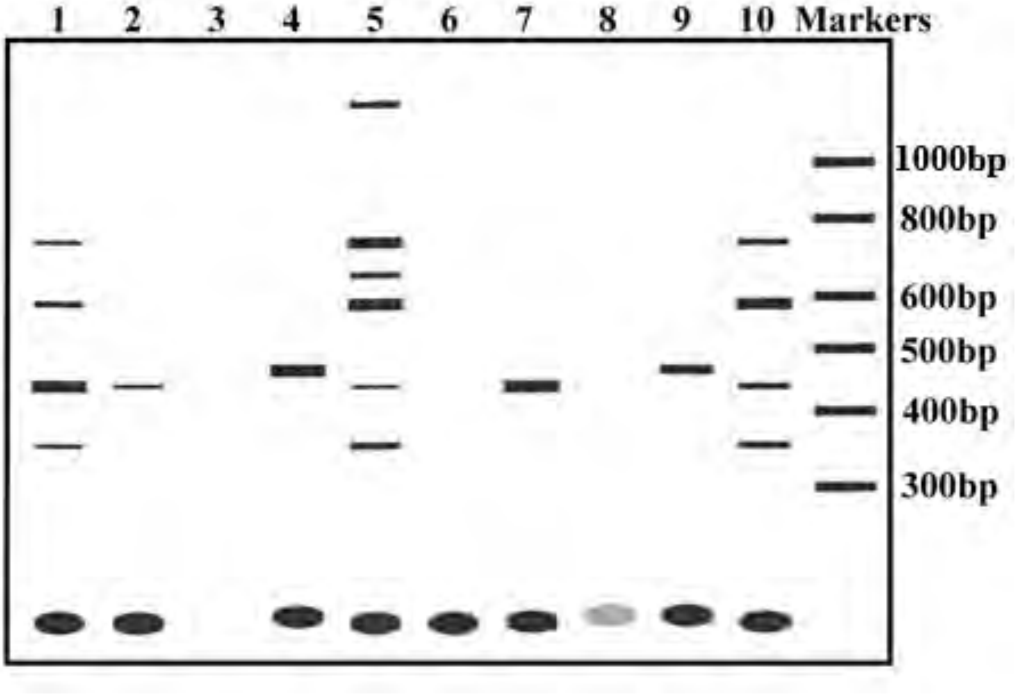
A number of PCR reactions were carried out attempting to optimise the amplification of a particular segment of chromosomal DNA. The reaction conditions were as follows:

Reaction number	Chromosomal DNA added	Primers added	Annealing temperature	Mg^{2+} concentration
(i)	Yes	short pair	55 °C	1.5 mM
(ii)	Yes	long pair	55 °C	1.5 mM
(iii)	Yes	short pair	65 °C	1.5 mM
(iv)	Yes	long pair	65 °C	1.5 mM
(v)	Yes	short pair	45 °C	1.5 mM
(vi)	Yes	one long primer only	55 °C	1.5 mM
(vii)	Yes	none	55 °C	1.5 mM
(viii)	No	short pair	55 °C	1.5 mM
(ix)	Yes	short pair	55 °C	3.5 mM
(x)	Yes	short pair	55 °C	7.5 mM

Taq DNA polymerase was added to all reactions, and amplification was for 25 cycles using the following program: 94 °C for 1 minute (the denaturation or melting step); the stated annealing temperature for 50 seconds; 75 °C for 30 seconds (the polymerisation step). The short primers were each 15 residues long, and the long primers 30 residues. The short and long forward primers were co-terminal at their 3'-ends, and the short and long reverse primers were likewise co-terminal at their 3'-ends. Primers were added in large excess.

At the end of the reaction the products were separated by agarose gel electrophoresis in a gel containing ethidium bromide, and the products visualised by the fluorescence of the bound ethidium under UV illumination. The result is depicted below. However, the reaction tubes had been carelessly muddled up before loading the samples on the gel.

Match the lanes (1) – (10) with the reactions (i) – (x) described above, explaining your reasoning. Note that for some lanes it may not be possible to unambiguously assign that lane to a single unique set of reaction conditions.



Notes

Week 2 – Questions

What percentage of the colonies on your negative control plate were white?

What percentage of the colonies on your ligation plate were white?

How can blue and white colonies arise on the control plate?

How could blue colonies arise on the ligation plate?

Nucleic Acid Melting

From your graph of A_T/A_0 against temperature determine the T_M of dsDNA in TE buffer alone and in the presence of 0.1 M NaCl and 2 M urea.

Rationalise the effects of NaCl and $MgCl_2$ upon the stability of the dsDNA.

Explain the effects of urea upon the stability of dsDNA.

What is different about the melting profile of tRNA compared with dsDNA? Explain the differences that you observe.

Week 3 – Questions

Estimate the molecular weight of the His_6-MBP-POU domain fusion protein that you have produced. How does this compare with the expected size (N.B. His_6-MBP contains 398 amino acids; an average amino acid has a molecular weight of ~110 Da)?

What is the concentration of your purified protein sample?

In mg/ml:

In mmol/litre:

Estimate the degree of purity of your protein (in %). What assumptions are you making?

How much pure protein could you make from 1 litre of *E. coli* culture?

What could be the nature of the other proteins that co-elute with the POU domain? What is the difference between the two wash buffers and what is the difference between the contaminants that these buffers are removing?

What steps would you suggest to purify the POU domain further?

How does the separation achieved by gel electrophoresis differ from that of gel filtration?

Week 4 – Questions

What is the approximate K_d of the purified POU domain for the octamer motif?

What is the approximate K_d of the purified POU domain for the mutant octamer motifs/random DNA (obtain results of the other 2 mutants from neighbouring groups)?

Mutant 1

Mutant 2

Mutant 3

Random DNA

What can you conclude about the relative importance of Pou_H and Pou_S subdomain binding to the octamer motif?

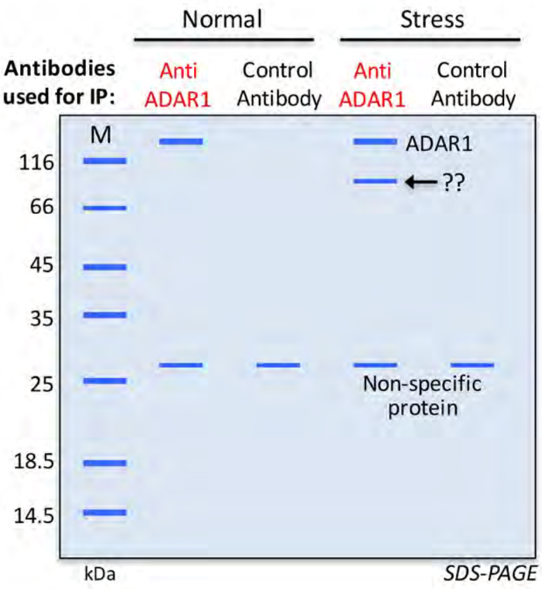
What other techniques can be used to measure and detect specific interactions between DNA and DNA-binding proteins?

Which of these techniques are applicable to RNA-binding proteins?

Week 5: BMB Experimental Exercise – Using Online Databases as Tools

This exercise will guide you through using some online tools that would be used in the lab to answer specific experimental questions or to analyse data. There are numerous approaches that could be used to do the task set out here – the methods outlined are just examples of the way you may choose to approach the question.

Your lab is interested in a specific protein (Adenosine Deaminase that Acts on RNA – ADAR1) that plays a novel role in stress granules in mammalian cells. Stress granules are cytoplasmic foci that form during stress conditions, and which enable to cell to survive stresses. Although it is known that stress granules contain an assortment of proteins and RNA, the function of ADAR1 in stress granules is not clear. To gain some insight into the role of ADAR1 in stress granules, various experiments have been performed, including an immunoprecipitation assay that has been used to identify any human proteins that interact with recombinant ADAR1. The results of this experiment are shown here:



This experiment showed that a protein of unknown identity (~100 kDa) interacted specifically with ADAR1 under stress conditions – the corresponding protein band was excised from the polyacrylamide gel and peptide sequences were determined using mass spectrometry. This gave rise to the following peptides:

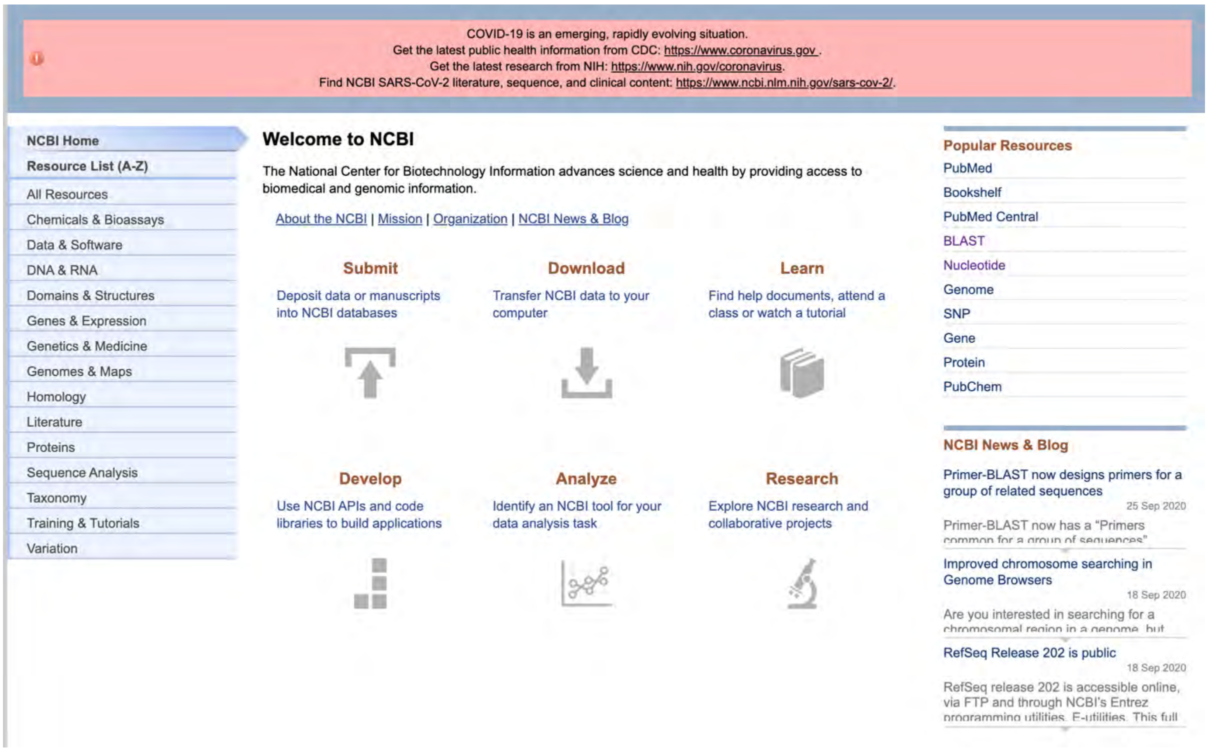
1) VHFTAERSSYYK
2) TIHLSSIRPPRLEGENTQDK
3) DTPDEPWAFPAR
4) AGNFIGWLHIDGANLSVLLVEHALSK
5) ETCLITFLLAGIECPR
6) ALLLPDYYLVTVMLSGIK
7) PLYDIPYMFEAR
8) SSYYKSLLSAEEAAK

Summary of what you will achieve in this exercise – you will aim to:

- Determine the origin of the peptides – what is your protein of interest?
- Find the nucleotide sequence encoding your protein
- Identify the cDNA encoding your protein and the ORF
- Design primers for cloning the ORF into an expression plasmid
- Find siRNAs to knockdown your protein of interest

1. Investigating the origin of the peptides

The first thing you want to do is to determine the identity of the protein(s) that the peptides come from. There are various websites you can use to do this. One such site is NCBI (National Center for Biotechnology Information – http://www.ncbi.nlm.nih.gov/).



COVID-19 is an emerging, rapidly evolving situation.
Get the latest public health information from CDC: https://www.coronavirus.gov .
Get the latest research from NIH: https://www.nih.gov/coronavirus.
Find NCBI SARS-CoV-2 literature, sequence, and clinical content: https://www.ncbi.nlm.nih.gov/sars-cov-2/.

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Resource List (A-Z)
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Variation

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NCBI News & Blog

Primer-BLAST now designs primers for a group of related sequences
25 Sep 2020
Primer-BLAST now has a "Primers common for a group of sequences"

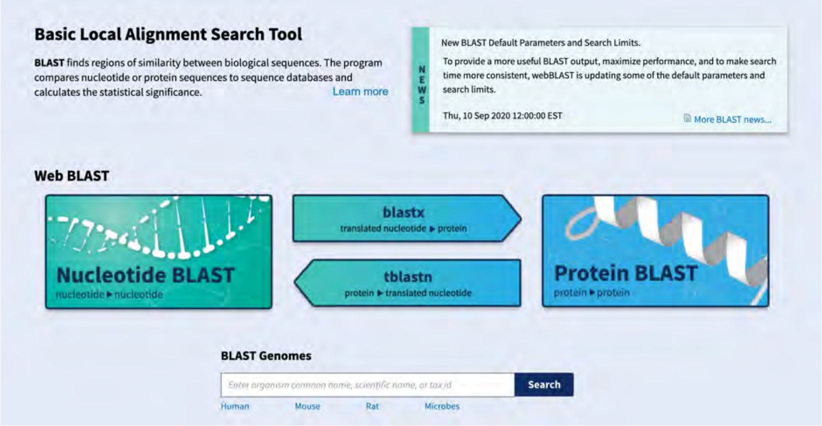
Improved chromosome searching in Genome Browsers
18 Sep 2020
Are you interested in searching for a chromosomal region in a genome, but

RefSeq Release 202 is public
18 Sep 2020
RefSeq release 202 is accessible online, via FTP and through NCBI's Entrez programming utilities, E-utilities. This full

First choose one of the peptides listed above (start by looking at the first peptide: VHFTAERSSYYK), and then use the 'BLAST' (**B**asic **L**ocal **A**lignment **S**earch **T**ool) function to look for proteins that contain that peptide sequence. BLAST can be found within the list at the top right-hand side of the page above.

BLAST finds regions of similarity between biological sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance.

Click on "BLAST", and you will go to the following window, where you will have the choice of doing either a Nucleotide BLAST or a Protein BLAST.



Basic Local Alignment Search Tool

BLAST finds regions of similarity between biological sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance. Learn more

NEWS

New BLAST Default Parameters and Search Limits.

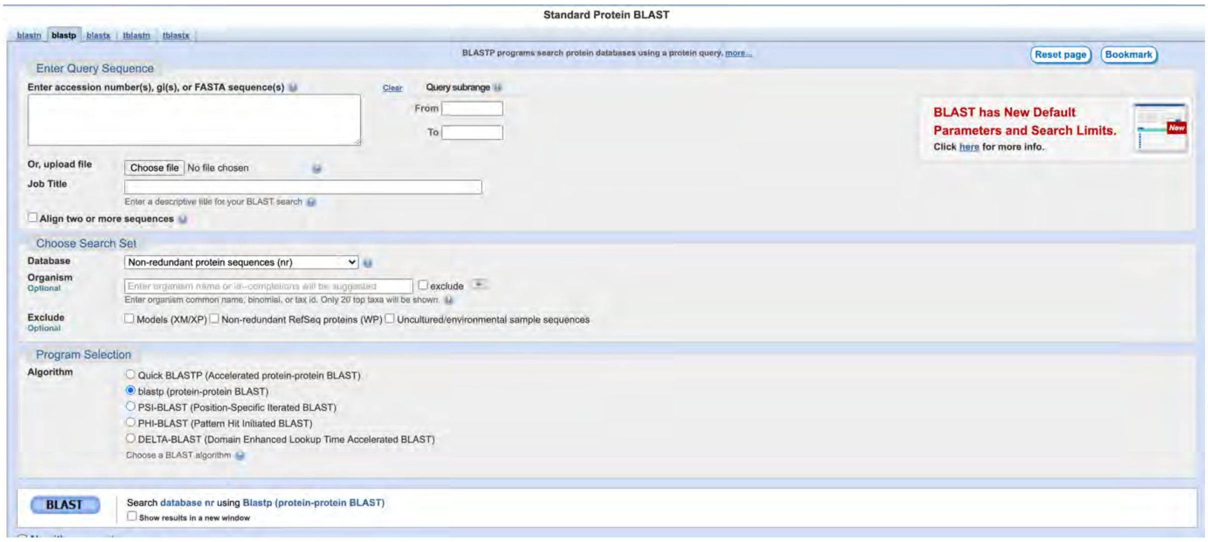
To provide a more useful BLAST output, maximize performance, and to make search time more consistent, webBLAST is updating some of the default parameters and search limits.

Thu, 10 Sep 2020 12:00:00 EST

More BLAST news...

Web BLAST
Nucleotide BLAST
nucleotide ▸ nucleotide
blastx
translated nucleotide ▸ protein
tblastn
protein ▸ translated nucleotide
Protein BLAST
protein ▸ protein
BLAST Genomes
Enter organism common name, scientific name, or tax id
Search
Human
Mouse
Rat
Microbes

Click on "**Protein Blast**", and you will be taken to this page:



Standard Protein BLAST

BLASTP programs search protein databases using a protein query. more...

Enter Query Sequence

Enter accession number(s), gi(s), or FASTA sequence(s)

Or, upload file: No file chosen

Job Title: Enter a descriptive title for your BLAST search

☐ Align two or more sequences

BLAST has New Default Parameters and Search Limits. Click here for more info.

Choose Search Set

Database: Non-redundant protein sequences (nr)

Organism (Optional): ☐ exclude

Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown.

Exclude (Optional): ☐ Models (XM/XP) ☐ Non-redundant RefSeq proteins (WP) ☐ Uncultured/environmental sample sequences

Program Selection

Algorithm:
- ○ Quick BLASTP (Accelerated protein-protein BLAST)
- ● blastp (protein-protein BLAST)
- ○ PSI-BLAST (Position-Specific Iterated BLAST)
- ○ PHI-BLAST (Pattern Hit Initiated BLAST)
- ○ DELTA-BLAST (Domain Enhanced Lookup Time Accelerated BLAST)

Choose a BLAST algorithm

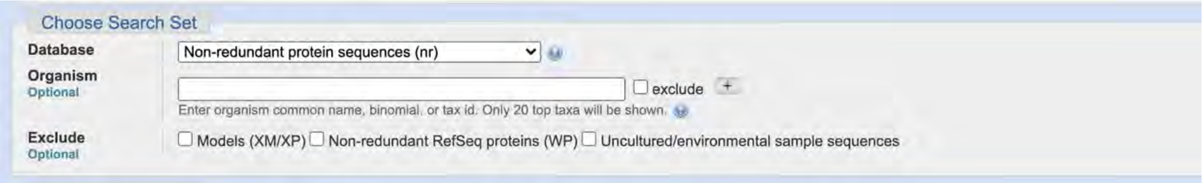
Search database nr using Blastp (protein-protein BLAST)

☐ Show results in a new window

blastn
blastp
blastx
tblastn
tblastx
Reset page
Bookmark
Clear
Query subrange
From
To
Choose file
Enter organism name or id--completions will be suggested
New
BLAST

Enter your query sequence into the box ('Enter Query Sequence') – this can be entered as the accession number(s) (e.g. Genbank Accession number), gi(s), or FASTA sequence(s) – see glossary at end). Or in our case, we will just enter one of the peptide sequences ('bare sequence').

Once you have done that, you should look at the various search parameters. First, decide which database you want to search from the top dropdown menu. We will use the 'Non-redundant protein sequences (nr)' for this exercise – this database contains non-redundant sequences (i.e. with duplicated sequences removed) from both non-curated (e.g. GenBank/GenPept, trEMBL) and curated databases (e.g. RefSeq, SwissProt, PIR, PDB)).



Choose Search Set

Database: Non-redundant protein sequences (nr)

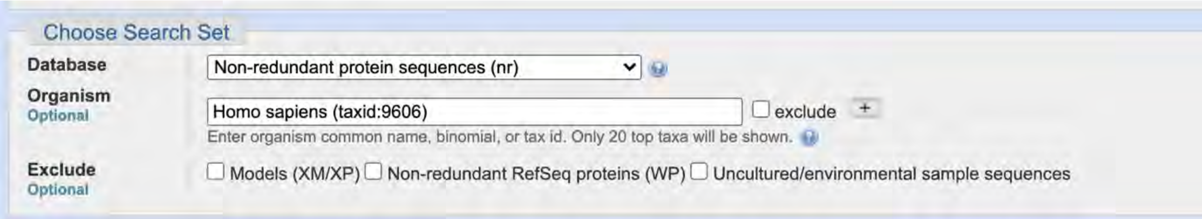
Organism (Optional): ☐ exclude

Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown.

Exclude (Optional): ☐ Models (XM/XP) ☐ Non-redundant RefSeq proteins (WP) ☐ Uncultured/environmental sample sequences

Once you have done that, you can choose whether to search the entire data base, or whether you want to limit your search to a particular organism ('Choose Search Set').

If you leave the box blank, the search will be unrestricted. As we are looking for a human sequence, we could restrict the search to only look at human proteins – so type '*Homo sapiens*') in the box, as follows:



Choose Search Set

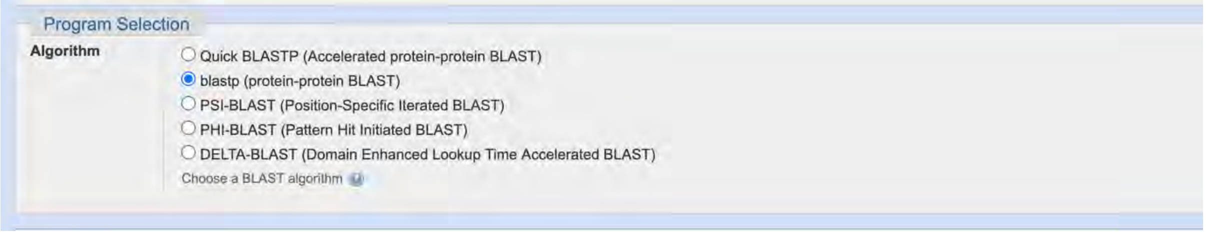
Database: Non-redundant protein sequences (nr)

Organism (Optional): Homo sapiens (taxid:9606) ☐ exclude

Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown.

Exclude (Optional): ☐ Models (XM/XP) ☐ Non-redundant RefSeq proteins (WP) ☐ Uncultured/environmental sample sequences

You can also alter the algorithm for the search if necessary – click on the '?' symbol to find out more about these functions. We are going to use 'blastp (protein-protein BLAST)' to search the database to find proteins that contain the peptide sequence.



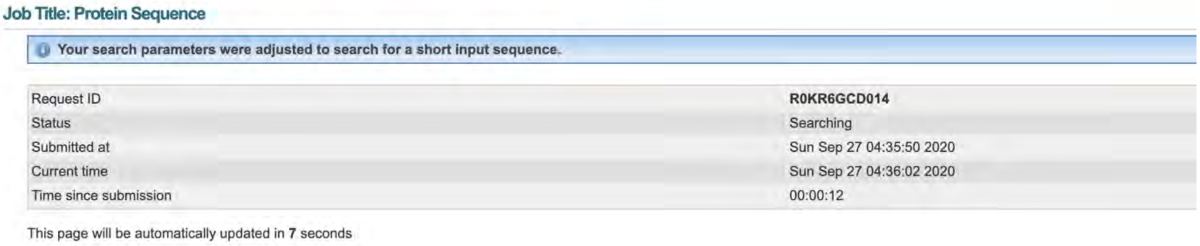
Program Selection

Algorithm

○ Quick BLASTP (Accelerated protein-protein BLAST)
● blastp (protein-protein BLAST)
○ PSI-BLAST (Position-Specific Iterated BLAST)
○ PHI-BLAST (Pattern Hit Initiated BLAST)
○ DELTA-BLAST (Domain Enhanced Lookup Time Accelerated BLAST)

Choose a BLAST algorithm

When searching with such a short sequence, you will see the message that 'Your search parameters were adjusted to search for a short input sequence'. It will take a few moments for the results of your search to be generated.



Job Title: Protein Sequence

Your search parameters were adjusted to search for a short input sequence.

Request ID	R0KR6GCD014
Status	Searching
Submitted at	Sun Sep 27 04:35:50 2020
Current time	Sun Sep 27 04:36:02 2020
Time since submission	00:00:12

This page will be automatically updated in 7 seconds

When the search is complete, you will see a graphical summary of the proteins matching the peptide sequence (with colour-coded alignment scores), and below that you will see the list of proteins that match your query sequence.

Q1: What is the top hit?

Q2: What is the identity score (you will find this information in the 5th column on the right-hand side)?

Click on the top hit to see the alignment between your query sequence and the protein identified. It is important to look at this to see the matches between amino acids –

sometimes the identity may be less than 100%, but this is the result of conservative amino acid changes (e.g. Glu (E) for Asp (D)).

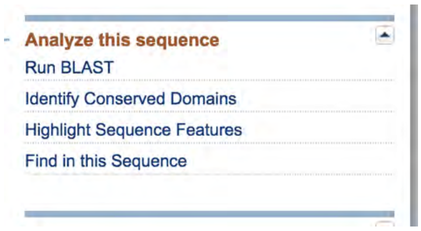
You may notice that there are a number of matches at the top of the list that match the query sequence 100%. This is typically because there are multiple entries in the database of the same thing but that are from different sources – and may be called different things depending on the context in which they were found. Some will be described as ‘ unnamed protein product’ – for example, these sequences may come from a massed sequencing project.

Then click on the ‘sequence id’ (above the alignment) to find out more about the protein. Make a note of the protein name and the GenBank Accession number (‘Accession’), which you will find toward the top of the page (combination of 3 letters and 5 numbers – see glossary). You will need this information later.

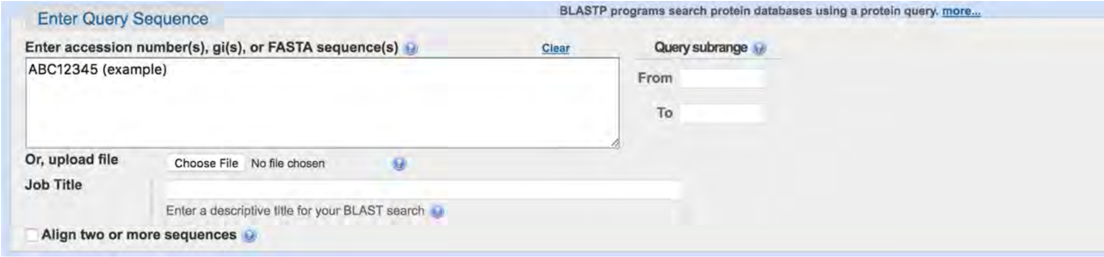
Q3: How many amino acids are shown for the protein of interest?

Q4: How long would the mRNA be encoding that number of amino acids?

You next want to check whether the other peptide sequences obtained also match this sequence – that is important in order to determine whether the protein identified is the protein of interest. Go to the box on the right-hand side of the page labelled ‘Analyze this sequence’, then click on ‘Run BLAST’.


Analyze this sequence
Run BLAST
Identify Conserved Domains
Highlight Sequence Features
Find in this Sequence

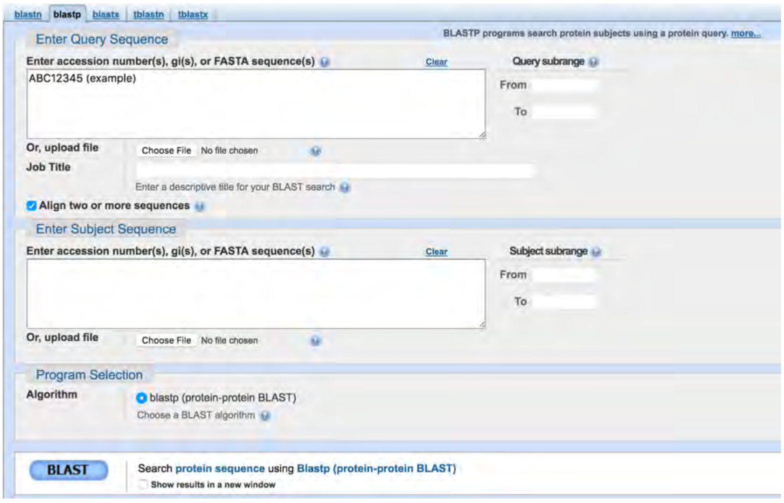
This will take you back to the search screen seen previously. This time however, the GenBank Accession number for the protein sequence identified will already be in the search box.


Enter Query Sequence
BLASTP programs search protein databases using a protein query. more...
Clear
Query subrange
From
To
Or, upload file
Choose File
No file chosen
Job Title
Enter a descriptive title for your BLAST search
Align two or more sequences

Enter accession number(s), gi(s), or FASTA sequence(s): ABC12345 (example)

At the bottom of that box, click on ‘align two or more sequences’.

This will give you a second sequence box for the 'Subject Sequence'.


blastn
blastp
blastx
tblastn
tblastx
Enter Query Sequence
BLASTP programs search protein subjects using a protein query. more...
Enter accession number(s), gi(s), or FASTA sequence(s)
Clear
Query subrange
From
To
Or, upload file
Choose File
No file chosen
Job Title
Enter a descriptive title for your BLAST search
Enter Subject Sequence
Enter accession number(s), gi(s), or FASTA sequence(s)
Clear
Subject subrange
From
To
Or, upload file
Choose File
No file chosen
Program Selection
Algorithm
Choose a BLAST algorithm
BLAST
Search protein sequence using Blastp (protein-protein BLAST)
☐ Show results in a new window

ABC12345 (example)

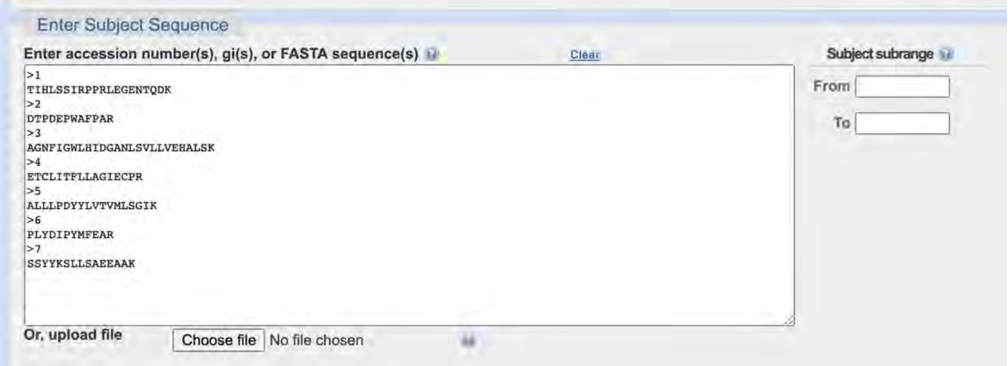
☑ Align two or more sequences

● blastp (protein-protein BLAST)

You can copy one of the other peptide sequences into the 'Enter Subject Sequence' box – then carry out a BLAST search to check whether the sequence is contained within the protein sequence identified (look at the alignment). Do this for each peptide sequence in turn until you are sure that most or all of the peptides are derived from this protein.

***Note**: if you were using this search function for sequences that have NCBI identifiers (e.g. accession or gi numbers) or are using FASTA-formatted sequences, multiple query sequences are allowed – but it is important to include the list of identifiers (accession or gi numbers) as one per line, or the group of FASTA sequences with each beginning line and starting with the ">" sign.*

So if we apply this idea to our list of peptides, the search would look something like this….


Enter Subject Sequence
Enter accession number(s), gi(s), or FASTA sequence(s)
Clear
Subject subrange
From
To
Or, upload file
Choose file
No file chosen

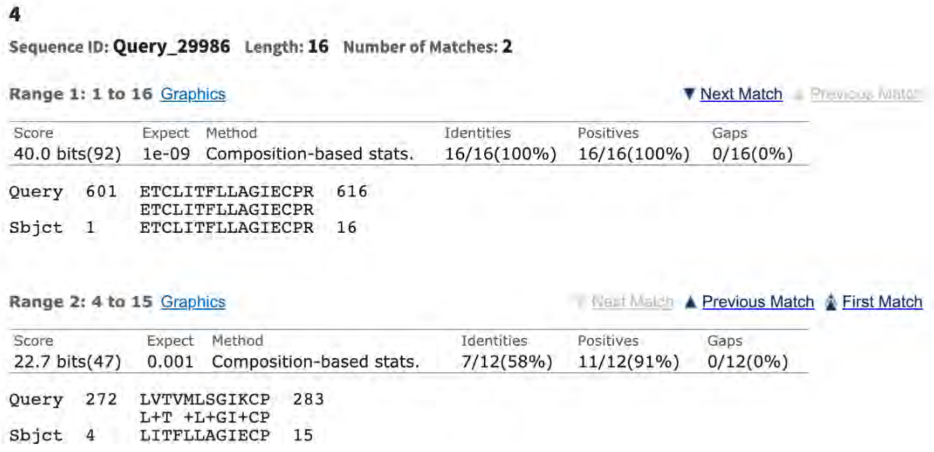
```
>1
TIHLSSIRPPRLEGENTQDK
>2
DTPDEPWAFPAR
>3
AGNFIGWLHIDGANLSVLLVEHALSK
>4
ETCLITFLLAGIECPR
>5
ALLLPDYYLVTVMLSGIK
>6
PLYDIPYMFEAR
>7
SSYYKSLLSAEEAAK
```

This means that the sequences will **each** be blasted against the protein sequence **independently**. If you simply put all the sequences in the search box as a list (without the '>'), they would be treated as a single long peptide.

Q5: If you had started with a different peptide for your initial BLAST search, would you have got the same initial result? If not, why?

Once the Blast is complete, have a look at the sequence matches. It's important to note that the report will show you all possible matches… so for example, if a peptide matched a sequence **perfectly**, you will see it has a 100% match. If it happens to match another bit of the sequence **imperfectly**, this will also be shown, but with a lower % match.

e.g.



```
4
Sequence ID: Query_29986  Length: 16  Number of Matches: 2

Range 1: 1 to 16

Score          Expect  Method                      Identities    Positives     Gaps
40.0 bits(92)  1e-09   Composition-based stats.    16/16(100%)   16/16(100%)   0/16(0%)

Query  601   ETCLITFLLAGIECPR  616
             ETCLITFLLAGIECPR
Sbjct  1     ETCLITFLLAGIECPR  16

Range 2: 4 to 15

Score          Expect  Method                      Identities    Positives     Gaps
22.7 bits(47)  0.001   Composition-based stats.    7/12(58%)     11/12(91%)    0/12(0%)

Query  272   LVTVMLSGIKCP  283
             L+T +L+GI+CP
Sbjct  4     LITFLLAGIECP  15
```

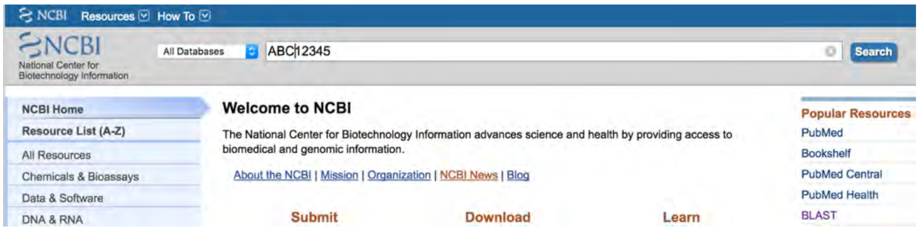
In this example, you can see that the 16 residue peptide has a 100% match with the query sequence between amino acids 601-616 (query), but an imperfect match (58% identity) with the query sequence between amino acids 272-283.

Having checked whether the peptides are all derived from the same protein, this will enable you to decide whether the protein identified via the BLAST searches corresponds to the protein band that interacted with your protein of interest (ADAR1) in the immunoprecipitation assay. If you are confident that this is the case, your next steps will aim to look more closely at the interaction between the proteins. To do this, it will next be necessary to clone the DNA encoding the protein identified.

2. Finding the nucleotide sequence encoding your protein

To find the nucleotide sequence encoding the protein you have identified, you need to go back to the page that you looked at earlier that gave information about the protein. One way of doing this is to enter the GenBank Accession number noted above into the search box at the top of the front page of NCBI (http://www.ncbi.nlm.nih.gov/).

I.e.


NCBI
Resources
How To
NCBI
National Center for Biotechnology Information
All Databases
ABC12345
Search
NCBI Home
Resource List (A-Z)
All Resources
Chemicals & Bioassays
Data & Software
DNA & RNA
Popular Resources
PubMed
Bookshelf
PubMed Central
PubMed Health
BLAST
About the NCBI | Mission | Organization | NCBI News | Blog
Submit
Download
Learn

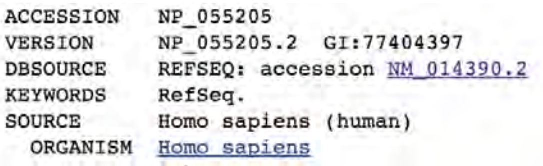
Welcome to NCBI

The National Center for Biotechnology Information advances science and health by providing access to biomedical and genomic information.

In the dropdown menu at the left of the search box, scroll down and select 'Protein', and then 'Search' – this will take you back to the page showing the details of the protein you have identified.

Towards the top of this page, there is information about the name of the protein, the number of amino acids determined, the organism, references etc. Further down there is information about any domains that have been identified within the protein (via homology), and then the actual amino acid sequence is given. Copy the protein sequence you have identified for reference later (e.g. copy and paste into a word document).

If you click on the accession number (2 letters, 6 numbers) toward the top of the page next to the text 'DBSOURCE', this will take you to the page showing the mRNA (cDNA) that encodes the protein shown. Make a note of the GenBank accession number for the mRNA, as you will need this later.



```
ACCESSION   NP_055205
VERSION     NP_055205.2  GI:77404397
DBSOURCE    REFSEQ: accession NM_014390.2
KEYWORDS    RefSeq.
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
```

Q6: What is the difference between the mRNA sequence and the genomic sequence?

Q7: Why is it important to think about that when you are trying to obtain the sequence encoding the protein of interest?

If you look towards the top of this page you will see the length of the mRNA encoding your protein of interest (in bases).

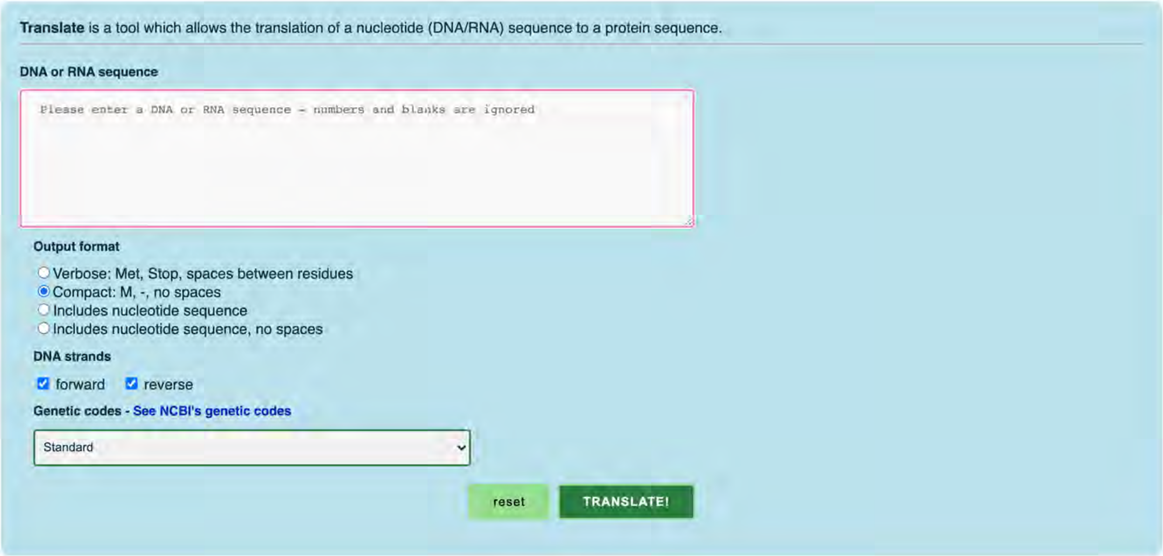
Q8: How does this number compare with the predicted length of the mRNA encoding your protein of interest, as calculated above? If it is not the same, how can you explain the discrepancy?

Note: If you look further down the page, it gives some references for the sequence, which tells us that the cDNA sequence shown has been identified as a part of a large sequencing project at the RIKEN Genomic Sciences Centre (http://protein.gsc.riken.go.jp/), where cDNAs have been cloned into a pBluescript II cloning vector (pBluescript II SK plus) and subsequently sequenced. This means that the sequences given may not be annotated correctly – and if you look at the information given, it tells us that there is 'non-experimental evidence, no additional details recorded' and that the 'start codon is not identified'. You need to bear this information in mind when you start analyzing the cDNA sequence for cloning and expression.

3. Identifying the Open Reading Frame (ORF)

At the bottom of the page you will see the nucleotide sequence encoding your protein of interest. Copy this sequence, as you will need it later. As noted above, this sequence is longer than expected to encode the protein you have found. It is therefore important to identify the Open Reading Frame (ORF) within the sequence that encodes the protein of interest.

To do this, we can use the ExPASy Bioinformatics Resource Portal to translate the sequence (http://web.expasy.org/translate/).



Translate is a tool which allows the translation of a nucleotide (DNA/RNA) sequence to a protein sequence.

DNA or RNA sequence

Please enter a DNA or RNA sequence – numbers and blanks are ignored

Output format

○ Verbose: Met, Stop, spaces between residues
● Compact: M, -, no spaces
○ Includes nucleotide sequence
○ Includes nucleotide sequence, no spaces

DNA strands

☑ forward ☑ reverse

Genetic codes - See NCBI's genetic codes

Standard

Copy the complete cDNA sequence from the NCBI page and paste it into the box on the ExPASy site, as shown above. You can alter the output format from 'Verbose' ('Met', 'Stop', spaces between residues) to 'Compact' ('M', '–', no spaces), or to include the nucleotide sequence. There is also the option to change the genetic code used for translation, but for this exercise we will just use the standard code. Once you have chosen the options you prefer (choose either 'Verbose' or 'Compact'), click on the 'Translate Sequence' button at the bottom of the page.

You will now see a page where the nucleotide sequence has been translated in all 6 possible reading frames.

Q9: Why is it important to do translate the cDNA in 6 frames?

Q10: How will you know which one is correct?

Have a look at the results and see which reading frame is most likely to be correct – use the protein sequence noted above to help. You already know the approximate size of the protein you are looking for – you know this from the protein sequence obtained *in silico*, but also from the initial gel showing the results of the immunoprecipitation assay (above).

Q11: Which reading frame is correct?

If you compare the predicted open reading frame (ORF) sequence to the amino acid sequence obtained earlier using NCBI, you will see that the translated sequence predicted ***appears*** to be truncated at the N-terminus by 54 amino acids. Make a note of the first and last few amino acids of the translated protein (determined using ExPASy).

Q12: Why does the predicted sequence not include the first 54 amino acids as part of the ORF?

Q13: How could you verify that the translated sequence is correct?

Assuming that the translated sequence you have obtained is correct, you go on with your analysis.

4. Identifying the cDNA sequence corresponding to the ORF

As the ORF is encoded by only part of the cDNA sequence you obtained above, you need to identify the part of the cDNA sequence that corresponds to the ORF.

You can do this by inspection – go back and translate the cDNA again in ExPASy but this time choose the ‘output format’ to ‘Include the nucleotide sequence’.

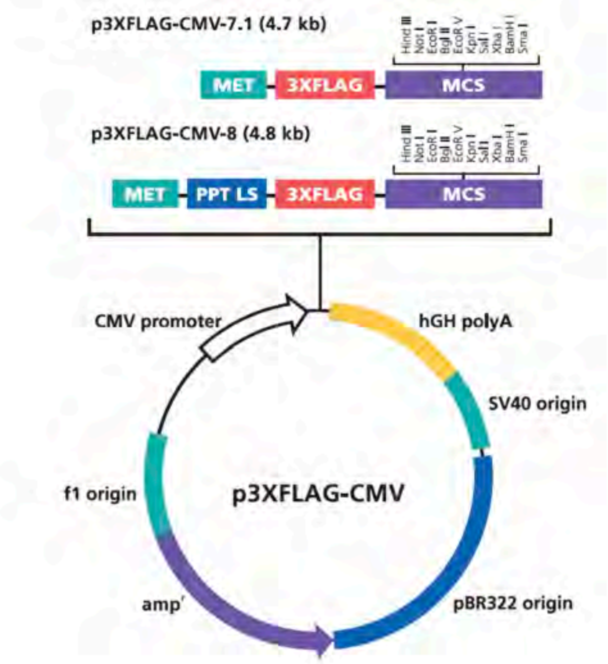
Once you have the translated sequence, scroll down until you find the correct reading frame, as determined above, then find:

- The **start codon** (ATG; use the first few amino acids noted above to help you).
- Next find the **stop codon** at the end of the ORF (TAA) (use the information you have regarding the length of the sequence to help you with this – and check that the amino acid residues preceding the TAA are the same as those noted above).

Use this information to trim the excess 5’ and 3’ nucleotides off the cDNA sequence obtained from NCBI (that you made a copy of earlier) – you should then have a sequence that only encodes the ORF for your protein of interest. If you want to be sure you’ve done this correctly, use the translate tool from ExPASy to translate your final cDNA sequence – you should get a translated sequence that only contains the amino acids corresponding to the ORF.

5. Cloning the cDNA encoding the ORF into a suitable expression vector

The next step of your experiment is to clone the cDNA encoding the ORF of your newly identified protein into an expression vector suitable for expression in mammalian cells (e.g. HeLa cells – see glossary). The vector you choose is p3xFLAG-CMV™-7.1(Sigma-Aldrich; 'p3xFlag'), which contains a sequence encoding a triple Flag tag (epitope tag) – this will ultimately be fused to your protein of interest (see diagram below).


p3XFLAG-CMV-7.1 (4.7 kb)
Hind III
Not I
EcoR I
Bgl II
EcoR V
Kpn I
Sal I
Xba I
BamH I
Sma I
MET
3XFLAG
MCS
p3XFLAG-CMV-8 (4.8 kb)
Hind III
Not I
EcoR I
Bgl II
EcoR V
Kpn I
Sal I
Xba I
BamH I
Sma I
MET
PPT LS
3XFLAG
MCS
CMV promoter
hGH polyA
SV40 origin
f1 origin
p3XFLAG-CMV
amp^r
pBR322 origin

Q14: Looking at the overview of the plasmid, above, will this generate a fusion protein (between the 3xFlag tag and your protein of interest) with an N- or C-terminal 3xFlag tag?

Q15: Is the relative location of the 3xFlag tag important when designing your cloning strategy? Why?

You initially design a strategy for cloning the cDNA into p3xFLAG. This will be carried out as follows:

- PCR primers will be designed for amplification of the cDNA encoding the protein of interest. Restriction enzyme recognition sites will be incorporated into the primers to enable directional cloning into p3xFLAG.
- The template for PCR will be generated by Reverse Transcription (RT; using an oligo(dT) primer or random hexamer primers), where RNA harvested from HeLa cells is used as a template for the RT reaction.

  Alternatively, it may be possible to order a clone from the I.M.A.G.E. consortium (see glossary) that contains your sequence of interest, which can then be used as a PCR template. Information regarding the availability of I.M.A.G.E. clones can be found via NCBI or equivalent databases.

6. Designing Primers

a) Firstly, using the cDNA sequence determined above, design a pair of primers (Forward and Reverse) that will be used to amplify the entire ORF (starting with the ATG start codon, and ending with the TAA stop codon). The primers should be written 5’-3’.

Forward primer sequence:

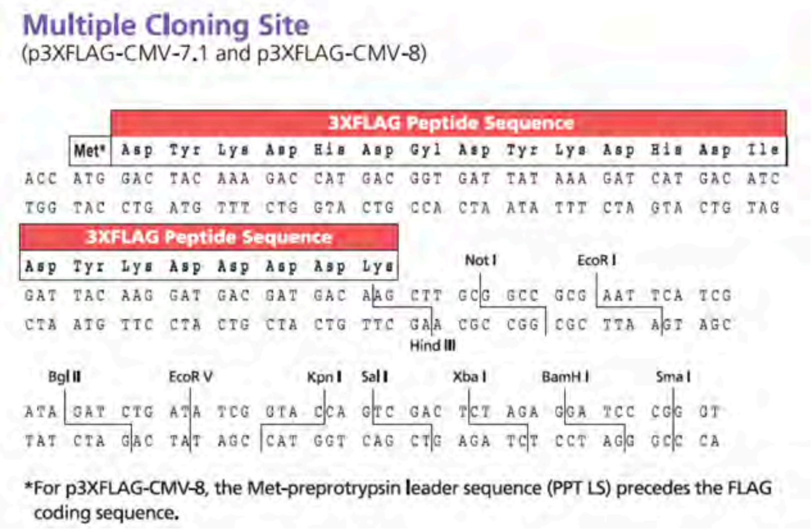
Reverse primer sequence:

Using the map of the Multiple Cloning Site (MCS; below) and the cDNA sequence determined above encoding your novel protein, you choose restriction sites that are suitable for cloning your cDNA into the expression plasmid. In this case, *Eco*RI and *Bam*HI restriction sites are chosen for cloning.

EcoRI
5′...G▼AATTC...3′
3′...CTTAA▲G...5′

***Bam*HI**
5′...G▼GATCC...3′
3′...CCTAG▲G...5′

Q16: What do you need to think about when choosing restriction sites for cloning?


Multiple Cloning Site
(p3XFLAG-CMV-7.1 and p3XFLAG-CMV-8)

3XFLAG Peptide Sequence
Met* Asp Tyr Lys Asp His Asp Gyl Asp Tyr Lys Asp His Asp Ile
ACC ATG GAC TAC AAA GAC CAT GAC GGT GAT TAT AAA GAT CAT GAC ATC
TGG TAC CTG ATG TTT CTG GTA CTG CCA CTA ATA TTT CTA GTA CTG TAG

3XFLAG Peptide Sequence
Asp Tyr Lys Asp Asp Asp Asp Lys
GAT TAC AAG GAT GAC GAT GAC AAG CTT GCG GCC GCG AAT TCA TCG
CTA ATG TTC CTA CTG CTA CTG TTC GAA CGC CGG CGC TTA AGT AGC

ATA GAT CTG ATA TCG GTA CCA GTC GAC TCT AGA GGA TCC CGG GT
TAT CTA GAC TAT AGC CAT GGT CAG CTG AGA TCT CCT AGG GCC CA

*For p3XFLAG-CMV-8, the Met-preprotrypsin leader sequence (PPT LS) precedes the FLAG coding sequence.

b) You then incorporate the restriction enzyme recognition sites into the primer sequences you designed above. i.e.

Forward primer sequence:

Reverse primer sequence:

When you make a fusion protein, you must **ensure that the reading frame is maintained between the tag and the protein of interest**.

Q17: Do you need to add any additional nucleotides to your primer sequences in order to maintain the reading frame?

Q18: Why is this necessary?

c) Write down the primer sequences (5’ to 3’), including any modifications you need to make to maintain the reading frame:

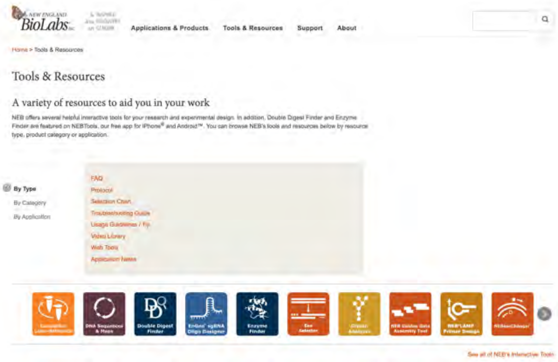
Forward primer sequence:

Reverse primer sequence:

Q19: If the tag was C-terminal rather than N-terminal, what additional changes to the primers would be made (in addition to modifications related to frame)?

Q20: When adding restriction enzymes onto primers, additional residues are often added 5’ of the restriction enzyme recognition sequences. Why is this necessary?

New England Biolabs Inc. are a common source molecular biology reagents used in the lab, and they also have numerous online 'tools and resources' that are helpful when designing cloning strategies (https://www.neb.com/tools-and-resources).


Applications & Products
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About

Home > Tools & Resources

Tools & Resources

A variety of resources to aid you in your work

NEB offers several helpful interactive tools for your research and experimental design. In addition, Double Digest Finder and Enzyme Finder are featured on NEBTools, our free app for iPhone® and Android™. You can browse NEB's tools and resources below by resource type, product category or application.

By Type
By Category
By Application
FAQ
Protocol
Selection Chart
Troubleshooting Guide
Usage Guidelines / Tip
Video Library
Web Tools
Application Notes
DNA Sequences & Maps
Double Digest Finder
EnGen sgRNA Oligo Designer
Enzyme Finder
NEB Golden Gate Assembly Tool
NEB LAMP Primer Design
NEBaseChanger
See all of NEB's Interactive Tools

Shown below is part of a table taken from the New England Biolabs online resources, which has information regarding the efficiency of cleavage for different restriction enzymes when cutting close to the end of DNA fragments (https://www.neb.com/tools-and-resources/usage-guidelines/cleavage-close-to-the-end-of-dna-fragments).

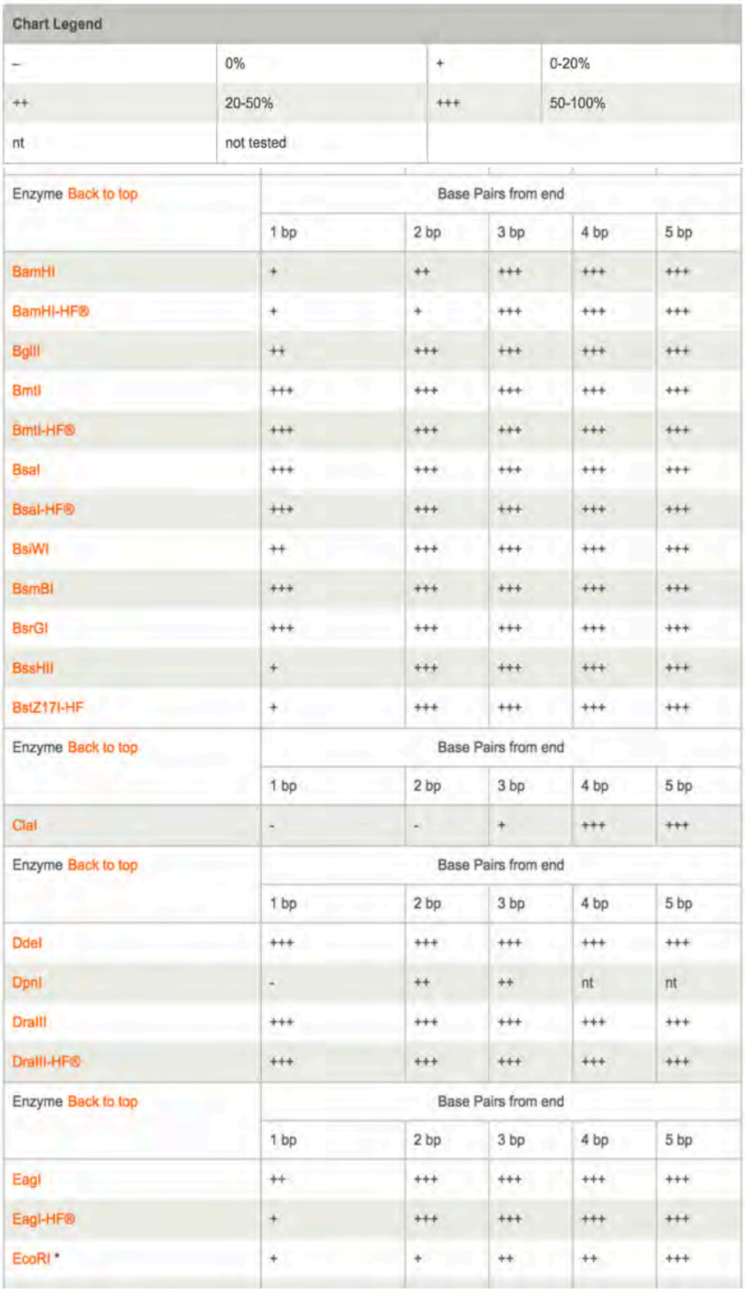


Chart Legend			
–	0%	+	0-20%
++	20-50%	+++	50-100%
nt	not tested		

Enzyme Back to top	Base Pairs from end				
	1 bp	2 bp	3 bp	4 bp	5 bp
BamHI	+	++	+++	+++	+++
BamHI-HF®	+	+	+++	+++	+++
BglII	++	+++	+++	+++	+++
BmtI	+++	+++	+++	+++	+++
BmtI-HF®	+++	+++	+++	+++	+++
BsaI	+++	+++	+++	+++	+++
BsaI-HF®	+++	+++	+++	+++	+++
BsiWI	++	+++	+++	+++	+++
BsmBI	+++	+++	+++	+++	+++
BsrGI	+++	+++	+++	+++	+++
BssHII	+	+++	+++	+++	+++
BstZ17I-HF	+	+++	+++	+++	+++
Enzyme Back to top	Base Pairs from end				
	1 bp	2 bp	3 bp	4 bp	5 bp
ClaI	-	-	+	+++	+++
Enzyme Back to top	Base Pairs from end				
	1 bp	2 bp	3 bp	4 bp	5 bp
DdeI	+++	+++	+++	+++	+++
DpnI	-	++	++	nt	nt
DraIII	+++	+++	+++	+++	+++
DraIII-HF®	+++	+++	+++	+++	+++
Enzyme Back to top	Base Pairs from end				
	1 bp	2 bp	3 bp	4 bp	5 bp
EagI	++	+++	+++	+++	+++
EagI-HF®	+	+++	+++	+++	+++
EcoRI *	+	+	++	++	+++

Q21: Using this table, how many nucleotides should be added to each of your primer sequences?

d) Using this information, write down the modified primer sequences (5' to 3'):

Forward primer sequence:

Reverse primer sequence:

7. RT-PCR

Using your new primers, you carry out the RT-PCR reaction in order to amplify the insert DNA for cloning into p3xFlag. You subsequently digest the amplified DNA with *Eco*RI and *Bam*HI, and then clone it into the p3xFlag vector that has similarly been digested with *Eco*RI and *Bam*HI.

Q22: How could you check that your final clone is correct?

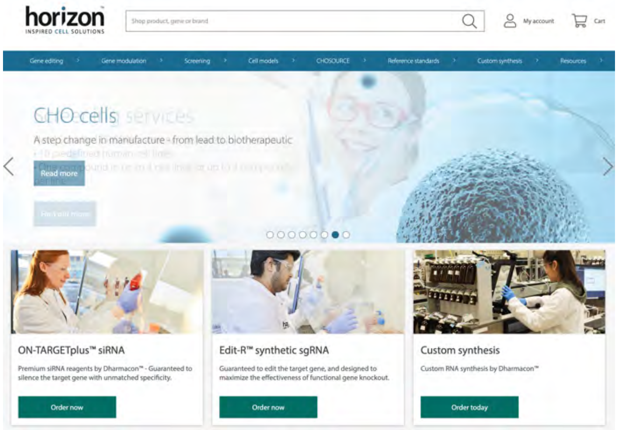
You thereby obtain your expression vector that will be used to express your 3xFlag-tagged protein of interest in mammalian cells, which will be used to study the interaction between ADAR1 and its newly identified interacting protein partner. These analyses will in turn provide insights into the role of ADAR1 and the novel protein in stress granules.

8. RNAi to knockdown your protein of interest

In addition to overexpressing your protein of interest, it is often informative to investigate a biological process in the **absence** of your protein. 'Knockdown' of your protein in mammalian cells (e.g. HeLa cells) is often achieved using RNA interference (RNAi; see Dr Scadden's lectures). You therefore need to design or acquire some siRNAs (small interfering RNAs – see Dr Scadden's lectures) that are optimised to target the mRNA encoding your protein. There are numerous suppliers of siRNAs (e.g. Horizon (formerly Dharmacon), Sigma, Qiagen), and there are now many 'pre-tested' siRNAs available to target many human mRNAs. If an siRNA is not available, a custom siRNA can be designed using various websites that use suitable algorithms for siRNA design.

horizon INSPIRED CELL SOLUTIONS | Dharmacon

For this exercise, you will use Horizon (https://horizondiscovery.com/), which provides specialist Dharmacon products related to gene expression, to obtain siRNAs to knockdown the protein interacting with ADAR1.


horizon
INSPIRED CELL SOLUTIONS
Shop product, gene or brand
My account
Cart
Gene editing
Gene modulation
Screening
Cell models
CHOSOURCE
Reference standards
Custom synthesis
Resources
CHO cells services
A step change in manufacture - from lead to biotherapeutic
Read more
ON-TARGETplus™ siRNA
Edit-R™ synthetic sgRNA
Custom synthesis
Order now
Order now
Order today

Premium siRNA reagents by Dharmacon™ - Guaranteed to silence the target gene with unmatched specificity.

Guaranteed to edit the target gene, and designed to maximize the effectiveness of functional gene knockout.

Custom RNA synthesis by Dharmacon™

There are various options for siRNAs depending on what cell type you use, what sort of sequence you are trying to target (e.g. mRNA or non-coding RNA), whether you want the siRNAs to be modified (which reduces off-target effects), etc. Sometimes the choice you make may simply come down to cost.

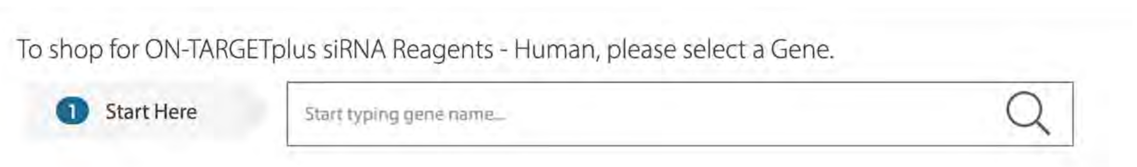
For this example, select 'ON-TARGETplus^{TM} siRNA' (modified siRNAs to reduce off-target effects). (https://horizondiscovery.com/en/products/gene-modulation/knockdown-reagents/sirna/PIFs/ON-TARGETplus-siRNA-Reagents-Human).

"**ON-TARGETplus siRNA Reagents - Human**

siRNA designed and modified for greater specificity

Guaranteed gene silencing and patented modifications to reduce off-targets; pre-designed ON-TARGETplus siRNA are available as individual or in SMARTpool format"

You can then 'Start your gene search' using the **human sequences** available:

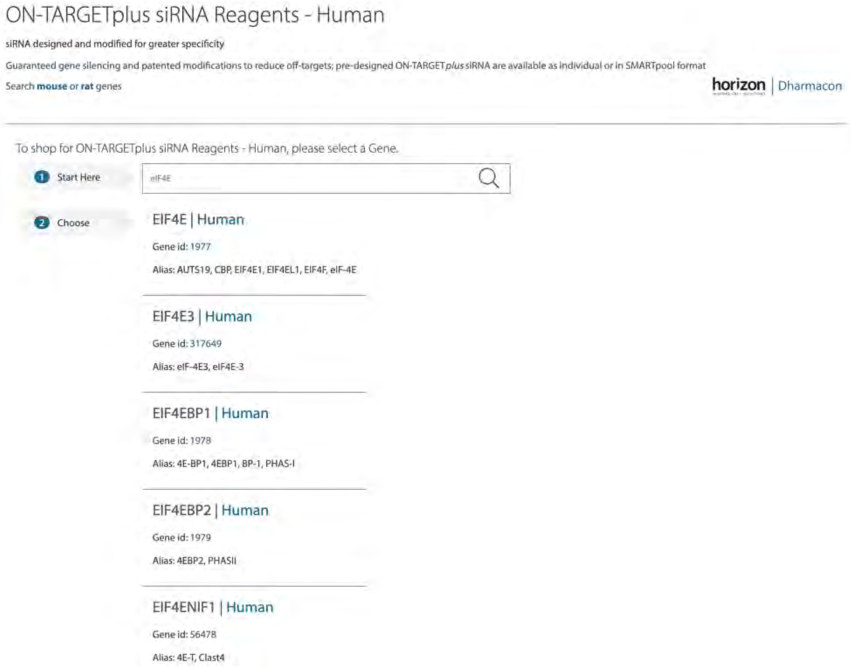

To shop for ON-TARGETplus siRNA Reagents - Human, please select a Gene.

1 Start Here
Start typing gene name...

You can then add the name of the gene you are looking to knock down.

Once the search is complete, you will get a list of gene(s) that match the gene name – check the gene you are expecting is shown – but note that many genes or proteins are described by multiple different names. If it is correct, click on the name of the sequence and this will take you to a page that shows you more information about the mRNA, and what siRNAs are available.

For example, if you searched for eIF4E, you would see the following information:



ON-TARGETplus siRNA Reagents - Human

siRNA designed and modified for greater specificity

Guaranteed gene silencing and patented modifications to reduce off-targets; pre-designed ON-TARGET*plus* siRNA are available as individual or in SMARTpool format

Search **mouse** or **rat** genes

To shop for ON-TARGETplus siRNA Reagents - Human, please select a Gene.

1 Start Here: eIF4E

2 Choose

EIF4E | Human
Gene id: 1977
Alias: AUTS19, CBP, EIF4E1, EIF4EL1, EIF4F, eIF-4E

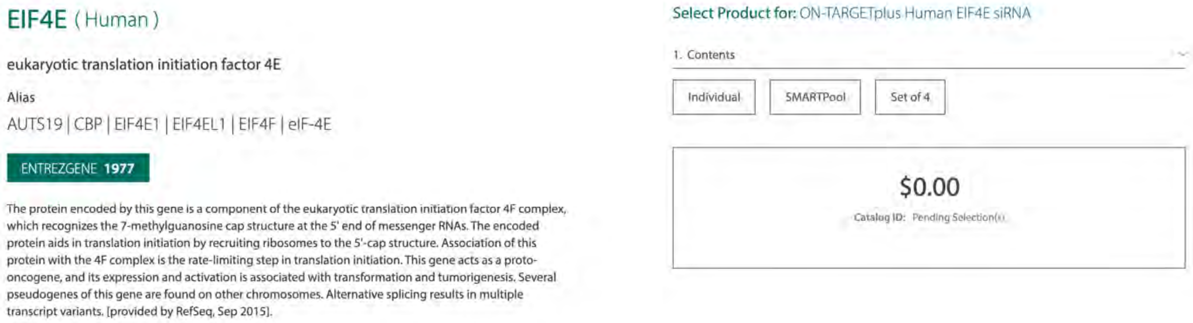
EIF4E3 | Human
Gene id: 317649
Alias: eIF-4E3, eIF4E-3

EIF4EBP1 | Human
Gene id: 1978
Alias: 4E-BP1, 4EBP1, BP-1, PHAS-I

EIF4EBP2 | Human
Gene id: 1979
Alias: 4EBP2, PHASII

EIF4ENIF1 | Human
Gene id: 56478
Alias: 4E-T, Clast4

When you click on a gene name (e.g. eIF4E | Human) you will find the various siRNA options.



EIF4E (Human)

eukaryotic translation initiation factor 4E

Alias
AUTS19 | CBP | EIF4E1 | EIF4EL1 | EIF4F | eIF-4E

ENTREZGENE 1977

The protein encoded by this gene is a component of the eukaryotic translation initiation factor 4F complex, which recognizes the 7-methylguanosine cap structure at the 5' end of messenger RNAs. The encoded protein aids in translation initiation by recruiting ribosomes to the 5'-cap structure. Association of this protein with the 4F complex is the rate-limiting step in translation initiation. This gene acts as a proto-oncogene, and its expression and activation is associated with transformation and tumorigenesis. Several pseudogenes of this gene are found on other chromosomes. Alternative splicing results in multiple transcript variants. [provided by RefSeq, Sep 2015].

Select Product for: ON-TARGETplus Human EIF4E siRNA

1. Contents

$0.00

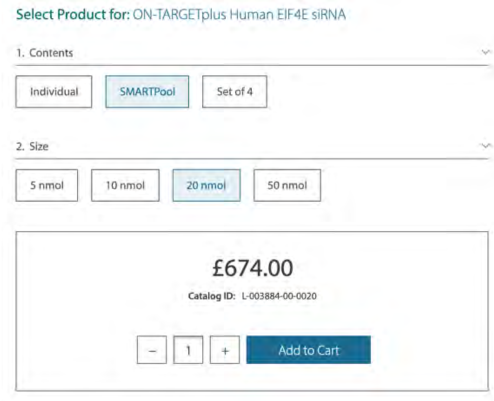
Catalog ID: Pending Selection(s)

In this case, you can order individual, SMARTPool, or Set of 4 siRNAs.

e.g. You chould choose ‘SMARTpool’ – this is a mixture of 4 siRNAs – the use of 4 different sequences simultaneously should increase the chance of getting good knockdown.

Alternatively, the use of individual siRNAs may be desirable to reduce costs, or to target a specific bit of the mRNA sequence (e.g. ORF or 5’/3’ untranslated region (UTR)).

You will notice that siRNAs are relatively expensive, and the costs vary enormously depending on your choices. Example:



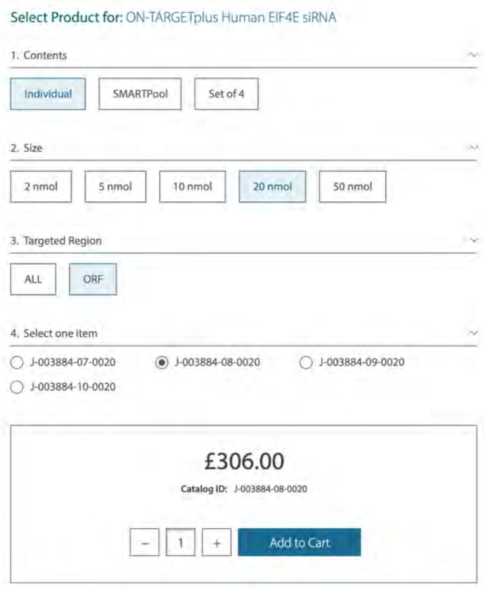
Select Product for: ON-TARGETplus Human EIF4E siRNA

1. Contents
Individual [✓ SMARTPool] Set of 4

2. Size
5 nmol 10 nmol [✓ 20 nmol] 50 nmol

£674.00

Catalog ID: L-003884-00-0020



Select Product for: ON-TARGETplus Human EIF4E siRNA

1. Contents
[✓ Individual] SMARTPool Set of 4

2. Size
2 nmol 5 nmol 10 nmol [✓ 20 nmol] 50 nmol

3. Targeted Region
ALL [✓ ORF]

4. Select one item
○ J-003884-07-0020 ● J-003884-08-0020 ○ J-003884-09-0020
○ J-003884-10-0020

£306.00

Catalog ID: J-003884-08-0020

You are thus able to order siRNAs that will be used to knockdown your protein of interest, and thereby gain further insights into its function in mammalian cells, and its potential role in stress granules.

Q23: How would you determine the efficiency of protein knockdown?

Q24: What could you do if the knockdown is inefficient?

Other databases

Other online databases can be used to look at other features of the novel ADAR1-interacting protein you have identified:

These are just a couple of examples, but there are many other resources that may be used.

(i) https://www.uniprot.org/ – use the GenBank accession number above to find your protein of interest, and then scroll through the page to find out more about the protein.

Example:

- Function – Gene Ontology (GO) analyses
- Domains and Repeats
- 3D structure
- etc.

Finding out as much as you can about your new protein will contribute to determining its cellular function.

(ii) https://www.ensembl.org/index.html – use the name of your new protein to search within ensembl. Alternatively, ensembl could similarly be used to perform BLAST searches etc. Ensembl contains a wealth of information, including:

- Analysis of gene expression – e.g. tissue specificity
- Splice variants
- Comparative Genomics etc.
- Gene Ontologies
- etc.

Glossary

A. GenBank

GenBank ® is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences (*Nucleic Acids Research*, 2013 Jan;41(D1):D36-42). GenBank is part of the International Nucleotide Sequence Database Collaboration , which comprises the DNA DataBank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and GenBank at NCBI. These three organizations exchange data on a daily basis.

The GenBank database is designed to provide and encourage access within the scientific community to the most up to date and comprehensive DNA sequence information. Therefore, NCBI places no restrictions on the use or distribution of the GenBank data. However, some submitters may claim patent, copyright, or other intellectual property rights in all or a portion of the data they have submitted.

B. Accession Numbers

An accession number in bioinformatics is a unique identifier given to a DNA or protein sequence record to allow for tracking of different versions of that sequence record and the associated sequence over time in a single data repository.

The International Nucleotide Sequence Database Collaboration DDBJ/EMBL/GenBank all receive sequence submissions, assign accessions, and exchange data so that all three groups represent the total collection. The accession assignment process is managed by prior agreement within the collaboration on which group will 'own' which accession prefix.

C. GenBank Accession Numbers

The format for GenBank Accession numbers are:

Protein: 3 letters + 5 numerals

(Example: AAF78782 corresponds to eIF4E from *Pisaster ochraceus*)

Nucleotide: 1 letter + 5 numerals OR 2 letters + 6 numerals

(Example: AF272369 corresponds to eIF4E from *Pisaster ochraceus*)

D. GI (gi) numbers

GI number (sometimes written in lower case, "gi") is simply a series of digits that are assigned consecutively to each sequence record processed by NCBI. The gi number bears no resemblance to the Accession number of the sequence record. The gi number provides a unique sequence identifier which is independent of the database source (e.g. nucleotide sequences from DDBJ/EMBL/GenBank, protein sequences from SWISS-PROT, PIR and many others.)

***Historical Note**: GI stands for "GenInfo Identifier." GenInfo was an early system used to access GenBank and related databases.*

E. FASTA sequence format

In bioinformatics, FASTA format is a text-based format for representing either nucleotide sequences or peptide sequences, in which nucleotides or amino acids are represented using single-letter codes. The format also allows for sequence names and comments to precede the sequences.

Example:

```
>MCHU - Calmodulin - Human, rabbit, bovine, rat, and chicken
ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTID
FPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREA
DIDGDGQVNYEEFVQMMTAK*
```

F. **HeLa cells** (https://en.wikipedia.org/wiki/HeLa)

A HeLa cell is a cell type in an immortal cell line used in scientific research. It is the oldest and most commonly used human cell line. The line was derived from cervical cancer cells taken on February 8, 1951 from Henrietta Lacks, a patient who died of her cancer on October 4, 1951. The cell line was found to be remarkably durable and prolific which warrants its extensive use in scientific research.

G. **I.M.A.G.E Clones** (https://www.sourcebioscience.com/life-science-research/clones/cdna-clones/image-consortium-cdna-clones/)

The I.M.A.G.E. Consortium was initiated in 1993 by four academic groups on a collaborative basis after informal discussions led to a common vision of how to achieve an important goal in the study of the human genome: the **I**ntegrated **M**olecular **A**nalysis of **G**enomes and their **E**xpression Consortium's primary goal is to create arrayed cDNA libraries and associated bioinformatics tools, and make them publicly available to the research community.

Utilizing high speed robotics, the I.M.A.G.E. consortium have arrayed over nine million individual cDNA clones into 384-well microtiter plates and have created sufficient replicas to distribute copies both to sequencing centers and to a network of five distributors located worldwide. Investigators who identify a clone of interest then contact any one of the distributors to obtain these clones – free of restriction – for further study.

(also see Dr Scadden's lectures for information about I.M.A.G.E. clones)

Notes

Notes

Week 6: Discussion of Practicals from Weeks 1–5

Two activities will take place in Week 6, Michaelmas Term:

a) A discussion of Practicals from Weeks 1–5 (11am (except Monday – 12pm))

Dr Darerca Owen (do202@cam.ac.uk)

The discussion will involve an overview of all the practical knowledge gained in weeks 1-5, with an opportunity to apply this to a theoretical protein.

More information about the discussion session will be provided on Moodle.

b) Chemistry Revision session

Dr Katherine Stott (Sanger Building – Room 3.53, Tel. 333669)
http://www.bioc.cam.ac.uk/uto/stott.html

- Revision sessions will be held for all students **who are not taking Part 1B Chemistry B**. Further details about these sessions (groups, venues, materials) will be available on Moodle prior to Week 6. The handout will be provided at a BMB lecture preceding the session.

- Additional materials will be made available on Moodle for students currently taking Chemistry B. The online handout will cover enzyme mechanisms from a chemistry viewpoint which you will cover in some detail in the Easter Term. Much of it is revision of concepts covered in IA. You should work through it by yourself, and compare your answers to those in the filled-in version that will appear on Moodle at the end of week 7.

Week 7 Practical

Journal Club

Journal clubs will be at 2pm every day except Monday (3pm).

Please see Moodle for more information regarding groups and venues.

Week 8 – Analysis of Protein Structure by Molecular Graphics

Objectives

The aim of this practical is to give you an overview of the current understanding of protein structure, using molecular graphics software as a tool to aid the learning process. Visualising and manipulating molecules with a sophisticated program, the ICM–Browser, will help you learn how to analyse protein architecture in a hierarchical way.

When this practical has been completed you should be able to:

- Recognise common elements of secondary structure, super-secondary structural motifs, protein folds and tertiary structures and relate them to possible protein functions.
- Use the ICM–Browser to view and manipulate protein structures.

Introduction

The complex nature of protein structures makes them extremely difficult to represent in two dimensions. Models must be built in three dimensions for a full appreciation of their structures and hence their functions. This can be done in two ways: by building physical models or by generating an interactive computerised graphical representation. Physical models have some benefits over computer models in that they are truly three dimensional, but computer models are far easier to analyse, distribute, manipulate and represent.

A protein model is stored on a computer as a file containing a list of Cartesian coordinates, in which each atom of the protein is defined by *x*, *y* and *z* coordinates in three-dimensional space. The bonds between the atoms are generally not stored in this file, but rather calculated by the computer algorithms used to display the structure. The precise way in which a structure is displayed using computer graphics can be varied so as to facilitate the observation of particular features of the protein being investigated.

The aim of this practical is for you to learn how to view protein structures and recognise features which often occur in them, rather than merely perceiving a collection of rather uninteresting and apparently random lines!

In the practical you will use an interactive molecular visualisation program, the ICM–Browser, to view protein structures presented in a series of electronic documents. Molecular graphics software of this sort is very easy to operate and is widely used in research. It enables you to generate a range of interactive pictures of molecules that highlight different aspects.

The practical is designed to guide you through a hierarchical approach to protein structure. It is divided into the following sections:

1) Analysing the covalent connectivity of a polypeptide chain
2) Analysing the secondary structure of proteins
3) Analysing protein super-secondary structures
4) Analysing functional motifs
5) Analysing the tertiary structure of proteins
6) Analysing the structural basis of protein function

As you progress through the sections, the protein structures you analyse will become increasingly complicated and may only be comprehensible if you have fully appreciated the previous sections.

The whole practical will be made available for internet access on the BMB Moodle site after the week's practical classes are finished. This should make it possible for you to go back and work through sections of the practical in more detail at a later date using computer facilities in your college. Details of how to install your own copy of the ICM–Browser are provided on the Moodle site.

References

Books

- *In Focus: Protein Structure* (1993) Darby, N.J. & Creighton, T.E., IRL Press.
- *Protein Structure* (1998) Branden, C. & Tooze, J., 2nd edition, Garland.
- *Proteins - Structures and Molecular Properties* (1993) Creighton, T.E., 2nd edition, Freeman.
- *Introduction to Protein Architecture* (2001) Lesk, A.M., Oxford.
- *Protein Structure and Function* (2009) Petsko, G.A. & Ringe, D., Oxford.
- *How Proteins Work* (2011) Williamson, M., Garland

Websites

- *More details about the ICM–Browser*

  http://www.molsoft.com/icm_browser.html

- *PyMOL – a different state-of-the-art open source molecular visualization system: a free standalone program (available for PC, Mac or Linux) that can produce publication quality ray-traced graphics*

  https://pymol.org/2/

- *Search for structures deposited in the Protein Data Bank*

  https://www.pdb.org

  https://www.ebi.ac.uk/pdbe

- *Hierarchic databases of protein structures*

  http://scop2.mrc-lmb.cam.ac.uk/

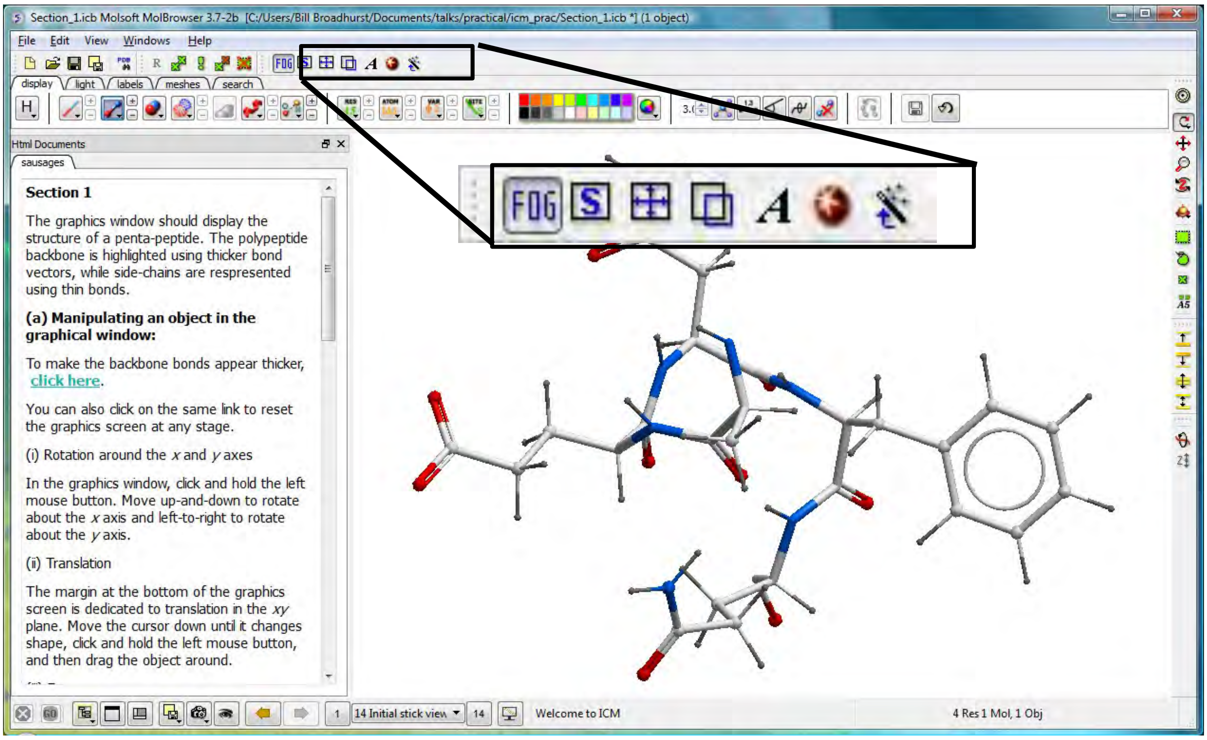
  http://www.cathdb.info

Section 1: Analysis of the covalent connectivity of the polypeptide chain

On the desktop of your workstation, open the folder with the tile “MolGraph_Practical”, and double click on the file “Section_1.icb”. This will launch the ICM–Browser, which will display two panels, one containing text, and the other showing a graphical model of a short polypeptide chain.

1(a) Manipulating an object in the graphical window

First, on the uppermost bar of icons, toggle off the “FOG” icon, which is switched on by default. You can improve the quality of the graphics on screen by selecting the “*A*” icon (to switch on antialiasing, thereby minimising lines with jagged edges), and also the neighbouring circular icon, to toggle on “high quality graphics”. If the browser appears to run slowly at any stage, you can toggle off these antialiasing and high-quality options.


Section_1.icb Molsoft MolBrowser 3.7-2b [C:/Users/Bill Broadhurst/Documents/talks/practical/icm_prac/Section_1.icb *] (1 object)
File Edit View Windows Help
FOG
display light labels meshes search
Html Documents
sausages
14 Initial stick view
Welcome to ICM
4 Res 1 Mol, 1 Obj

Section 1

The graphics window should display the structure of a penta-peptide. The polypeptide backbone is highlighted using thicker bond vectors, while side-chains are respresented using thin bonds.

(a) Manipulating an object in the graphical window:

To make the backbone bonds appear thicker, click here.

You can also click on the same link to reset the graphics screen at any stage.

(i) Rotation around the *x* and *y* axes

In the graphics window, click and hold the left mouse button. Move up-and-down to rotate about the *x* axis and left-to-right to rotate about the *y* axis.

(ii) Translation

The margin at the bottom of the graphics screen is dedicated to translation in the *xy* plane. Move the cursor down until it changes shape, click and hold the left mouse button, and then drag the object around.

If necessary, click on the link labelled “**click here**” to emphasize the polypeptide backbone by marking it with thick bond vectors; side-chain bonds between heavy atoms will be represented by thin lines, and bonds to hydrogen atoms by even thinner lines. You can click on the same link at any stage to reset the view displayed in the graphics panel.

Next, learn how to use the mouse to manipulate the image:

(i) *Rotation around the x and y axes.*

With the cursor inside the graphics panel, click and hold the left mouse button. Move the mouse up-and-down to rotate about the horizontal *x* axis and left-to-right to rotate about the vertical *y* axis.

(ii) *Translation along the x and y axes.*

The margin at the bottom of the graphics panel is dedicated to translation in the *xy* plane. Move the cursor down until it changes shape (into a four-way arrow), click and hold the left mouse button, and then practice dragging the object around.

(iii) *Zooming along the z axis.*

The left-hand margin of the graphics panel is dedicated to moving the object closer or further away along the z axis. Move the cursor left until it changes shape (into a magnifying glass), click and hold the left mouse button, and then zoom in and out.

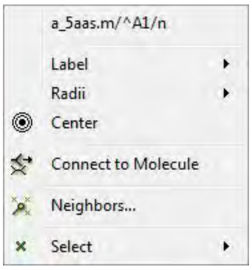
(iv) *Rotation about the z axis.*

The top margin of the graphics panel is dedicated to rotation around the z axis. Move the cursor up the screen until it changes shape (into two semi-circular arrows), click and hold the left mouse button, and then rotate the object clockwise and anticlockwise.

(v) *Clipping planes.*

For large molecules, it is often convenient to view part of an object in a "slab", hiding any atoms that lie in front and behind a pair of "clipping planes". These can be manipulated by clicking and dragging the cursor in the right-hand margin of the graphics panel. Play with the three settings in the upper, middle and lower segments of the margin to discover how they work.

1(b) Interrogating an atom



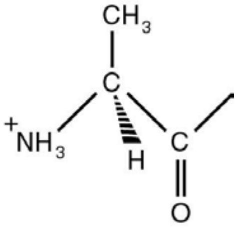
Clicking on an atom with the right mouse button will bring up a small menu. For now, concentrate on the first line, which gives a report on the atom you just selected. The report will indicate: the name of the structure file (e.g. "a_5aas.m"); the residue type (e.g. "A", meaning alanine); its number in the primary sequence (e.g. 1); and the element type and name of the atom (e.g. "n", meaning a backbone amide nitrogen atom).

1(c) Residue numbering

1.1. Ascertain the amino acid sequence of the penta-peptide and fill this into the boxes below, using the "three-letter code".

(***Hint***: *switch on automatic labelling of the residues by clicking on the link for part (c)*)

1.2. Draw out the covalent structure of the penta-peptide, following on from the first residue shown below. You may want to refer to the Appendix to look up the structures of the remaining residues. (***Hint***: *there's no need to get the stereochemistry right – for now just focus on the covalent structure of the peptide backbone and the side-chains*)

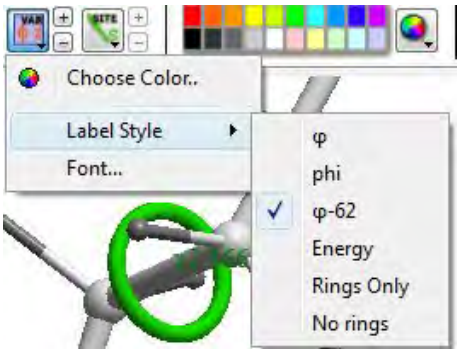


1(d) Hydrogen bonds

1.3. Click on the link for part (d) to display the single hydrogen bond present in the penta-peptide. Which atoms of which residues are linked by this hydrogen bond?

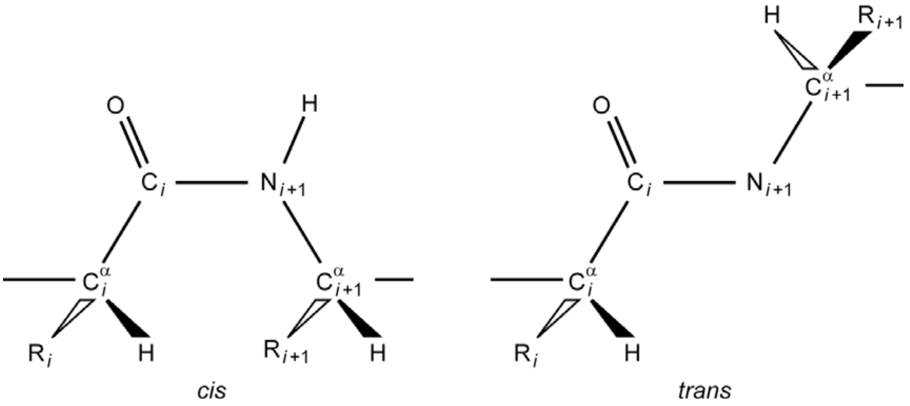
....

1(e) Backbone dihedral angles


Choose Color..
Label Style
Font...
φ
phi
✓ φ-62
Energy
Rings Only
No rings

Click on the link for part (e) to display a set of green circles which represent measurable backbone and side-chain dihedral angles. To switch on the values of these angles, click and hold on the "VAR φ 2" icon in the second bar at the top of the graphics panel. This will bring up a square menu; click on the "Label Style" option to bring up a secondary menu and choose the "φ-62" style. This will display the name of the angle followed by its value.

1.4. The partial double bond character of the peptide bond, which links the carbonyl carbon of residue (*i*) with the amide nitrogen of residue (*i* +1), constrains the CO, NH and both adjacent C^{α} atoms to lie in the same plane. Two planar orientations are possible, in which adjacent C^{α} carbons are fixed in either the *cis* or the *trans* configuration:


O
H
C_i
N_{i+1}
C^{α}_i
C^{α}_{i+1}
R_i
H
R_{i+1}
H
cis
H
R_{i+1}
C^{α}_{i+1}
O
C_i
N_{i+1}
C^{α}_i
R_i
H
trans

Which of these configurations do the residues in the penta-peptide possess? Why do you think this might be? (***Hint**: list the four values of ω measured in the graphical panel*)

....

....

....

1(f) Different representations

Next, try out various different representations of the penta-peptide (such as *wire*, *xsticks*, *ribbon*, *CPK*, etc.) by using the links in section (f).

Wire: this basic representation emphasizes the different types of bonds in molecules; it is good for small molecules such as drugs, but is probably not the best choice for looking at large protein assemblies.

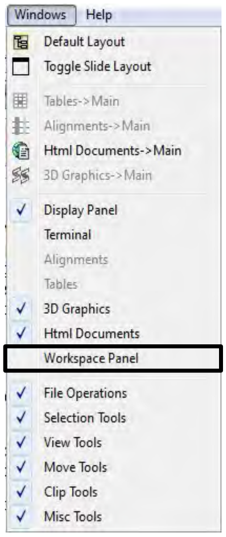
Xsticks: this ball-and-stick representation provides a "true" idea of the bonding present, but provides too much detail to help in understanding overall protein structures; however, it is useful for highlighting specific features of, for example, enzyme active sites.

Ribbon: this shows alpha-helices, beta-strands and connecting "loop" regions in their customary "cartoon" forms, providing a useful way of following the secondary structure and topology of a protein structure. Unfortunately, in this case there are not enough amino acid residues present for the graphics software to recognize a genuine alpha-helix, so it has decided that the whole penta-peptide must be a worm- like "loop". This representation will be much more useful in later, more complex examples.

CPK: this space-filling representation shows the different atoms with spheres that represent their true volumes, and avoids giving the impression that protein structures are full of empty space; however, the display is now too crowded to convey an accurate idea of how the protein works. CPK stands for "Corey-Pauling-Koltun", after the authors who first suggested this representation, along with the standard atomic colour scheme of white for hydrogen atoms, blue for nitrogen, black for carbon, and red for oxygen, etc.

Surfaces: these representations are solvent-excluded surfaces, formed by rolling a sphere the same size as a water molecule around the protein to indicate the volume that solvent molecules cannot penetrate; they are useful for giving a feel of what protein surfaces and binding sites look like to molecules they interact with.

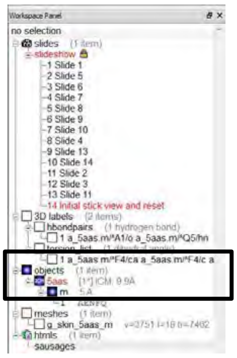
1(g) Optional extension material: the workspace panel


Windows
Help
Default Layout
Toggle Slide Layout
Tables->Main
Alignments->Main
Html Documents->Main
3D Graphics->Main
✓ Display Panel
Terminal
Alignments
Tables
✓ 3D Graphics
✓ Html Documents
Workspace Panel
✓ File Operations
✓ Selection Tools
✓ View Tools
✓ Move Tools
✓ Clip Tools
✓ Misc Tools

From the "Windows" label on the bar at the top of the ICM–Browser, select the "Workspace Panel" option. A new panel will appear on the left, but you may need to resize this, along with the adjacent "Html Documents" panel. The Workspace Panel offers a view under the bonnet of the program.

The penta-peptide structure can be found under the "objects" heading. Within this, you will find the amino acid sequence in one letter code: "AENFQ".

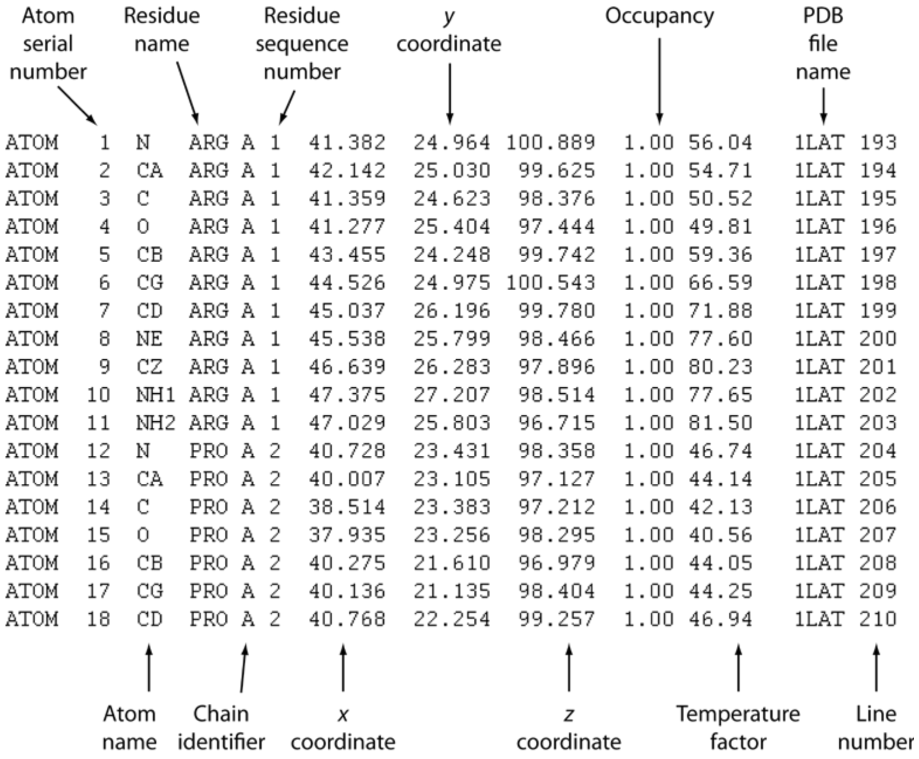
Use the cursor to select a couple of residues, such as "AE". The graphical display will highlight the selected atoms with green crosses and the connecting bonds will turn green. Change the colour of the selected atoms and bonds by choosing a shade from the colour palette on the second icon bar at the top of the program window. Initially, only the atoms will appear to change colour. However, deselect the atoms by clicking anywhere on the white space in the Workspace Panel and you will see that the bonds have also changed colour. What other transformations can you make using different icon button commands?


Workspace Panel
no selection
slides (1 item)
slideshow
1 Slide 1
2 Slide 5
3 Slide 6
4 Slide 7
5 Slide 8
6 Slide 9
7 Slide 10
8 Slide 4
9 Slide 13
10 Slide 14
11 Slide 2
12 Slide 3
13 Slide 11
14 Initial stick view and reset
3D labels (2 items)
hbondpairs (1 hydrogen bond)
1 a_5aas.m/^A1/o a_5aas.m/^Q5/hn
torsion_list
1 a_5aas.m/^F4/ca a_5aas.m/^F4/c a
objects (1 item)
5aas [1*] ICM: 9.9A
m 5A
1 AENFQ
meshes (1 item)
g_skin_5aas_m v=3751 l=18 t=7402
htmls (1 item)
sausages

1(h) PDB (Protein Data Base) files

The atomic coordinates of the penta-peptide you have been looking at are stored as a text file in a format specified by the Protein Data Bank, a computer-based archival database for macromolecular structures. The database was established in 1971 by Brookhaven National Laboratory, New York, as a public domain repository for resolved crystallographic structures. In 1999, the Protein Data Bank moved to the "Research Collaboratory for Structural Biology" (http://www.rcsb.org/pdb/). The database now contains structures elucidated by additional experimental methods, such as nuclear magnetic resonance spectroscopy and electron microscopy.

The format of a typical protein database (PDB) file is shown below. After a long header, which includes remarks concerning details of authorship, publication and the identity and preparation of the protein studied, the *x*, *y* and *z* coordinates of the atoms of each residue are listed. The unit for the coordinates is the Ångstrom (1 Å = 10^{-10} m = 0.1 nm).


Atom serial number
Residue name
Residue sequence number
y coordinate
Occupancy
PDB file name
Atom name
Chain identifier
x coordinate
z coordinate
Temperature factor
Line number

```
ATOM   1  N   ARG A 1  41.382  24.964 100.889  1.00 56.04   1LAT 193
ATOM   2  CA  ARG A 1  42.142  25.030  99.625  1.00 54.71   1LAT 194
ATOM   3  C   ARG A 1  41.359  24.623  98.376  1.00 50.52   1LAT 195
ATOM   4  O   ARG A 1  41.277  25.404  97.444  1.00 49.81   1LAT 196
ATOM   5  CB  ARG A 1  43.455  24.248  99.742  1.00 59.36   1LAT 197
ATOM   6  CG  ARG A 1  44.526  24.975 100.543  1.00 66.59   1LAT 198
ATOM   7  CD  ARG A 1  45.037  26.196  99.780  1.00 71.88   1LAT 199
ATOM   8  NE  ARG A 1  45.538  25.799  98.466  1.00 77.60   1LAT 200
ATOM   9  CZ  ARG A 1  46.639  26.283  97.896  1.00 80.23   1LAT 201
ATOM  10  NH1 ARG A 1  47.375  27.207  98.514  1.00 77.65   1LAT 202
ATOM  11  NH2 ARG A 1  47.029  25.803  96.715  1.00 81.50   1LAT 203
ATOM  12  N   PRO A 2  40.728  23.431  98.358  1.00 46.74   1LAT 204
ATOM  13  CA  PRO A 2  40.007  23.105  97.127  1.00 44.14   1LAT 205
ATOM  14  C   PRO A 2  38.514  23.383  97.212  1.00 42.13   1LAT 206
ATOM  15  O   PRO A 2  37.935  23.256  98.295  1.00 40.56   1LAT 207
ATOM  16  CB  PRO A 2  40.275  21.610  96.979  1.00 44.05   1LAT 208
ATOM  17  CG  PRO A 2  40.136  21.135  98.404  1.00 44.25   1LAT 209
ATOM  18  CD  PRO A 2  40.768  22.254  99.257  1.00 46.94   1LAT 210
```

Section 2: Analysis of protein secondary structure – alpha helices

Click on "**File"** and then "**Quit**" to exit the "Section_1.icb" file. Next, go back to the "MolGraph_Practical" folder and double click on the file named "Section_2.icb", which should show an α-helix that has been excised from the protein ROP. As before, the polypeptide backbone is highlighted using thicker bonds.

2.1. Click on the link for part (b) to display the hydrogen bonds as dotted lines interspersed with small spheres. Choose a residue near the middle of the α-helix. If a hydrogen bond starts at the carbonyl oxygen atom of residue (*i*), what is the sequence number of the residue that donates the amide hydrogen to the hydrogen bond? How many residues are there in a single turn of the helix?

2.2. Are the hydrogen bonds linear or bent?

(***Hint***: *to measure the O-H-N angle across a hydrogen bond, select the angle measurement tool from the second icon bar at the top of the window. Then, click on three atoms, such as the carbonyl O of N10, the amide H of F14, and the amide N of F14. The value of the bond angle will then be displayed in degrees. To remove any unwanted measurements, select the nearby icon that displays a red cross*).

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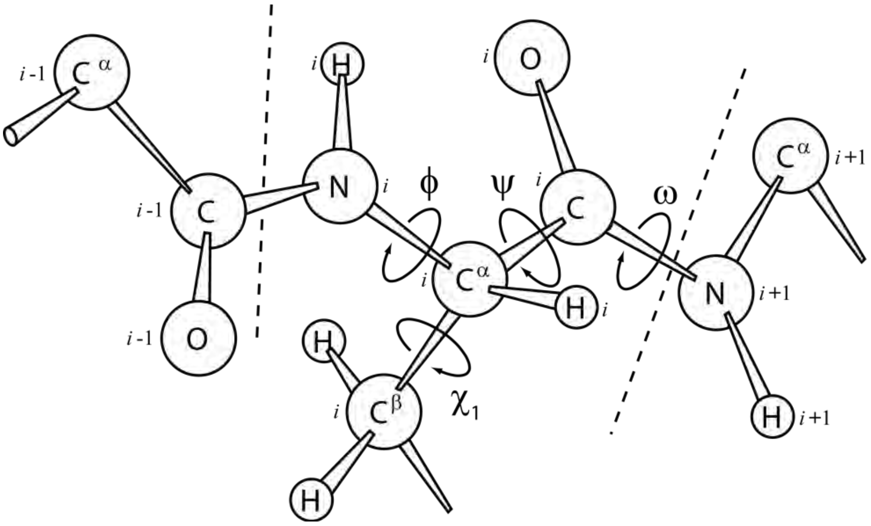
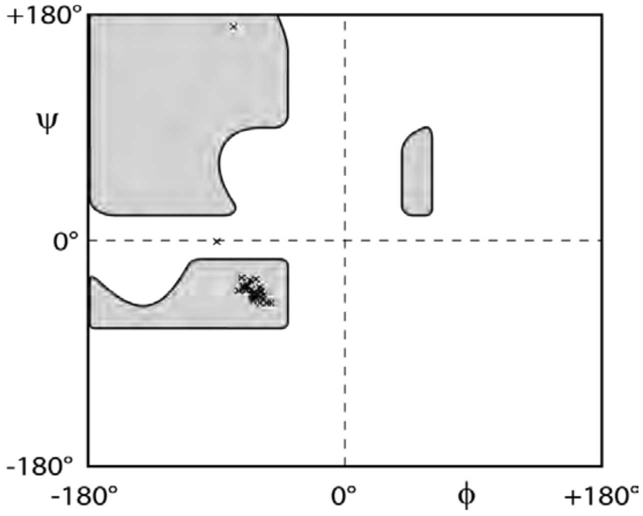


The figure above shows the definition of the three backbone *torsion angles* of residue (*i*): φ (for the angle made by the three bonds and four atoms C'_{i-1}–N_i–C^{α}_i–C'_i), ψ (for N_i–C^{α}_i–C'_i–N_{i+1}) and ω (for C^{α}_i–C'_i–N_{i+1}–C^{α}_{i+1}). The side-chain conformation is described by χ_1, χ_2, etc., where χ_1 is the torsion angle around the bond between C^{α} and C^{β}. A useful way of analysing the backbone conformation of a polypeptide is to plot φ against ψ for each residue, creating what is known as a *Ramachandran plot*. Only certain values of φ and ψ are permitted, because many rotations about these bonds lead to unfavourable steric interactions between atoms of the same and neighbouring residues. A Ramachandran plot for the α-helix shown in the Section 2 file is displayed below, with the "allowed regions" shaded grey. Note how the values of φ and ψ for the α-helix cluster into a small region of the plot.



2.3. Click on the link for part (c) to display to display circles that represent the backbone and side-chain dihedral angles, and then switch on the values using the "VAR φ 2" icon, the "Label Style" option, and the "φ-62" style. List the backbone and side-chain dihedral angles for residue Met-11.

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2.4. Click on the link for part (d) to display the structure without distracting side-chains. Of the 30 backbone carbonyl oxygen and amide nitrogen atoms in this helix, only 7 amides (excluding the N-terminal amine) and 7 carbonyls are NOT involved in hydrogen bonds. Where in the helix are these?

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2.5. Taking into account the answer to the last question, how would you expect the free energy of formation of a helical turn between residues 1-5 to compare with that for forming one between residues 5-9 or 10-14? (***Hint***: *remember that $\Delta G = \Delta H - T\Delta S$.*)

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2.6. Why do you think that proline residues are very rarely found in helices? When they are found in helices, can you account for why they tend to appear at the ends of these structures?

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2.7. Click on the link for part (e) to display the structure in ribbon format and coloured from blue at the N-terminus to red at the C-terminus. Is the coiling of the helix left-handed or right-handed?

(***Hint***: *imagine the helix is a screw and you are screwing it into a piece of wood; given that your right hand twists more naturally clockwise and your left hand twists more naturally anti-clockwise, would it be easier to use your left or right hand to turn the screw?*)

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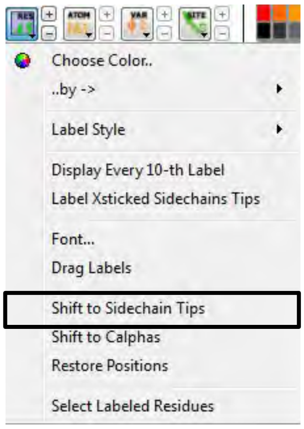
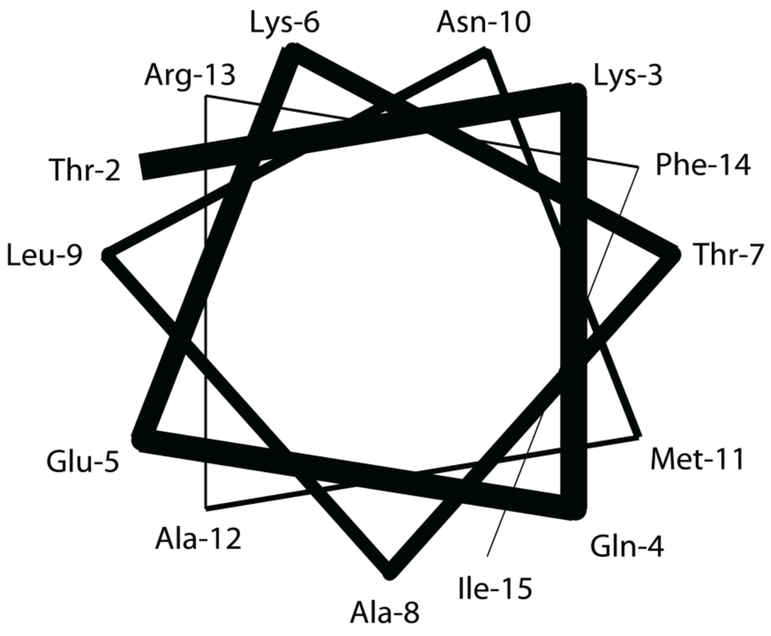

Choose Color..
..by ->
Label Style
Display Every 10-th Label
Label Xsticked Sidechains Tips
Font...
Drag Labels
Shift to Sidechain Tips
Shift to Calphas
Restore Positions
Select Labeled Residues

2.8. Click on the link for part (f) to display the first few turns of the helix in *xsticks* format, oriented so that you are looking along it with the N-terminal end facing you. You may find it useful to move the residue labels to the ends of the side-chains by holding the cursor down on the "RES *A5*" button in the second icon bar at the top of the window, and then selecting "Shift to Sidechain Tips".

The *helical wheel* diagram below represents this view. Fill in the nature of each of the amino acid residues indicated (i.e. +ve/–ve, polar, hydrophobic, etc.; refer to the Appendix if you need to).

N.B.: ignore residue 1, as it is not in an α-helical conformation.


Lys-6
Asn-10
Arg-13
Lys-3
Phe-14
Thr-2
Leu-9
Thr-7
Glu-5
Met-11
Ala-12
Gln-4
Ile-15
Ala-8

2.9. What do you notice about the distribution of hydrophobic and hydrophilic residues in the helical wheel representation?

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2.10. What explanation can you suggest for this distribution (taking into account the fact that the helix is part of a larger globular protein)?

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2.11. An α-helix has a net dipole moment. Why do you think this is, and which way do you think it is pointing?

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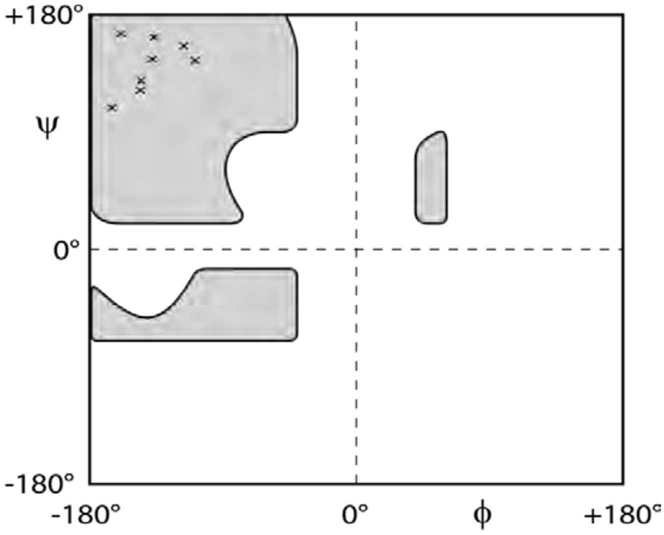
Section 3: Analysis of protein secondary structure – beta strands

The file, "Section_3.icb" shows two regions of polypeptide chain that are hydrogen bonded together to form an *anti-parallel* β-sheet. The distribution of the φ and ψ angles is now very different from that displayed by an α-helix (compare the figure below with that on page 148).



Notice that in an antiparallel β-sheet the hydrogen bonds are almost parallel to each other, are perpendicular to the direction of the strand, and form large and small hydrogen bonded 'rings'. These hydrogen bonded 'rings' are related by a two-fold rotational symmetry axis perpendicular to the plane of the sheet.

(***Hint**: to spot this pseudo-symmetry, try executing a rotation of 180° around the z axis*)

3.1. Click on the link for part (b) to display a pair of β-strands with *parallel* contacts. To answer the following question, you may need to toggle back and forth between the links for part (a) and part (b). Can you spot three differences between the hydrogen bonding patterns in the parallel β-sheet and the anti-parallel β-sheet? How might these differences affect the relative stabilities of parallel and anti-parallel β-sheets?

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Click on the link for part (c) to display the side-chain atoms of the antiparallel β-sheet in a space-filling representation. Hydrophobic side-chains (i.e. either Val, Ile, Leu, Phe, Trp, Met, Ala or Cys) are coloured in blue. Notice how the side-chains of adjacent residues in the sequence of each strand point in approximately opposite directions, so that the polypeptide backbone appears to be sandwiched between two side-chain layers. You might want to make this more apparent by selecting "Shift to Sidechain Tips" here.

3.2. Compare the properties of the amino acid side-chains on either side of the β-sheet: what do you observe? Why might this be?

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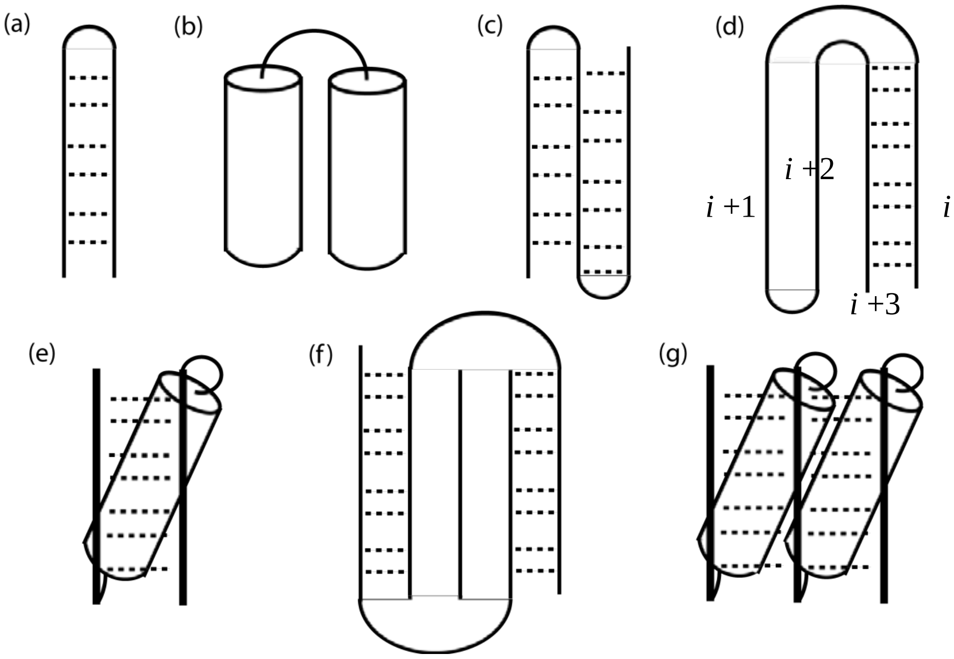
Section 4: Super-secondary structure – recurrent structural motifs in globular proteins.

The way in which the β-strands and/or α-helices of a protein are connected together in space is referred to as the *topology* of the protein. In general, the arrangement of these units of secondary structure is such that distinct *layers* of structure are formed. It is between these layers of structure that the hydrophobic cores of proteins lie, protected from water to a large degree by the framework formed by the polypeptide backbone.

Certain topologies that are local with respect to the sequence tend to recur in a large number of different proteins, even where there is no evidence of an evolutionary relationship. These are commonly referred to as *super-secondary structures*. The figure below shows schematic representations of some of the more common super-secondary structures.

Simple topology diagrams of (a) a beta-turn; (b) an alpha-alpha unit (coiled-coil); (c) a beta-meander; (d) a Greek key motif; (e) a beta-alpha-beta motif; (f) a double Greek key motif; and (g) a Rossmann fold.



Section 4: Super-secondary structures

Open the file "Section_4.icb" to view what is perhaps the simplest example of super-secondary structure: a β-hairpin in which two β-strands are linked via a tight β-turn (see panel (a) on the previous page).

4.1. Examine the hydrogen bonding in the β-turn. How distant in sequence are the two groups that form the hydrogen bond at the apex of the turn? How does this periodicity compare with that of the α-helix you were examining in Section 2 (i.e. in question 2.1)?

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Click on the link for part (b) to display the α-helical equivalent of the β-hairpin (see panel (a) in the figure on page 152). Although there is obviously no main chain hydrogen-bonding between the two α-helices, they are closely packed in an aligned fashion. Such arrangements of α-helices are often referred to as *coiled-coils* and in some proteins the interacting helices can be very long (e.g. leucine zippers and fibrous proteins such as α-keratin or myosin).

The link for part (c) reveals an elaboration on a simple β-hairpin called a β-meander (see panel (c) on page 152). β-meanders consist of an array of β-strands, each linked by β-turns (or sometimes more extensive loops), in which β-strand (i) is always hydrogen-bonded to β-strand (i+1). This link shows a three-stranded meander, but many more strands can occur in a β-meander.

4.2. The β-meander shown here is part of a larger globular protein in which there are two β-sheets that pack together to form a 'sandwich', the filling being the hydrophobic core. By examining the structure, decide whether residue 18 is likely to be on the hydrophilic exterior or the hydrophobic interior.

(***Hint**: Click on the link for part (d) to view the side-chains coloured according to hydrophobicity, and then select "Shift to Sidechain Tips" once again*)

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The link in part (e) will show you a more complex topology that is extremely common in a number of apparently unrelated proteins, commonly referred to as a 'beta-alpha-beta' unit (see panel (e) on page 152). Here the sequence of the protein contains first a β-strand, then an α-helix, and then another β-strand which is hydrogen-bonded to the first such that they form a parallel β-sheet.

4.3. Beta-alpha-beta units have a handedness, just like α-helices and β-sheets, and the handedness is always the same. By following the path of the polypeptide backbone of the entire unit, can you assess the handedness of the beta-alpha-beta unit?

(***Hint**: orient the molecule so that the blue strand at the N-terminus travels across the screen from left to right, with the α-helix below and the red C-terminal strand behind, i.e. into the screen.*)

The beta-alpha-beta motif is an example of a multi-layer structure: the helix is part of one layer, while the β-strands participate in a second layer. The connections between the β-strands and the α-helix cross from one layer to another and are therefore referred to as *cross-over connections*. With extremely few exceptions, cross-over connections involving β-strands are always of the same handedness.

An intriguing topological arrangement of β-strands that recurs in a large number of proteins is the *Greek key motif*. The simplest definition of a Greek key is that it consists of an array of four consecutive β-strands in which β-strand (*i*) is hydrogen-bonded to β-strand (*i*+3), as shown in panel (d) on page 152. Sometimes all four strands lie in the same layer of structure (making a 4,0 Greek key); sometimes three strands are in one layer and one is in a different layer (a 3,1 Greek key); alternatively, two of the β-strands form a β-sheet in one layer, while the other two form another β-sheet in a separate layer (a 2,2 Greek key). The term *Greek key* is coined from the fact that two-dimensional representations of the motif make a pattern similar to one that is often found on ancient Greek vases. Click on links (f) and (g) to view examples of 3,1 and 2,2 Greek keys, with the relevant strands coloured in yellow.

4.4. A common variation on the four-stranded Greek key is the five-stranded, or "double" Greek key (see panel (f) on page 152). The double Greek key motif can be found in immunoglobulins, electron transport proteins, viral coat proteins, inhibitors and toxins, as well as enzymes.

To view an example of a double Greek key coloured from blue at the N-terminus to red at the C-terminus, click on link (h). The five consecutive β-strands in this motif are arranged in space such that two different pairs of β-strands have strand (*i*) hydrogen bonded to strand (*i*+3), creating two overlapping single Greek key arrangements: one for strands 1 to 4, and another for strands 2 to 5. The β-strands in the double Greek key topology form two distinct β-sheets (one with three strands and one with two) in two separate layers. As a result, some of the β-strands in the same β-sheet are connected via β-strands in the opposite β-sheet by cross-over connections. How would you classify the two Greek keys shown in this link using the scheme outlined? For example, do strands 1 to 4 form a 4,0 Greek key? What about strands 2 to 5?

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Section 5: Functional motifs

Before moving on to discuss the tertiary structures of proteins, it is worth discussing another group of recurring super-secondary structures that are all *functional* motifs. These local topologies are found in a number of proteins with quite unrelated functions, but which bind the same substrate (be it DNA, Ca^{2+}, NADH, etc.). Here you will briefly examine three types of functional motif: the zinc finger and helix-turn-helix motifs from two DNA-binding proteins and the Rossmann fold from a nucleotide-binding protein.

Open the file "Section_5.icb" to view the glucocorticoid receptor bound to DNA (PDB code: 1GLU). In fact this view displays a homo-dimer of the zinc finger DNA-binding domain of GR. These particular zinc fingers contain four cysteine residues (shown in *xsticks* format) co-ordinating each zinc atom (shown as red spheres). Other varieties of zinc finger may have two cysteines and two histidines co-ordinating each zinc atom.

5.1. How many zinc atoms are bound per monomer?

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5.2. Which groove in the DNA (i.e. major or minor) does this protein seem to interact with?

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5.3. Do the zinc atoms appear to play a direct role in interacting with the DNA?

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Click on link (b) to display the 434 Cro repressor bound to DNA (PDB code: 3CRO). This protein is also a homo-dimer. Helix-turn-helix motifs are shown in green and yellow, one in each protomer. The green coloured section is termed the "recognition helix" and the "positioning helix" is shown in yellow.

5.4. Which groove in the DNA does the recognition helix interact with? Do any residues interact with the *other* groove?

(***Hint**: to see additional interactions with the DNA, click on link (c)*)

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Link (d) displays the enzyme lactate dehydrogenase (PDB code: 9LDT). An arrangement of β-strands and α-helices known as the Rossmann fold (see panel (g) on page 152) is picked out in the ribbon representation. This fold is found in a number of enzymes and is involved in binding nucleotides; in this case it binds to NADH, highlighted in both *xsticks* and CPK space-filling spheres). It has been suggested that the recurrence of this fold may be the result of convergent as well as divergent evolution. The Rossmann fold is made up of two overlapping beta-alpha-beta units, although most textbooks refer to the Rossmann fold as a super-secondary structure in its own right.

Section 6: Analysis of the tertiary structure of proteins

Just as with secondary structure and super-secondary structures, the tertiary structures of proteins can be grouped into a number of distinct classes depending on their overall folds. Two levels of tertiary structure classification are used, one which reflects the usage and distribution of secondary structure in the protein, and another more detailed classification that is topology-based. The former (due to M. Levitt and C. Chothia) groups all globular proteins into 5 distinct classes:

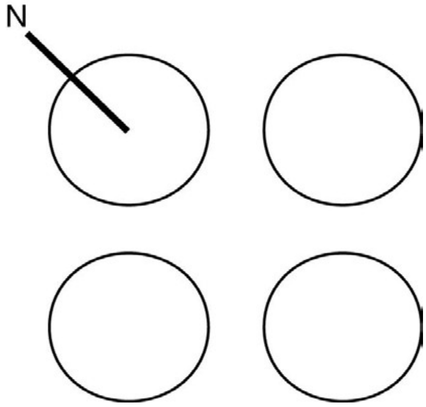
(i) **α**, in which the vast majority of the protein is α-helical (e.g. myoglobin)
(ii) **β**, where the vast majority of the protein is β-sheet (e.g. immunoglobulin domain)
(iii) **α+β**, in which there are distinct contiguous α-helical and β-sheet regions in the sequence of the protein (e.g. lysozyme)
(iv) **α/β**, where the sequence of the polypeptide chain alternates between α-helices and β-strands (e.g. triose phosphate isomerase)
(v) **cross-linked irregular domains**, which are small protein regions, often rich in cysteine residues; binding to metal ions or disulphide bridges are used to cross-link different parts of the domain together, and as a result these proteins often possess irregular secondary structure (e.g. ferredoxin).

More detailed topological descriptions of the overall tertiary folds of proteins can be used to identify different structural families. Understanding these classifications is useful, as modern protein structure databases can require users to define the types of fold they are interested in. Several hierarchical classifications of the entire set of known protein structures are available on the internet (e.g. *SCOP*, "Structural Classification of Proteins", based in Cambridge; or *CATH*, "Class, Architecture, Topology, Homology", based in London).

The following snapshots illustrate examples of proteins with particularly well-known tertiary folds: a "four-helix bundle", a "TIM-barrel" and an "immunoglobulin fold". For each of these proteins, use the protein structural analysis skills you have acquired so far to class the protein as being either α, α/β, etc., and to identify any super-secondary structures.

Open the file "Section_6.icb" to view cytochrome c', a four-helix-bundle protein (PDB code: 2CCY), coloured from blue at the N-terminus to red at the C-terminus.

6.1. The figure below shows a simplified top view of the protein, such that each circle represents one of the four helices. Show the connectivity between the helices in the sequence on the diagram. Use ***bold*** lines to represent connections between helices at the top of the protein and *dotted* lines to show connections at the bottom. The result will be a two-dimensional topology diagram of the protein. This procedure is worthwhile because different four helix bundle assemblies can be connected in different ways.



6.2. Do you recognise the prosthetic group in this protein?

(***Hint**: right click on the green sphere and on an atom in the prosthetic group to get more information.*)

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What can you say about the environment that the prosthetic group is in?

(***Hint**: click on link (b) to view a representation showing atoms in CPK space-filling mode, with non-polar residues shaded in purple, and polar residues in yellow. This view uses a clipping plane to slice through the structure. You can move the clipping plane backwards and forwards using the control in the lower portion of the right-hand margin of the window.*)

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Link (c) shows a TIM barrel from the protein triosephosphate isomerase (PDB code: 1TRE). This is one of the most elegant structural motifs (and for enzymes one of the most common).

6.3. To which structural class does this protein belong (α, α/β, etc.)? What is the repeated super-secondary structural motif in this protein?

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6.4. Link (d) reveals the structure of a "constant" domain from the heavy chain of immunoglobulin G (PDB code: 1IGT). To which structural class does this protein belong (α, α/β, etc.)? Which super-secondary structural motifs can you find?

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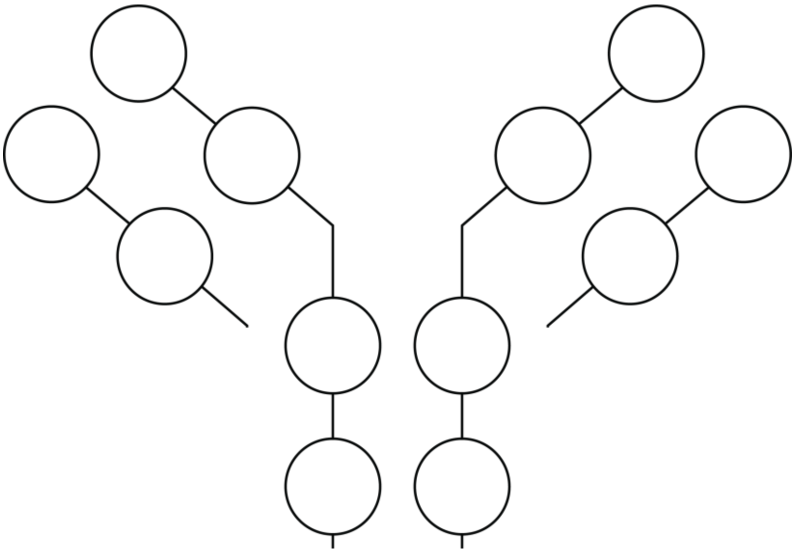
Large proteins are generally found to be made up of a number of smaller globular units, called *domains*, that are linked together by lengths of polypeptide chain. A domain is rather hard to define: they can be independent units that are simply tethered by flexible polypeptide linkers, or they can interact structurally to varying degrees. A domain typically includes only residues that are consecutive in the peptide chain, and such domains can often be separated from each other by proteolytic cleavage. Well-defined domains are often considered as autonomous folding units, because such individual units will fold independently of each other. However, other domains are not so structurally independent.

Link (e) illustrates protein quaternary structure, showing the four cross-linked polypeptide chains of immunoglobulin G (PDB code: 1IGT). Label the different chains on the diagram on page 158 (blue and magenta correspond to the "light chains", while the "heavy chains" are displayed in red and green). Note how each chain is composed of a number of domains that possess the same immunoglobulin fold you examined in link (d).

6.5. On the diagram below, sketch the inter-subunit cysteine bridges to show how the different chains are covalently linked.

(***Hint**: click on link (f) to identify the residues involved in cysteine bridges, and discriminate between intra-subunit and inter-subunit disulphide bond connections.*)



Section 7: Analysis of the structural basis of protein function

In this section you will examine not just the structure of a protein but also its function. The graphics may at first seem distressingly complicated! However, give your eyes a minute or two to adjust and remember to use the various means of displaying structures that you have learned so far to simplify the views where necessary.

Open the file "Section_7.icb" to view the structure of chymotrypsinogen (PDB code: 2CGA), the *zymogen* or *inactive precursor* of the digestive enzyme chymotrypsin. Chymotryspin belongs to a family of serine proteases that includes trypsin and elastase; all three of these enzymes are found in the mammalian pancreas and have very similar structures.

7.1. To which structural class does the protein belong (i.e. (α, α/β, etc.)? Chymotrypsinogen possesses two similar domains (residues 1-123 and 124-245), which are related by an approximate two-fold rotational symmetry. How could this kind of structure evolve?

(***Hint**: click on link (b) to rotate the structure by 90° around the x axis, and then click link (a) to move back again. What do you notice?*)

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Link (c) highlights the cysteine residues of chymotrypsinogen, which are all paired to form cysteine bridges, cross-links between different regions of the protein backbone.

7.2. How many bridges are there, and which residues do they connect? On the diagram below, show how the cysteine bridges form cross-links between different regions of the polypeptide backbone. According to the definition of the domain boundaries suggested in question 7.1, are any bridges formed between the two domains?

(***Hint***: *the domain boundaries are residues 1-123 and 124-245.*)

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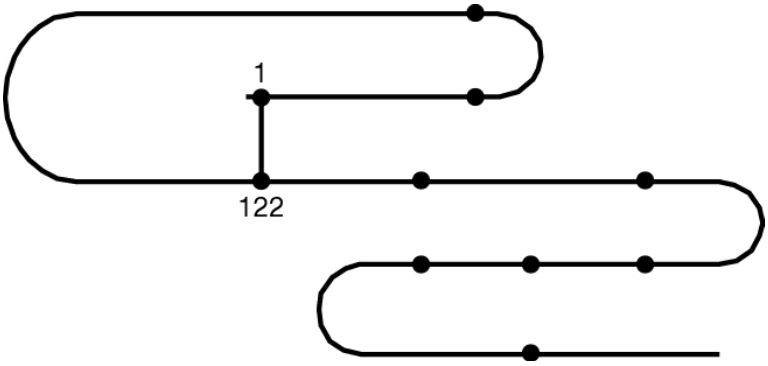

7.3. Cysteine bridges are common in extracellular proteins, but not in intracellular proteins. Why do you think this might be?

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Chymotrypsinogen is converted into an enzymatically active form, called π-chymotrypsin, when the peptide bond between residues 15 and 16 is cleaved by trypsin. Further cleavages are then made by another π-chymotrypsin molecule, removing residues 14, 15, 147 and 148, to make a stable form of the enzyme, which is called α-chymotrypsin. The locations of these residues can be observed by following link (d).

7.4. Comment on the location of the cleavage sites. Will any of the three new polypeptide chains be connected by cysteine bridges?

(***Hint***: *use your answer to question 7.2.*)

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Click on link (e). The activation of the zymogen involves localised conformational changes. Cleavage between residues 15 and 16 creates a positively charged backbone amino-terminal group on residue Ile-16 (marked with a yellow sphere), which turns inwards and forms a *salt bridge* with the side-chain of Asp-194 in the interior of the molecule (marked with a magenta sphere). This electrostatic interaction triggers other changes, creating a cavity for binding to the substrate that is not fully formed in the precursor chymotrypsinogen.

7.5. How far will the amino nitrogen of Ile-16 have to move in order to interact with the carboxyl side-chain of Asp-194?

(***Hint****: activate the distance measurement tool from the second icon bar at the top of the window. Clicking on a pair of atoms will then display the distance between them in Ångstroms. Estimate the distance by clicking on the "N" of Ile-16, followed by the "OD" of Asp-194.*)

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Link (f) shows an overlay of cartoons of chymotrypsinogen (in green) and activated α-chymotrypsin (in red), which can be used to identify where conformational changes have occurred. The side-chains of Asp-194 and Ile-16 are highlighted in both structures.

7.6. How far *does* the new N-terminus at Ile-16 move in order to interact with Asp-194?

(***Hint****: note the distance between the red and green spheres for Ile-16.*)

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7.7. What else changes? (***Hint****: Look at the side-chain of Asp-194.*)

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Link (g) displays important parts of the active site of chymotrypsin in orange, with a peptide substrate shown in purple (PDB code: 8GCH).

7.8. Comment on the location of the orange active site residues.

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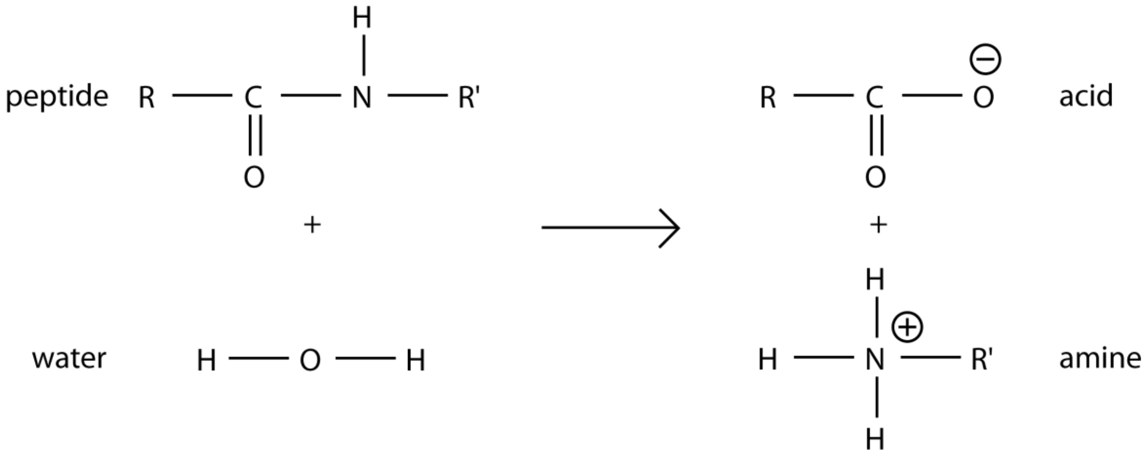
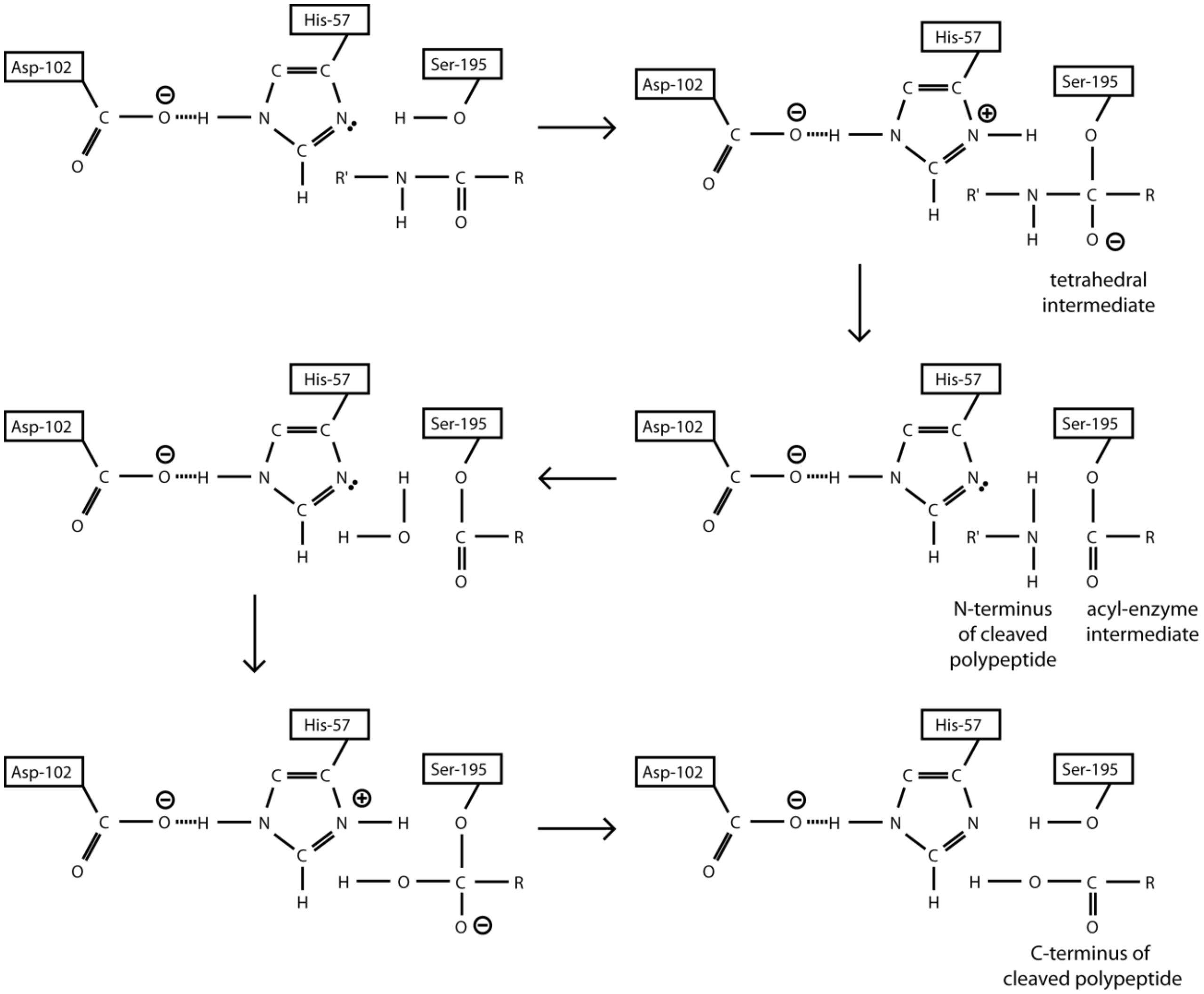
Chymotrypsin acts as a hydrolase, cleaving the peptide backbone by hydrolysis and catalysing the overall reaction:



The serine proteases acquired their name because they contain a serine residue with an unusually reactive side-chain, which attacks the carboxyl group of the substrate. The reactivity of this residue (Ser-195 in chymotrypsin) depends on the arrangement of the serine side-chain with two other polar side-chains, all approximately in a straight line. This so-called "catalytic triad" has Ser-195 at one end of the line, His-57 in the middle, and Asp-102 at the far end, all highlighted in orange in link (g). These side-chains form a charge transfer network in which Asp-102 polarizes the imidazole ring of His-57, which then acts as a proton shuttle, attracting the hydrogen ion from Ser-195 while it makes a nucleophilic attack on the substrate, forming a tetrahedral oxyanion intermediate (see scheme below).



7.9. Click on link (h) and comment on the possible role of the nearby backbone amide atoms from residues Gly-193 and Ser-195, which are highlighted using yellow CPK spheres.

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The serine proteases have no absolute specificity, but they do have strong preferences for cleaving the peptide bonds adjacent to certain amino acid residues. All three enzymes, chymotrypsin, trypsin and elastase, have a "specificity pocket" which accommodates the side-chain of the residue of the substrate that precedes the bond to be cleaved. The specificity of each enzyme depends on the size and nature of three crucial residues that line the edges of the pocket, 189, 216 and 226 (using chymotrypsin residue numbering), which are highlighted using *xsticks* and CPK representations in link (i). The structure is of a complex between α-chymotrypsin and a peptide substrate. The tryptophan side-chain of the substrate binds into the specificity pocket, which is illustrated here using an “accessibility surface”. The surface is coloured according to ligand binding properties: white – neutral surface; green – hydrophobic surface; red – hydrogen bonding acceptor potential; and blue – hydrogen bond donor potential.

7.10. Account for the sequence cleavage preferences shown in the table below:

Enzyme:	**Side-chain preference:**	**189:**	**216:**	**226:**
Chymotrypsin	Large & hydrophobic (Phe, Tyr, Trp)	Ser	Gly	Gly
Trypsin	Positively charged (Lys, Arg)	Asp	Gly	Gly
Elastase	Small & hydrophobic (Ala)	Ser	Val	Thr

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Link (j) shows a complex between bovine α-chymotrypsin (in pink and grey) and a serine protease inhibitor protein (in purple), the third domain of turkey ovomucoid (PDB code: 1CHO). The complex has an association constant, K_a, of 3.2×10^{11} M^{-1}. The inhibitor acts by forming a hydrogen bond to the side-chain of Ser-195, the crucial residue in the active site of the enzyme. Mutating residue Leu-18 of the inhibitor to methionine lowers the K_a to 1.8×10^{11} M^{-1}, while a further substitution of Gly-32 of the inhibitor to aspartic acid reduces the K_a to 4.0×10^{8} M^{-1}.

7.11. Can you account for these results on the basis of the structure of the complex?

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Section 8: Some interesting structures

If you have reached this far, you might like to look at a few more protein structures.

Link (a) shows the porcine RNase inhibitor (PDB code: 2BNH), a leucine-rich repeat protein. This is perhaps one of the most aesthetically pleasing proteins in the database: being a scientist shouldn't preclude one from finding some protein structures beautiful!

Link (b) displays the structure of a nucleosome (PDB code: 1AOI), the fundamental unit for

DNA packaging found in chromatin. The DNA double helix is wrapped around a central protein core that contains eight histone molecules.

Link (c) shows the first of an infamous pair of viral proteins: haemagglutinin from Influenza A (PDB code: 5HMG), which binds to polysaccharide chains on the surface of target cells and then mediates fusion of the virus envelope with membranes of the host cell. Link (d) shows its partner in crime: neuraminidase (PDB code: 2BAT), which clips off the ends of polysaccharide chains that would otherwise prevent new virus particles from escaping the infected cell. Together, these molecules control the infectivity of the virus, hence the designation of dangerous mutant strains with names like H1N1.

To view HIV protease (PDB code: 1HIV) click on link (d). Unlike human enzymes, this dimeric aspartate protease can cleave polypeptide chains between a phenylalanine and a tyrosine or proline. Drugs that disable it have been designed by analysing structures like this one using molecular graphics.

Finally, link (e) displays a theoretical model of a section of a lipid bilayer and some surrounding water molecules. This should be considered alongside the structure of an integral membrane protein, such as bacteriorhodopsin (PDB code: 1QHJ) shown in link (f). This protein is a member of an important family called the seven-transmembrane (7TM) receptors, which regulate many physiological processes and transduce signals from hormones, neurotransmitters, chemokines, calcium ions, and even photons. Most of the medicines sold throughout the world target 7TM receptors either directly or indirectly. Link (g) shows a recent structure of a G-protein coupled receptor (the β2- adrenergic receptor) bound to a heterotrimeric G-protein (PDB code 3SN6). Structures of this sort promise to reveal crucial insights into signal transduction pathways.

What next?

Why not set up the ICM-Browser on a computer back in your college and explore some protein structures by yourself using the list of useful web sites given on page 142. You can use the program to make your own high-quality images. Scientific publications of structural studies are increasingly using software of this sort (including an internet plug-in named ActiveICM, which is based on the ICM-Browser used here) to supplement more traditional text-based descriptions with interactive displays of notable features and functional aspects of proteins. For more details, you could read the following open-access articles:

"A new method for publishing three-dimensional content." (2009) Raush, E., Totrov, M., Marsden, B.D. & Abagyan, R. *PLOS One*, e7394.

"SGC – Structural biology and human health: a new approach to publishing structural biology results." (2009) Lee, W.H., Atienza-Herrero, J., Abagyan, R. &Marsden, B.D. *PLOS One*, e7675.

A series of interactive articles can be accessed at the website of the Structural Genomics Consortium: http://www.thesgc.org/iSee/

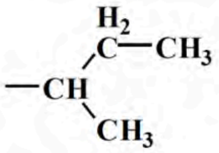
The ICM-Browser itself possesses several other powerful features that there wasn't time to look at in this practical session, including a PDB search engine, a protein sequence alignment tool, and the ability to superimpose similar protein structures, to display electron density maps from X-ray crystallography studies, and to display the nuclear Overhauser effect distance restraints obtained in solution NMR experiments.

Notes

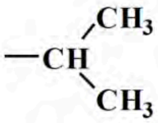
Appendix 4: The 20 common amino acids:

Listed in order of decreasing hydrophobicity (more or less).

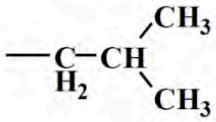
I Ile Isoleucine



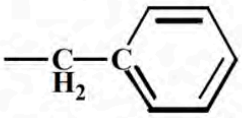
V Val Valine



L Leu Leucine



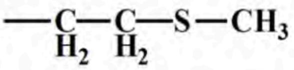
F Phe Phenylalanine



Relatively large, non-polar side-chains. Often (but by no means always) found in the structural core of water-soluble proteins.

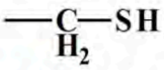
Since they tend to stick together, exposed hydrophobics are often important in molecular recognition – e.g. protein-protein interaction, substrate specificity.

M Met Methionine



$-\underset{H_2}{C}-\underset{H_2}{C}-S-CH_3$

C Cys Cysteine



$-\underset{H_2}{C}-SH$

Fairly hydrophobic. Cys often occurs paired as disulphide bridges. Free Cys is often involved in metal coordination or enzyme activity (it is very nucleophilic).

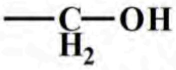
A Ala Alanine $-CH_3$

Non-polar but small – quite often found on protein surface.

G Gly Glycine $-H$

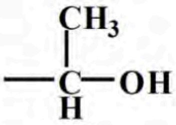
Unique in having no side-chain – often occurs in tight spots where a side-chain would get in the way.

S Ser Serine



$-\underset{H_2}{C}-OH$

T Thr Threonine



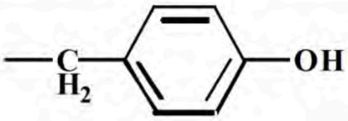
$-\overset{CH_3}{\underset{H}{C}}-OH$

The hydroxyl OH must normally form hydrogen bonds – but this is quite often achieved by looping back to the backbone, so they can often be found buried.

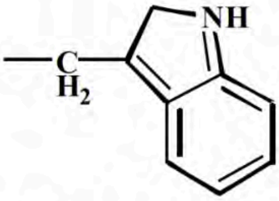
Serines are often catalytically important (they are nucleophiles).

The hydroxyl OH can be used to link modifying groups – e.g. phosphorylation

Y Tyr Tyrosine

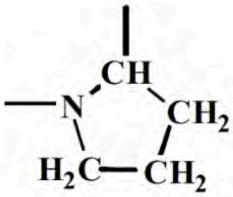


W Trp Tryptophan



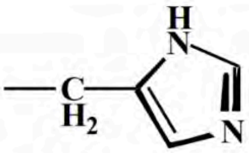
Bulky and largely non-polar but with hydrogen bonding functions – so appear quite often at surfaces.

P Pro Proline



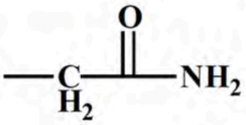
Unique ring structure – backbone N has no H. Unique geometrical properties – usually breaks α-helices, often found in tight turns.

H His Histidine



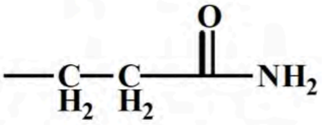
Ring can be protonated: the pK is about 6 but varies with environment. Positively charged at low pH. Reversible protonation is often important in catalysis.

N Asn Asparagine



$-CH_2-C(=O)-NH_2$

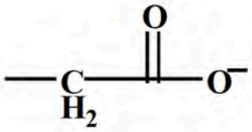
Q Gln Glutamine



$-CH_2-CH_2-C(=O)-NH_2$

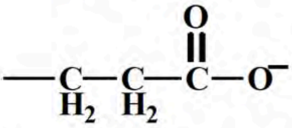
Neutral but very polar – usually found on surfaces. Asn can be glycosylated.

D Asp Aspartate



$-CH_2-C(=O)-O^-$

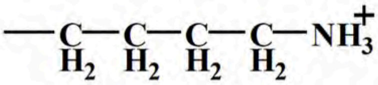
E Glu Glutamate



$-CH_2-CH_2-C(=O)-O^-$

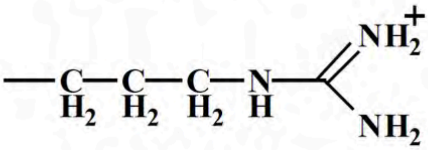
Negatively charged at neutral pH and often found on surface.
Also often involved in catalysis.
If buried, must be paired with positively charged residue to form a *salt bridge*.

K Lys Lysine



$-CH_2-CH_2-CH_2-CH_2-NH_3^+$

R Arg Arginine



$-CH_2-CH_2-CH_2-NH-C(=NH_2^+)-NH_2$

Positively charged at neutral pH. Occasionally involved in catalysis.

Week 1 (Lent) Portrait of an Enzyme: Chymotrypsin – A Model Serine Protease

Objectives

In this practical we want you to obtain hands-on experience of setting up and carrying out experiments to explore enzyme behaviour and of alternative ways of analysing the results. The experimental manipulations themselves are simple but you will need to work out how to construct the reaction mixtures and allow for possible limitations imposed by the nature of the enzyme substrate.

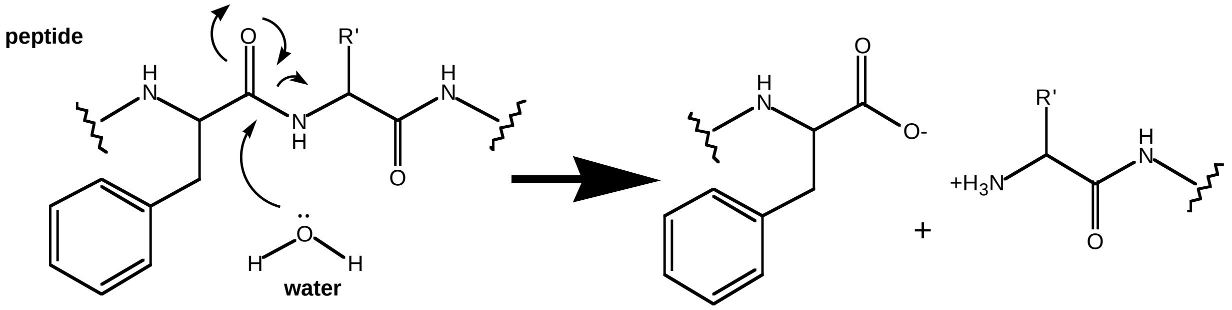
At the end of this practical you should be able to perform the following tasks:

- Plan and execute protocols for measuring reaction velocity over a range of substrate concentrations using spectrophotometer and recorder.
- Devise and carry out an experiment to investigate the potency of an irreversible inhibitor.
- Analyse kinetic data using the direct linear plot and linear transformations of the Michaelis-Menton equation.

Background (Berg, J. M., Tymoczko, J. L. and Stryer, L. *Biochemistry*. Freeman, 7th edn, 2012 pp. 237-260, 263-274)

The serine proteases play greatly diverse roles in a broad range of biological processes, ranging from dietary protein turnover to coagulation, fibrinolysis, complement-mediated cell killing, fertilisation and embryonic development. Their inappropriate activation can exacerbate, if not cause, many pathophysiological effects in diseases such as metastasis in cancer, myocardial infarction and cystic fibrosis. Chymotrypsin (CT), because of its ease of isolation and paradigmatic kinetic behaviour, has been extensively studied and thus provides a good model of this class of protease.

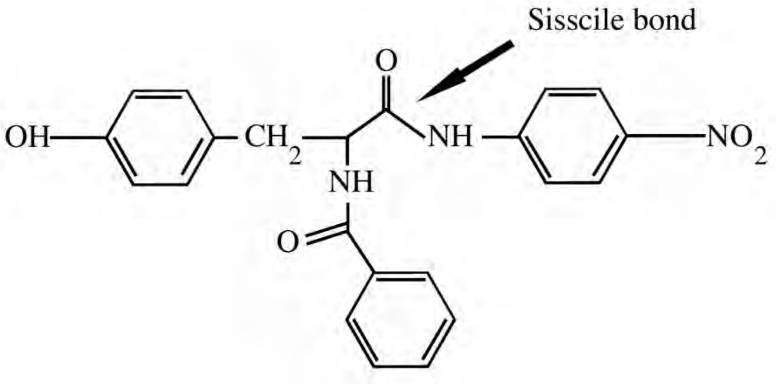
Chymotrypsin is a ~25 kDa enzyme consisting of 3 polypeptide chains linked together through interchain S-S bonds. As a digestive enzyme, it is selective for cleavage of peptide bonds on the carboxyl side of aromatic amino acids and large hydrophobic residues. The reaction will look something like this:



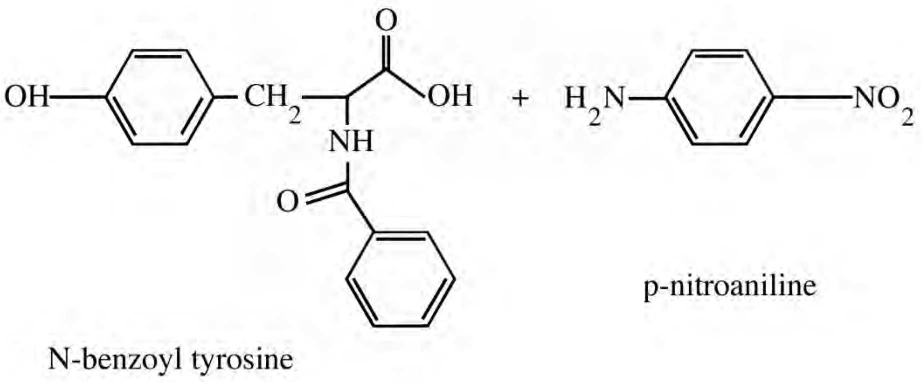
In vitro it can also cleave ester bonds and there is some evidence that this may be the major physiological role of many serine "proteases".

For kinetic analysis, it is most common to use substrates that yield coloured products, but for very sensitive detection one can also use fluorogenic substrates. (*What property makes the fluorogenic compounds more sensitive than the chromogenic ones?).*

In this practical you will be using the amide N-benzoyltyrosyl p-nitroanilide (BTNA) as the substrate for CT:



The products are thus:



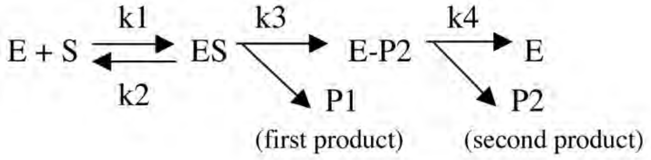
(You will be detecting the increase in [p-nitroaniline] spectrophotometrically. Why would you expect the U.V.-visible spectrum of p-nitroaniline to differ from that of BTNA?)

Kinetic studies have established that there are two stages to the mechanism of amide (or ester) substrate hydrolysis catalysed by CT:

- **Stage 1**: acylation, where an acyl-enzyme intermediate is produced, releasing stoichiometric amounts of amine (or alcohol).
- **Stage 2**: deacylation, where the enzyme-substrate intermediate is hydrolysed to yield free carboxylate under regeneration of the free enzyme.

The second step closes the enzymatic cycle. Only after the second stage is complete can the enzyme bind another substrate molecule.

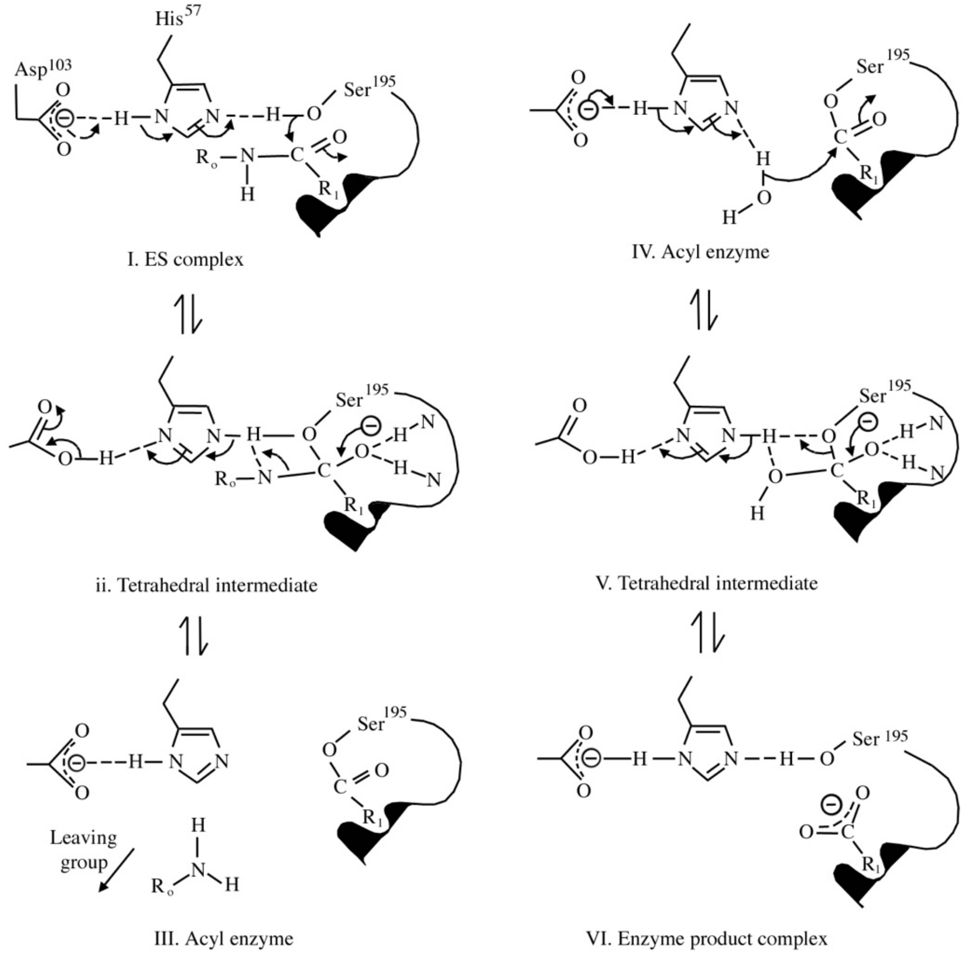
Formally therefore, the reaction can be written as:



The residues involved in catalysis form the famous "catalytic triad" in which **Ser-195** provides the attacking nucleophilic oxygen and becomes acylated during catalysis; **His-57** accepts the proton from the -OH of Ser-195 and donates it to the nitrogen atom of the susceptible peptide bond which is thus cleaved. (Its role was demonstrated using an affinity label: tosyl-L-phenylalanyl-chloromethylketone (TPCK)). This compound alkylates one of the ring nitrogens of His-57 blocking the ability for His to act as a base.

[**Proof**: (i) its reactivity can be protected against by adding a substrate and (ii) the rate of inactivation by TPCK is dependent on pH in nearly the same way that catalysis rate varies with pH].

The **Asp-102** orients the imidazole ring of His-57 and helps neutralise the charge that develops on His-57 during the transition state. The essence of catalysis by CT is the relative stabilisation of the tetrahedral transition-state over the substrate and product.



I. ES complex

ii. Tetrahedral intermediate

III. Acyl enzyme

IV. Acyl enzyme

V. Tetrahedral intermediate

VI. Enzyme product complex

Simple Kinetics

Why study enzyme kinetics?

Enzymes are amazingly efficient catalysts but all enzymes are not equal: the rate of an enzyme-catalysed reaction varies over several orders of magnitude, depending on enzyme, substrate and cellular or tissue location. Moreover, to avoid chemical chaos, all enzyme-catalysed reactions must be under tight control for a cell to operate effectively. This means that the catalytic competence of an individual enzyme molecule may also vary. Obviously, for a good understanding of how enzymes co-operate within cellular mechanisms, we need to be able to analyse and discuss their kinetic properties. In the particular case of Chymotrypsin, kinetic analyses also helped establish an understanding of its mechanism of action (see the discussion in Stryer).

The simplest enzyme system we could analyse is one with a single substrate and the simplest analysis we could do is to look at the dependence of the rate of reaction on the concentration of that substrate. Although many (if not most) enzymes are considerably more complicated, knowledge of this simple system enables us to describe the more general case in a straightforward manner.

Derivation of Michaelis-Menten equation

At a fixed enzyme concentration, the initial rate of substrate hydrolysis will increase hyperbolically with substrate concentration, displaying a nearly linear rise at low enzyme occupancy but falling off as a higher proportion of the enzyme molecules in solution become occupied by the substrate. At maximal occupancy, the rate is governed by how fast the enzyme can cleave the bound substrate (hence zero-order kinetics – i.e. rate independent of substrate concentration).

These properties were formally defined by Leonor Michaelis and Maud Menten, back in 1913 deriving what is known as the Michaelis-Menten (MM) equation. Let's go briefly through the derivation:

Take the following mechanism (**S** is substrate, **E** is enzyme and **P** is product):

$$\mathrm{E + S} \underset{\mathrm{k2}}{\overset{\mathrm{k1}}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{\mathrm{kcat}} \mathrm{E + P}$$

The rate **v** of the production of product **P** is:

$$\mathbf{v} = \mathbf{k_{cat}}[\mathbf{ES}] \qquad \text{(II)}$$

How can we determine the concentration of [ES]? To solve this in terms of known quantities, we need to define [ES] in terms of the rate constants k_1 k_2 and k_{cat} and the concentrations [S] and [E].

From first principles we can express the rate of change of [ES], d[ES]/dt, as the difference between the rates of the reactions that produce and remove it.

$$\frac{d[ES]}{dt} = k_1[E]\cdot[S] - (k_2 + k_{cat})\cdot[ES] \qquad \text{(III)}$$

If we work under conditions where the substrate is at a much higher concentration than enzyme ([S] >> [E] and [S] >>[ES]) we obtain conditions of steady state where the formation of ES is balanced by its breakdown so that [ES] remains constant:

$$\frac{d[ES]}{dt} = 0$$

By substituting in Equation (III), we reduce the problem to simple algebra

hence $k_1[E]\cdot[S] = (k_2 + k_{cat})\cdot[ES]$

solving for [ES]

$$[ES] = \frac{k_1[E]\cdot[S]}{(k_2 + k_{cat})} = \frac{[E]\cdot[S]}{\frac{(k_2 + k_{cat})}{k_1}} = \frac{[E]\cdot[S]}{K_m} \qquad \text{(IV)]}$$

The expression $\frac{(k_2 + k_{cat})}{k_1}$ is known as the **Michaelis constant**, or **K_m**.

Now we've replaced one unknown ([ES]) by two unknowns. So, what are [E] and [S]?

We are restricting ourselves to conditions where [S] >> [E] and [S] >>[ES]. Therefore [S], the free substrate concentration approximates to $[S]_{T,}$ the total concentration of S that is added to start the reaction.

But [E], the concentration of free enzyme is not >> [ES] so we need to define it in terms of $[E]_T$ and [ES]:

Since $[E]_T = [E] + [ES]$ then $[E] = [E]_T - [ES]$

Now we can rewrite equation (IV) as

$$[ES] = \frac{([E]_T - [ES])\cdot[S]}{K_m} = \frac{[E]_T[S]}{K_m} - \frac{[ES]\cdot[S]}{K_m} \qquad \text{(V)}$$

A little algebra and – voilà –

$$[\mathbf{ES}] = \frac{[\mathbf{E}]_\mathbf{T}[\mathbf{S}]}{\mathbf{K_m} + [\mathbf{S}]} \qquad \text{(VI)}$$

Inserting expression (VI) into equation (II) we get:

$$v = \frac{k_{cat}[E]_T[S]}{K_m + [S]} \quad \text{or} \quad \mathbf{v} = \frac{\mathbf{V_{max}}[\mathbf{S}]}{\mathbf{K_m} + [\mathbf{S}]} \qquad \text{(VII)}$$

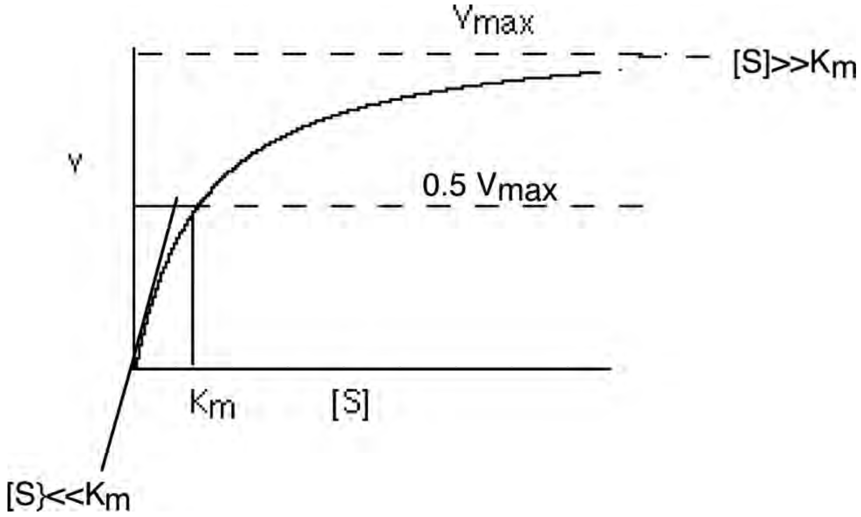
Note that $V_{max} = k_{cat}\cdot[E]_T$

Equation (VII) is the Michaelis-Menten equation, derived using the steady state approximation (which was not what Leonor and Maud did – they made the assumption that E and S are in equilibrium with ES ($k_2 >> k_{cat}$, in which case $K_m = \frac{k_2}{k_1}$ so that K_m is an indication of affinity of the ES complex.)

Properties of Michaelis Menten equation

The values of V_{max} and K_m can be determined by varying the substrate concentration and by measuring the velocity.

Does Equation VII predict the observations of Leonor and Maud? **YES**. How so?
Look at the graph below, and think how it is described by Equation (VII)



The MM equation describes a rectangular hyperbola.

It's helpful to look at two zones:

(i) When [S] << K_m

$$v = \frac{V_{max}[S]}{K_m + [S]} \longrightarrow v = \left(\frac{k_{cat}}{K_m}\right)[S][E]_T$$

so the reaction is first-order in [S] and the rate will increase linearly with an increase in [S]. The term $\frac{k_{cat}}{K_m}$ is the corresponding second-order rate constant (in $M^{-1}s^{-1}$) and is a measure of catalytic efficiency. The preference of an enzyme for different substrates is expressed via the k_{cat}/K_m value.

Since $K_m = \frac{(k_2 + k_{cat})}{k_1}$ we can rewrite this expression as:

$$\frac{k_{cat}}{K_m} = \frac{k_{cat} \cdot k_1}{(k_2 + k_{cat})}$$

so the maximal rate of reaction will be ultimately limited by the value of k_1, which at its fastest is restricted by the diffusion encounter between [S] and [E] just like in any ordinary bimolecular interaction between two molecules.

(ii) When [S] >> Km

$$v = \frac{V_{max}[S]}{K_m + [S]} \longrightarrow v = V_{max}$$

so the reaction is zero-order in [S], and the rate is independent of [S].

All of the enzyme molecules are loaded by substrate so the rate of the reaction will be maximal, governed simply by the rate constant of catalysis k_{cat} and the enzyme concentration $[E]_T$. This provides the means of deriving the **turnover number**, the number of catalytic cycles the enzyme can perform per unit time.

If not all enzyme is occupied then the fraction of active sites filled can be defined as

$$f_{ES} = \frac{v}{V_{max}} = \frac{[S]}{[S] + K_m}$$

Methods for estimating K_m and V_{max}

By fitting data to a hyperbola

Computer iteration techniques can be used to fit data directly to the MM hyperbola (Equation VII), using non-linear regression. This is probably the best method, as measurement errors can be taken into account properly and estimates of the errors in the results can be derived without difficulties. While producing good results, such approaches might be little intuitive. Historically, a great deal of effort has gone into devising manual, paper plot methods, which are easy to use and give reliable values. These are still worth knowing about – if you want to illustrate the effects of some experimental variable on V_{max} and K_m, then a plot of a linear transformation of the MM equation is much easier to understand at a glance and therefore in this practical you will be concentrating on manual methods.

By re-arranging the MM equation into linear forms.

K_m and V_{max} can be estimated from straight line plots after converting Equation VII to a variety of linear forms. Remember that the general equation for a straight line is:

$$y = \text{slope} \times x + \text{intercept}$$

or $$y = mx + c$$

The common analysis methods based on x/y-axis plots are summarised below:

1/v	*vs*	**1/[S]**	Lineweaver-Burk or double reciprocal plot
[S]/v	*vs*	**[S]**	Hanes-Woolf plot
v	*vs*	**v/[S]**	Eadie-Hofstee plot
v/[S]	*vs*	**v**	Scatchard plot

All of these plots have a statistical bias (for example because of the use of reciprocals of velocity), which should be allowed for by appropriate " weighting" of a linear regression. This is an important (but understandably) often rather neglected topic amongst non-specialists. The most widely-used plot, the Lineweaver-Burk, gives the worst distortions of experimental error but often the best-looking straight line when fitted by eye.

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

Equation for Lineweaver-Burk plot

The safest plot to use is [S]/v *vs* [S] – the Hanes-Woolf plot – which gives the least distortion of error. The corresponding equation is:

$$\frac{[S]}{v} = \frac{1}{V_{max}} \cdot [S] + \frac{K_m}{V_{max}}$$

Equation for Hanes-Woolf plot

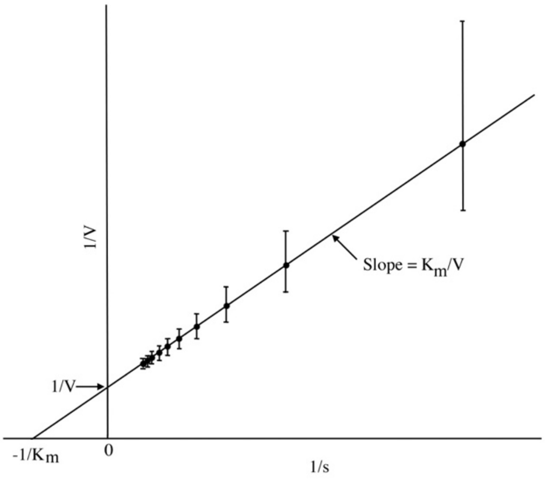


Fig2. Plot of 1/v against 1/s from Eqn (2), showing the effect of of the reciprocals on errors of ±0.05 V/max (Cornish-Bowden 1976)

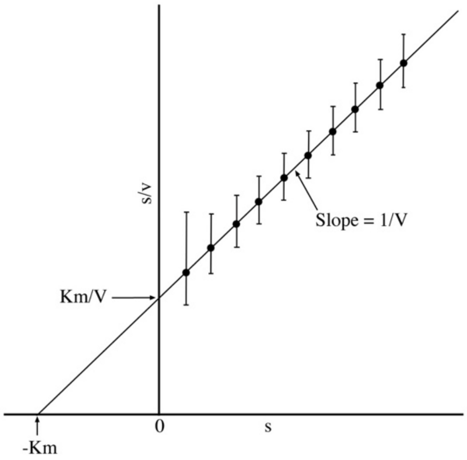


Fig 3. Plot of s/v against s from Eqn (3) with error bars ± 0.05 Vmax (Cornish-Bowden. 1976)

The Direct Linear plot

A rather different approach is the "Direct Linear" or Cornish-Bowden plot.
This re-arranges the MM equation so the V_{max} becomes the "y" variable and K_m the "x" variable. This takes a little thinking about to get used to but is very powerful:

$$V_{max} = \left(\frac{v}{[S]}\right) \cdot K_m + v$$

Equation for Direct Linear plot

So for any ([S],v) pair, we can plot V_{max} against K_m as a straight line linking an intercept of **-[S]** on the K_m axis and of **v** on the V_{max} axis. This line gives all values of V_{max} and K_m that satisfy the particular ([S],v) pair. If straight lines are drawn like this for several observations, they should intersect at a common point – the unique values of V_{max} and K_m that satisfy all of the observations. With real data you get a mesh of intersections, each of which is an estimate of V_{max} and K_m. The median values provide an estimate of the true magnitudes.

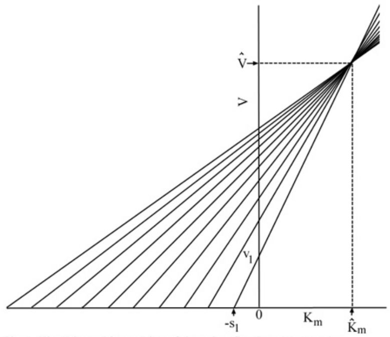


Fig.5. The Direct Linear Plot of data that fits Eqn (1) exactly. (Eisenthal and Cornish-Bowden, 1974; see also Cornish-Bowden, 1976.)

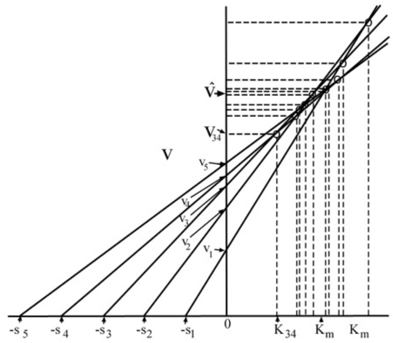


Fig.6. The Direct Linear Plot of data containing errors (Eisenthal and Cornish-Bowden,1974; see also Cornish-Bowden, 1976). $\hat{K}_m$ and $\hat{V}$ are the median, best fit, values

The "mesh" may be rather "loosely woven", as compared to the tight clustering of points when the same data set is subjected to the double reciprocal plot. What are the implications of this, do you think?

Computers can help

We want you to manually *plot* your own data during the practical class to compare all these different ways of estimating V_{max} and K_m. If you would like you can fit your data at home on a computer using excel spreadsheet, and compare with the manual analysis.

Depending on substrate type, the initial kinetics of chymotrypsin can deviate from steady state.

Note that the mechanism for which we solved the MM equation is simpler than that written for the chymotrypsin reaction. If the rate constant for the release of P1 (the alcohol or amine product is slow compared to that for hydrolysis of the acyl-intermediate to release P2, then MM laws apply right from the start). *(Why?)* If the release of P1 is faster than the release of P2 (k_3>k_4) then we have an awkward situation where there might be a burst of product in the first round of catalysis before the steady state is entered, something looking like this: *(How about our reaction with BTNA?)*

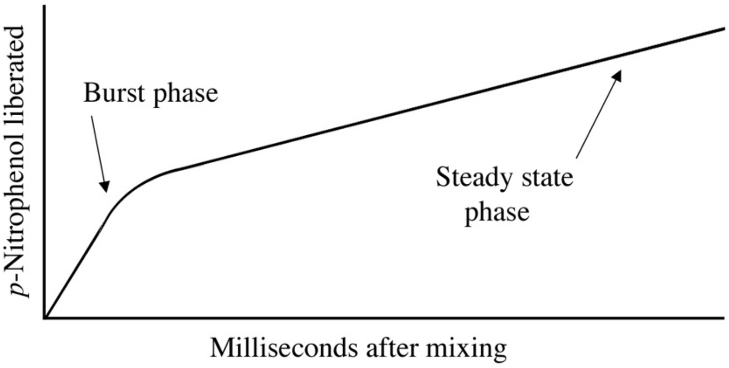
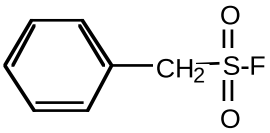


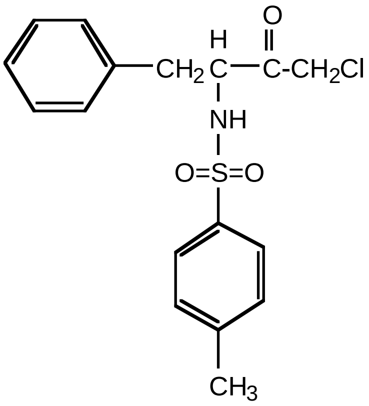
Figure 9-36
Two phases in the formation of *p*-nitrophenol are evident following the mixing of chymotrypsin and *p*-nitro-phenyl acetate

Irreversible inhibitors

Many irreversible and reversible inhibitors of chymotrypsin have been synthesised. Two irreversible inhibitors will be used in the practical: In PMSF, nucleophilic attack displaces F^- ions. In TPCK, specificity is conferred by the phenyl ring (a substrate analog) that binds in the active site and there is a Cl^- ion as the leaving group.



Phenylmethylsulphonylfluoride
(PMSF)



Tosyl-L-phenylalanylchloromethyl
ketone (TPCK)

Safety Note: These should be treated with respect! After all, they are irreversible and PMSF is not very specific!

An irreversible inhibitor reacts with an enzyme in two stages. First it binds reversibly to form a non-covalent complex (EI), and then a covalent reaction occurs to give a modified form of enzyme (EI*). Aficionados are welcome to develop the rate equation from the following mechanistic scheme.

$$E + I \underset{ki2}{\overset{ki1}{\rightleftharpoons}} EI \xrightarrow{ki3} EI^*$$

We can define a constant (k_{ass}) that gives an estimate of the efficacy of the inhibitor, taking into account both the strength of its reversible association with the enzyme and the rate of inactivation. The units of k_{ass} are $M^{-1}s^{-1}$ – it is a second order rate constant.

$$k_{ass} = \frac{k_{i1} \cdot k_{i3}}{k_{i2}}$$

How can we measure the "strength" of interaction of an irreversible inhibitor with the enzyme? If it produces a coloured intermediate, or is labelled, then it's easy – you simply mix it with the enzyme and follow the fate of the label (direct route).

When the reaction of the inhibitor with the enzyme does not itself yield a detectable product, one must study the rate at which it inhibits the ability of the substrate to produce product (indirect route). Deriving the overall rate constant for the interaction between I and E is now tricky. One approach is to incubate the enzyme with an *excess* concentration of the inhibitor, and follow how the activity of the enzyme falls with time by assaying samples at intervals. The initial rate of product formation is measured over as short a time as possible so that [S], [ES] and [EI] all remain roughly constant. Using an excess of [I] forces the above reaction to become pseudo 1st order. Therefore, assuming that [S]>>[E] and [I]>>[E] one can derive the expression:

$$\ln(\text{residual enzyme activity}) = -k_{obs} \cdot t$$

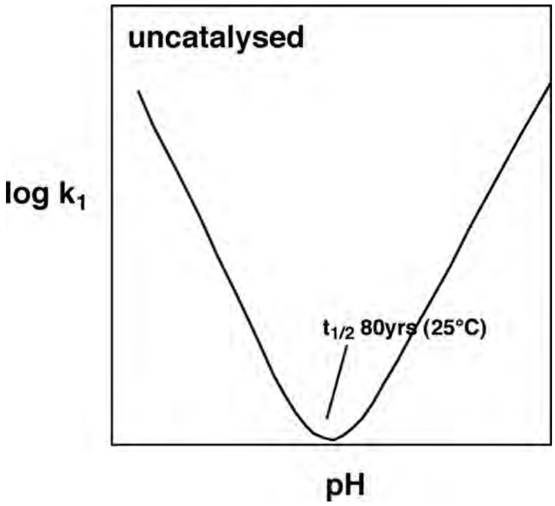
from which one can approximate the rate of inhibition k_{ass} from the relationship:

$$k_{ass} = \frac{k_{obs}}{[I]}$$

pH Dependence of Chymotrypsin Catalysis

Some catalytic groups on amino acid side chains are ionisable and exist in different ionic forms at different pH values, where they may or may not contribute to catalysis. All this shows up in effects of pH on rates or binding. Dependencies on pH are important pieces of information to understand enzyme mechanism as well as the physiological context of enzyme action.

The starting point for discussion of the pH dependence of an enzymatic reaction is the pH-rate profile of the background reaction, amide hydrolysis.

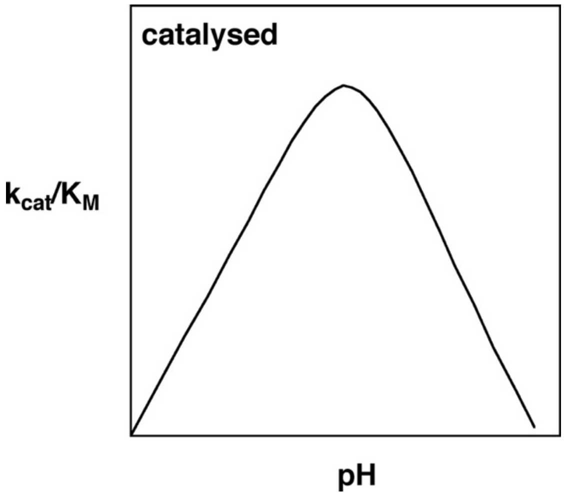


Catalysis is defined as transition state stabilisation. Thus neutralising the energetically costly build-up of charges in the transition state and making the reacting groups more powerful will accelerate the reaction. This is achieved for the non-enzymatic reaction at the extremes of pH:

- At low pH: protonation of the carbonyl group and amide leaving group helps to offset the build up of negative charge
- At high pH: making more OH^- (a better nucleophile than H_2O) available speeds up the reaction.

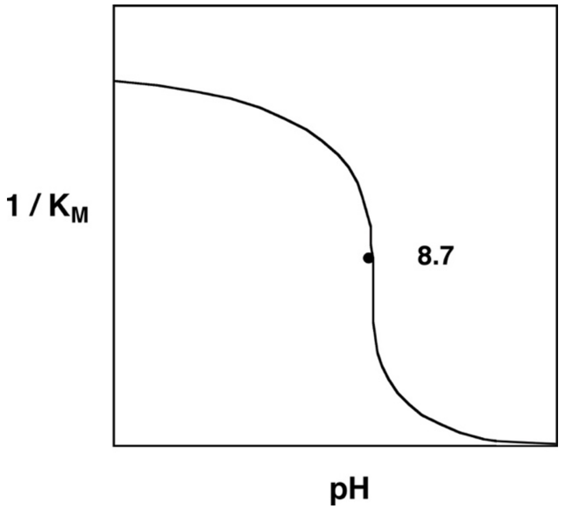
When we look at pH-rate profiles for enzymatic reaction, we have to distinguish what parameter is plotted against pH.

This is the pH rate profile for the **k_{cat}/K_M** parameter (rate at low [S]), with an uncharged peptide as a substrate:



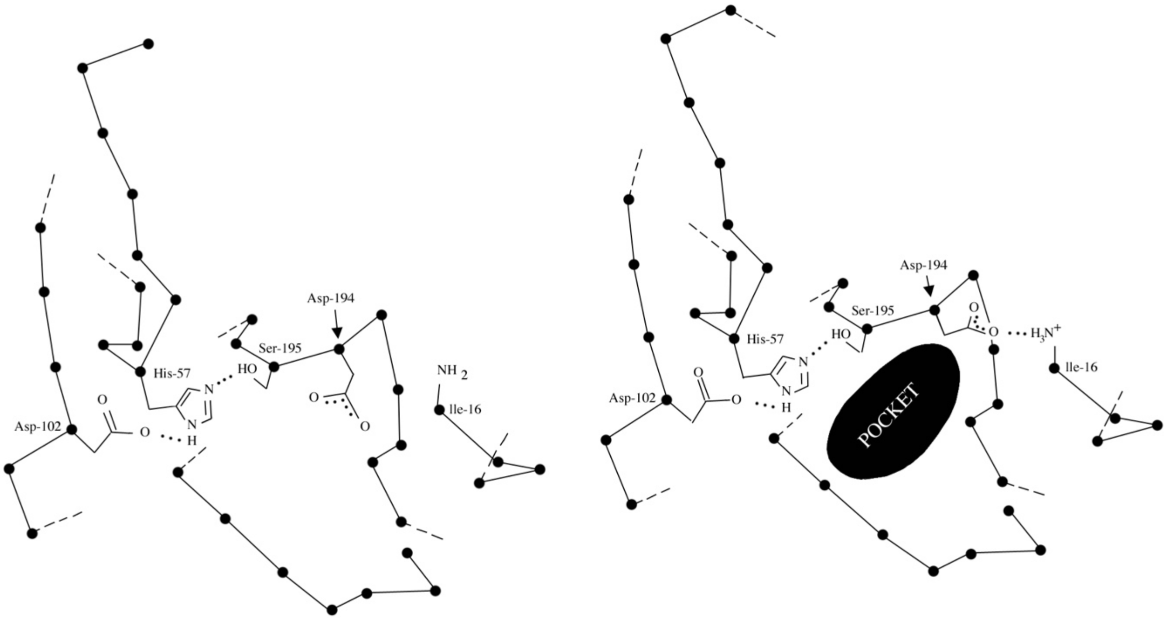
This k_{cat}/K_M pH rate profile turns out to be the aggregate of two effects. You will measure part of the k_{cat} vs pH profile in this practical.

pH dependence for $1/K_M$:



In the first approximation we interpret K_M as a binding constant (which is strictly only correct for $k_2 >> k_{cat}$). The pH dependence of K_M would then reflect differential interactions at various pHs resulting from ionisations in free enzyme and free substrate. A molecular explanation for this observation was only possible after the structure of chymotrypsin was elucidated:

Conformational Changes


Asp-194
Ser-195
His-57
HO
N
N
H
O
O
O
Asp-102
NH 2
Ile-16
Asp-194
Ser-195
His-57
HO
N
N
H
O
O
O
H_3N^+
Ile-16
Asp-102
POCKET

pH 10 (inactive)

pH 7 (active)

Figure 5-4

At high pH the *B*-carboxylate group of aspartate-194 blocks the specificity pocket of chymotrypsin. At neutral pH, the protonated isoleucine amino group forms an ion pair with the aspartate carboxylate group, which opens the pocket and allows the hydrophobic side chains of specific substrates to bind. The diagrams also show the carboxylate group of aspartate-102 which holds the imidazole group of histidine-57 in place. The imidazole can then accept a proton from the hydroxyl group of serine-195 when this hydroxyl group attacks the substrate.

Enzyme Kinetics – Experimental

The substrate concentrations and assay procedure

In order to obtain an accurate estimate of K_m and V_{max} it is necessary to use a range of substrate concentrations that straddle K_m. You will be given the chromogenic amide substrate N-benzoyltyrosyl p-nitroanilide (BTNA). The enzyme-catalysed reaction can be followed spectrophotometrically by changes in absorbance at 400 nm. The molar absorption coefficient of the product, p-nitroanilide, is 10,000 $M^{-1}cm^{-1}$ (or 10 $mM^{-1}cm^{-1}$).

It is very important NOT TO CROSS CONTAMINATE YOUR REACTIONS so PLEASE take care when constructing different mixtures. The water solubility of the reagents is limited: *just before* inserting the cuvette in the spectrophotometer, cover it with parafilm and once more invert it several times to achieve good mixing (don't shake as this will generate bubbles).

Stock solutions are:

- BTNA, 5 mM in DMSO
- Buffer, 100 mM Tris/Tris HC1, adjusted to pH7.4 at room temperature containing 20% ethanol
- α- Chymotrypsin, 4 mg/ml in 1 mM HC1
- PMSF (10 mM) in isopropanol
- TPCK (30 mM) in DMSO

Safety note: The inhibitors should be treated with respect! After all, they are irreversible and PMSF is rather non-specific!

1. For spectrophotometer and computer operation see instructions on next page.

2. Pre-prepare cuvettes containing mixtures of the substrate BTNA + buffer to obtain a range of concentrations (we suggest 7 or 8) between 25-600 μM, in a final volume of 1 ml. Think carefully about the distribution of concentrations you use *(why?)*.

   It is vital to make sure that the substrate and buffer are mixed *thoroughly* at this stage. If you hold the cuvette up to the light you will see that the BTNA will fall to the bottom. Mix properly by covering the cuvette with parafilm and invert several times (don't shake).

3. Insert a cuvette into the photometer for blanking (see spectrometer instructions). Once the blanking is complete, add 10 μl of α- chymotrypsin solution to one of your pre-prepared cuvettes to start the reaction, mix and place the cuvette into the spectrometer. **Again, you need to mix *thoroughly*, but this time you also need to be quick! Teamwork is the best approach here. Each cuvette needs blanking!**

A trial run or two is sensible to make sure the enzyme is alive and to establish a concentration range where the enzyme is not saturated and the substrate remains soluble. Remember also to cater for possible non-enzymatic hydrolysis of the substrate *(how?)*

4. Keep each reaction going for a few minutes so that you can measure *bona fide* linear rates from the slope of the curve.

5. Alter one of the following parameters and investigate its effects on the reaction kinetics. It is best again to use a set of at least 5 substrate concentrations for each new value of your chosen parameter. You will have a good idea of the range from first experiment. This should give information on how V_{max} and K_m are altered. Different groups should try different conditions, so that a range is covered. Ask our demonstrators which one you should do.

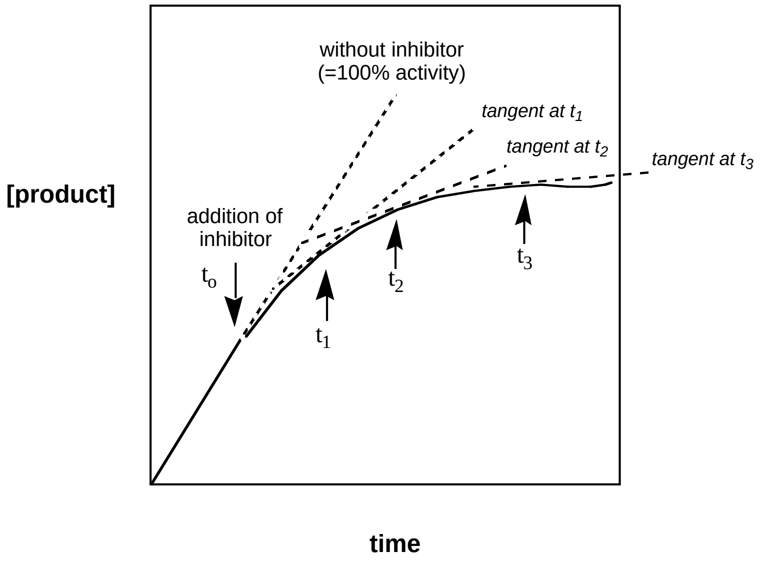
- pH (either 6.5 or 8.0).
- Enzyme concentration (say 5 μl or 20 μl of stock solution).

In order to get things done in time, do only one alternative set of conditions. Come to some agreement with groups on the benches around you so that you can get results from other sets of conditions. In any case put your values on the whiteboard.

6. For the inhibitor experiments, choose **either** (a) PMSF **or** (b) TPCK.

Start the reaction with 150-200 μM substrate (choose a concentration where the reaction is not too fast to allow convenient monitoring) and establish the initial rate in the absence of inhibitor (record for a maximum of ~30 sec).

Then add PMSF so that the final [PMSF] is ~0.4-0.5 mM (or TPCK to a final concentration of ~0.5 mM) and observe the reduction of the rate as time proceeds. As the inhibitor binds, less and less active enzyme will be available and the cleavage rate of BTNA will go down. Determine initial rates for a number of time points from the tangents on the time course. Compare the slopes of the tangents against the initial rate in the absence of the inhibitor and express these in %-activity of the initial rate (100% activity).



Plot the residual %-activity against time – you will see an exponential decay in activity from the original 100% without inhibitor. You can obtain the pseudo 1st order rate constant k_{obs} for the reaction of enzyme and inhibitor by linearising the exponential plot in a semi logarithmic fashion [ln(residual %-activity) *vs* time] – the slope will be the observed rate k_{obs} measured in the presence of a large excess of inhibitor. You can convert this into the rate of inhibition k_{assoz} by dividing k_{obs} by the inhibitor concentration [I] in your solution (watch the units !!!).
If it's all over too quickly or there is little response, adjust the inhibitor concentration accordingly.

7. Turn to your assessment sheet for calculations and write your obtained values on the whiteboard for comparison and to discuss the effects of varying the reaction conditions.

……

Enzyme Kinetics – Use of the Computer

Control of spectrophotometer via computer using 'Resolution software'

1. Open 'Resolution software'; this will take control of the spectrophotometer. *There is no need to press any buttons on the spectrophotometer.*
2. Open the 'Application' tab. Click on 'Kinetics' – this will open a new measurement window.
3. Go to the 'Acquire' menu – set wavelength to **400 nm**; select absorbance mode; set the pathlength to 10 mm.
4. Press the 'Timing' button. Here you can change the maximum recording time, and also the step size – set this to 1 s.
5. To take a measurement, first insert a blank (buffer + BTNA) and click 'Take reference'. After this, the start button is highlighted. Add the enzyme and press 'Start'. (NB: this will have to be done for each concentration). The measurement will begin and the results will be displayed in the measurement window. If the vertical y-axis is too short, the data will not be displayed, but will still be saved, and displayed in the result window. *If the x-axis is too short, the data recording will stop at the end of the x-axis. Therefore, it is better to choose a longer time and stop the recording early.*
6. Once the measurement is finished, choose 'Yes' to save the data. This will transfer the data to a result window.
7. In the result window, it is possible to add a title to label the graph (either 'Design' or 'Layout' tab). The result window will also add two vertical lines, with readings for the x and y values. These can be used to calculate the gradient. (NB: need to x 60 to get into min). Alternatively, choose 'Add linear section' and 'linear' to calculate the gradient. (Chords and tangents can be chosen, useful for the inhibitor data). The results from this are displayed below the graph window; *click on the **linear sections tab** and use the value in the '**Slope**' column.*
8. During the inhibitor measurements, there is no way to pause the run – remove the sample quickly, add the inhibitor, and return to the spectrophotometer. For these measurements, it will be necessary to increase the length of the acquisition to 5000 s.
9. Data from the result window can be exported to an excel file. Click on the circle (top left) and choose export.

Notes

Enzyme Kinetics – Question and Assessment Sheet

Preliminary questions: It will help you if you think about these before the practical starts!

(A) What property makes fluorogenic compounds more sensitive than the chromogenic ones?

(B) Why would you expect the U.V.- visible spectrum of p-nitroaniline to differ from that of BTNA?

(C) If the rate constant for the release of P1 (the alcohol or amine product) is slow compared to that for hydrolysis of the acyl-intermediate to release P2, why will MM laws apply immediately?

(D) What values for [S] have you chosen? Why?

(E) How can you cater for possible non-enzymatic hydrolysis of the substrate?

Experiment 1: Establishing the kinetic properties of Chymotrypsin

10 µl of 4 mg/ml Chymotrypsin, pH 7.4

Calculate the volumes of 5 mM BTNA and buffer required to make up reactions with your chosen [BTNA]:

[BTNA] (µM)	Vol. BTNA (µl)	Vol. Buffer (µl)	Vol. Chymotrypsin (µl)	Total Vol. (µl)
			10	1000
			10	1000
			10	1000
			10	1000
			10	1000
			10	1000
			10	1000
			10	1000

Record the initial rate of reaction at each substrate concentration in Abs s^{-1} and convert the rates to Abs min^{-1}:

[BTNA] (µM)	Rate (Abs s^{-1})	Rate (Abs min^{-1})

Plot your data manually using different linearisations methods as listed in the sections below.

Q1. Estimates from raw plot of v *vs* [S]

Plot the values of the initial rates for *Experiment 1* (pH 7.4, 10 µl E) against substrate concentration. (Direct v *vs* [S] plot). Estimate K_m and V_{max} from this plot.

BTNA, pH 7.4, 10 µl Chymotrypsin	**K_m** (µM)	**V_{max}** (Abs min^{-1})
Measured values		

Q2. Estimates from linearisations of the Michaelis-Menten equation

Plot the data from *Experiment 1* for yourselves using one (or more) of the linearisations of MM equation and derive K_m and V_{max} values for BTNA (enter the method used). Write your K_m and V_{max} values on the whiteboard.

Plotting method	**K_m** (µM)	**V_{max}** (Abs min^{-1})

Q3. Estimates from direct linear plot

Replot the data using a direct linear plot (Cornish-Bowden). It's quite difficult to get all the intersections on a single graph and if your data are scattered you will struggle to manage.

BTNA	**K_m** (µM)	**V_{max}** (Abs min^{-1})
Median values from direct linear plot		

Q4. Comment on how the K_m and V_{max} estimates vary between different plot modes. Does any method seem preferable and why?

Experiment 2: Comparing the kinetic properties of chymotrypsin under different conditions

Study the effects of **one** of the following experimental parameters:

- pH (either 6.5 or 8.5).
- Enzyme concentration (5 µl or 20 µl of 4 mg/ml Chymotrypsin).

Different groups should try different conditions, so that a range is covered. Ask the demonstrators which one you should do.

Experimental condition chosen:	
Vol. Chymotrypsin: µl	**Buffer pH:**

Choose at least 5 substrate concentrations and record the rates of reaction.

[BTNA] (µM)	**Rate** (Abs s^{-1})	**Rate** (Abs min^{-1})

Plot your data from Experiment 2 using the Michaelis-Menten plot and your preferred method of linerisation of the Michaelis-Menten equation. This would give information on how V_{max} and K_m are altered.

Q5. Compare your estimates of the kinetic properties of Chymotrypsin at pH 7.4 with the other conditions at which you and your colleagues on other benches have assayed:

pH	**Vol. Chymotrypsin** (µl)	K_m	V_{max}
7.4	10		
8.0	10		
6.0	10		
7.4	5		
7.4	20		

Q6. Is the behaviour of the enzyme affected by pH? From your knowledge of the mechanism of the reaction, would you expect a pH dependence?

Q7. How about the volume of enzyme used? Would you expect this to have an effect?

Experiment 3: Enzyme inhibition

Inhibitor (choose one): PMSF / TPCK

Calculate the volume of inhibitor stock solution required:

Substrate concentration ([BTNA]) used:

Time (s)	**Rate** (Abs s^{-1})	**% Enzyme Activity**	**ln (% Enzyme Activity)**
0 (no inhibitor)		100	

Q8. Plot the data obtained for the loss of enzyme activity with time in the presence of your inhibitor.

Plot [ln (value of residual enzyme activity) *vs* time] and derive $\mathbf{k_{obs}}$ and $\mathbf{k_{ass}}$ (see Green Sheets).

	$\mathbf{k_{obs}}$ (S^{-1})	$\mathbf{k_{ass}}$ (M^{-1} S^{-1})
PMSF		
TPCK		

Q9. Why might the values for the two inhibitors be different?

Q10. Calculate the turnover number for chymotrypsin action on BTNA.

The M_r of chymotrypsin is ~25,000 Da and you may assume that it is pure. To convert velocity from absorbance units per time, use the molar absorbance coefficient of the product, p-nitroanilide. Its value is 10,000 $M^{-1}cm^{-1}$ (or 10 $mM^{-1}cm^{-1}$). Use this to calculate the k_{cat} for BTNA as a substrate in units of s^{-1}.

Q11. How would you distinguish between the possibility that the inhibitor really inactivated the enzyme of that something toxic was added (like isopropanol in the case of PMSF) that "killed" the enzyme?

Linear graph paper

Linear graph paper

Week 2 (Lent) – Subcellular Fractionation of Mammalian Tissues

Glutamate Dehydrogenase, Lactate Dehydrogenase and Malate Dehydrogenase Activity of the Fractions

Objectives

This practical is intended to show you how the level and subcellular distribution of enzymes between mitochondria and cytoplasm can be investigated, starting with intact organs. You will learn how to calculate enzyme content of total homogenates from raw experimental data. A second objective is to investigate the tissue distribution of isoenzymes using non-denaturing gel electrophoresis.

At the end of this practical you should be able to perform the following tasks:

- Prepare a homogenate of rat organs and fractionate it by differential centrifugation.
- Carry out a spectrophotometric assay of NAD-linked dehydrogenases.
- Determine whether enzyme activity is latent.
- Calculate the total enzyme content of the organ homogenates and the recovery of enzyme activity.
- Prepare and load non-denaturing polyacrylamide gels and carry out an electrophoretic separation.
- Stain gels to reveal the location of lactate dehydrogenase isoenzymes.

Background

Cells contain a number of components, including organelles and various membrane systems as well as the fluid cytoplasm. The purpose of today's practical is to demonstrate that different enzymes may be localised in various parts of a cell; such differences in enzyme distribution may explain the different metabolic activities of the subcellular compartments.

Fractionation

After gentle disruption of cells, it is often possible to isolate, in a fairly pure state, some of the intracellular organelles. The procedure normally adopted for mammalian liver is one involving homogenisation of the organ in cold isotonic sucrose (0.25 M) followed by differential centrifugation of the homogenate. Intracellular components sediment at different rates, mainly due to their differences in size:

Component	Approximate diameter (nm)
Whole liver cells	20, 000
Red blood cells	8, 000
Nuclei	6, 000
Mitochondria	750
"Microsomes" (membrane fragments)	10 - 150
Ribosomes	10 - 15

A low-speed centrifugation can be used to remove unbroken cells, red blood corpuscles and nuclei; a subsequent higher-speed centrifugation will sediment the mitochondria; a very high-speed spin could sediment the microsomal fraction. The same treatment will give a similar fractionation of kidney components. When brain is studied there is an extra component, the "synaptosomes". These are vesicles, artificially created by the homogenisation, being pinched off nerve endings with some cytoplasm trapped inside. (***Note***: for tougher, more muscular, tissues, other procedures may be necessary).

In today's practical we can use the bench centrifuge (which will give a centrifugal force of up to 3,000 x gravity, although we do not need this top speed) to spin down debris, red cells and nuclei; the supernatant from this centrifugation can then be spun at 10,000 x gravity in a larger centrifuge. This second spin will sediment most of the mitochondria as a pellet, leaving as the supernatant the so-called "post-mitochondrial" material, which contains components smaller than mitochondria – i.e. mainly the final cytoplasmic contents but including the microsomes. Having collected these two fractions – the one mainly mitochondria, the other mainly cytoplasm – they can be examined for three well-characterised oxidative enzymes, glutamate, lactate and malate dehydrogenases. We will try to determine the quantitative subcellular distribution, and the recovery.

One important point about subcellular fractionation – the intracellular organelles are very sensitive to mishandling, their membranes being easily ruptured. It is essential that all fractionation steps are carried out in an isotonic medium The sucrose-Tris medium provided, which is 0.25 M sucrose, containing 2 mM EGTA, (a metal-chelating agent) and 0.01 M Tris-chloride (a buffer at pH 7.4) has the same osmotic potential as the contents of the mitochondria, thus preventing swelling damage. The material must at all times be kept ice cold (buckets of crushed ice are provided for you to stand your vessels in).

Enzyme Assays

General

The assays for the three enzymes will be carried out using a spectrophotometer to give a measure of the rate of the enzyme-catalysed reaction. Since the reaction rate may be taken to be proportional to the amount of enzyme present, the assays will give a quantitative measure of enzyme in the two subcellular fractions. We will refer to this as enzyme activity. *How is this different from the specific activity of an enzyme?*

In the assays described below, a variety of reagents are used. When an enzyme is located within a vesicle or organelle (e.g. a synaptosome or mitochondrion) it may not be possible to detect unless steps are taken to break down membranes which form permeability barriers between the enzyme (inside) and the substrates (added outside). In such instances the enzyme is said to be "latent". The addition of the non-ionic detergent Triton to give a final concentration of about 0.05% can disrupt membranes and may thus reveal latent enzyme activities.

If, however, the membrane surrounding the organelle is permeable to the added substrates, then an enzyme within an organelle can be assayed fairly quantitatively without the necessity for membrane disruption. Enzymes that are not found within organelles, or those that are associated with organelles but with their catalytic surfaces exposed to the exterior, would not be expected to show this phenomenon of latency.

Dehydrogenase assays

Lactate, malate and glutamate dehydrogenases all catalyse reversible reactions, using the coenzyme NAD as a hydrogen acceptor.

Lactate dehydrogenase catalyses:

$$\underset{\text{lactate}}{CH_3\cdot CHOH\cdot COOH} + NAD \Leftrightarrow \underset{\text{pyruvate}}{CH_3\cdot CO\cdot COOH} + NADH + H^+$$

while **malate dehydrogenase** catalyses:

$COOH{\cdot}CH_2{\cdot}CHOH{\cdot}COOH + NAD \Leftrightarrow COOH{\cdot}CH_2{\cdot}CO{\cdot}COOH + NADH + H^+$

malate oxaloacetate

The **glutamate dehydrogenase** reaction is:

$COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CHNH_2{\cdot}COOH + NAD + H_2O \Leftrightarrow$

glutamate

$COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}COOH + NH_3 + NADH + H^+$

2-oxoglutarate

In each of these cases the activity could be measured by following the production of NADH, which absorbs light in the near ultra-violet and can therefore be quantified spectrophotometrically. A more suitable assay for the dehydrogenases, however, is provided by measuring the reactions they catalyse in the reverse direction. This is because (i) the rates of the enzyme-catalysed reactions are 3-4 times faster in this direction and (ii) the equilibrium constants of many of the NAD-linked dehydrogenases favour the generation of NAD and reduced substrate, rather than NADH and oxidised substrate.

The assays you will use today therefore involve adding pyruvate or oxaloacetate or 2-oxoglutarate and ammonia to a solution containing NADH and a sample of tissue fraction, and measuring the rate at which the ultraviolet absorption decreases. The spectrophotometer is set to transmit light of a wavelength of 340 nm, where NADH solutions, but not NAD solutions, absorb maximally – See Appendix 3 on "Spectrophotometry". The concentrations of the reactants used are sufficient to saturate the enzymes being studied. We know the molar absorption coefficient of NADH (6220 $M^{-1}cm^{-1}$), so we can determine absolute enzyme activities in units of katals. (A katal is the amount of enzyme that converts 1 mole of substrate per second – see "calculations" section of white sheets.)

Study of isoenzymes by electrophoresis

Some enzymes exist in more than one form in tissues (isoenzymes). Differences between isoenzymes may be large or small (but they carry out the same catalytic reaction, so they must have a basic similarity in their active centre arrangements). These differences can sometimes be exploited to demonstrate the existence and number of isoenzymes present. Lactate and malate dehydrogenases exist in isoenzymic forms in mammals.

LDH is the product of two major genes, encoding LDH subunit polypeptides designated A and B. The enzyme is a tetramer, so 5 possible isoenzymes exist (A_4, A_3B_1, A_2B_2, AB_3, and B_4). The gene products (polypeptides A and B) differ in their electrical charge; the tetramers generated from them also differ in charge and can therefore be separated by electrophoresis. You can try to show the different LDH isoenzymes in a variety of rat tissue extracts by subjecting them to polyacrylamide gel electrophoresis (PAGE) and then staining the gel with a substrate mixture that turns purple in regions where LDH is found. The gels are run without the detergent SDS, and so the proteins are not unfolded and denatured but retain their native tertiary and quaternary structures.

Subcellular Fractionation – Experimental

The division of labour for the practical is as follows:

Each bench has two groups:

- **GROUP A** will carry out the tissue homogenisation and fractionation.
- **GROUP B** will make the gel and resolve the LDH isozymes.

Both groups will carry out the enzyme assays using the spectrophotometer.

Firstly, organise yourselves for the work:

In the main experiment we are primarily concerned with the intracellular location of the enzymes, although the approximate total amount of enzyme present can also be determined.

- **GROUP A** will be provided with either half a liver, a heart, a pair of kidneys, a brain or a pair of testes.
- **GROUP B** will make the gel to resolve the LDH isozymes at the same time as GROUP A carries out the fractionation.

The timetable for the groups should be:

• Making gels; obtaining and homogenising tissues	0 hour
• Low speed spin of extracts	+ 0.5 hour
• Loading gels; high speed spin of extracts	+ 1 hour
• Running gels; assaying enzymes	+ 2 hours
• Staining gels; calculating results	+ 3.5 hours

1. Procedure for cell fractionation (Group A)

Before starting operations, pre-cool the homogeniser, the bottle of sucrose-Tris-EGTA, and two empty 50 ml beakers by standing them in a bucket of crushed ice.

The organs will have come from rats that have previously been used to supply aortae for medical research into smooth muscle pathologies.

Take the relevant organ, and blot them on filter paper; the liver is supplied halved (for sharing), and the kidneys should have the transparent capsule layer and white hilum removed. Trim off any obvious bits of blood vessel, fat or other extraneous material. Place the organs in a beaker containing just enough sucrose to cover them.

Cut the half-liver (or pair of kidneys or the brain) into small pieces with scissors. From experience it has been found that the most efficient way of doing this is to wedge the beaker upright in the ice-bucket, and to chop rapidly at the organ with scissors vertical, holding each handle with a separate hand (ask for a demonstration if this is unintelligible). When the organ is well chopped, pour the contents of the beaker into a homogeniser (do not allow water from the outside of the beaker to get into the homogeniser). Add more sucrose-Tris-EDTA to the homogeniser until the level is just over halfway up the narrow portion. Total volume at this stage is about 35 ml.

Immerse the homogeniser in ice. Insert the plunger (make sure it is clean); do not, at first, attempt to push the plunger all the way to the bottom of the homogeniser, but rather by short strokes break up the tissue a little at a time until the bottom can be reached easily.

Then give 6 slow complete strokes to complete the homogenisation. Homogenisation is a very critical operation and should be done with the utmost care. Be especially careful not to raise the plunger too fast, otherwise a vacuum will be created which will play havoc with the mitochondria and may even cause the homogeniser to implode. The desirable consistency is similar to milkshake with no 'bits'.

Measure the volume of the homogenate using a measuring cylinder and note the value. Set aside about 1 ml of the homogenate in a snap-top centrifuge tube and put into the ice bucket (for later analysis – this is **Sample 1 – homogenate**), and divide the rest into two polypropylene 50 ml centrifuge tubes. Make sure the level in the two tubes is the same (carefully balancing by eye), by pouring from one to the other if necessary. Wash out the measuring cylinder with the STE solution (you will need the cylinder later). Load the balanced tubes into the bench centrifuge, opposite to one another. Switch on centrifuge (if it isn't already 'on'), bring the speed to 3,000 rpm on the screen, using the speed control knob. This speed will impose a centrifugal force of 1,500 g. Set the time control knob to 3 minutes, press the start button. The centrifuge will stop automatically, and the lid will open.

Remove your centrifuge tubes from the instrument carefully. This low speed of centrifugation should have centrifuged down debris (consisting of tissue fragments, some unbroken cells and large cell membrane fragments), red blood cells and some mitochondria (pinkish, red pink from the bottom of the tube upwards, if you hold the tube up to the light). The precipitate is not wanted, but the supernatant fluid from your two centrifuge tubes should be poured carefully (including all liquid plus the lightly packed mitochondria) into a single clean centrifuge tube. Measure the volume of this 1st supernatant fraction using a measuring cylinder and note the value. Set aside about 1 ml of the 1st supernatant fraction in a snap-top centrifuge tube and put into the ice bucket (for later analysis – this is **Sample 2 – 1st supernatant fraction**). *How is Sample 1 different from Sample 2?*

Make sure that your centrifuge tube is labelled for identification. Take the tube to the communal ice bucket provided; it will be taken by a demonstrator for centrifugation in a refrigerated centrifuge to spin at 10,000 x g for 10 minutes.

--------------------------------------break--

After the high-speed centrifugation, your tube will be returned to you. Handling it carefully, decant off the supernatant into a measuring cylinder. Keep the precipitate as well. Measure the volume of the supernatant in the cylinder. The precipitate in your centrifuge tube should be stirred up with a little fresh sucrose-Tris-EGTA; scraped from the bottom with a glass rod and transferred to the (sucrose washed) homogeniser. Resuspend the pellet using a couple of strokes in the homogeniser, and finally transfer to another measuring cylinder. Make up this resuspended pellet to exactly the same volume as that of the supernatant. Record the volume of each fraction (you will need this information for the calculations). These fractions are: post-mitochondrial supernatant fraction (**Sample 3**) and mitochondrial-enriched fraction (**Sample 4**).

The samples you will now have aliquoted into 1.5 ml tubes are as follows:

- **Sample 1** – homogenate – this is the dounced tissue prior to centrifugation
- **Sample 2** – 1st supernatant – the cleared version of the 1st homogenate
- **Sample 3** – the supernatant fraction after centrifugation of Sample 2.
- **Sample 4** – the pelleted and resuspended fraction after centrifugation of Sample 2.

Assays for enzymes can be carried out, as detailed below; each group should be able to investigate all three enzymes (in principle only six assays are required: three for cytoplasm, and three for mitochondria but you should also try to determine how much of the LDH enzyme was in the whole homogenate and look into the business of "latency" and the Triton effect.

Therefore, the measurements for 3 different enzyme activities are:

1) **Measurement 1** – Total activity – measure total activity of the homogenate (**Sample 1**) by first treating with Triton.
2) **Measurement 2** – **Sample 2** activity – measure the total activity of the 1st supernatant fraction by first treating with Triton.
3) **Measurement 3** – cytoplasmic activity (**Sample 3**) – activity of the post-mitochondrial fraction.
4) **Measurement 4** – Latent cytoplasmic activity: (**Sample 3**) – treat the cytoplasmic sample with Triton.**
5) **Measurement 5** – Mitochondrial activity (**Sample 4**).
6) **Measurement 6** – Latent mitochondrial activity (**Sample 4**) – mitochondrial fraction that has been Tritonised.**

***** Ask a demonstrator how these measurements can be combined with previous ones.***

2. Procedure for Enzyme Assays

a) Lactate dehydrogenase

Because we would like to have an idea about the percent recovery of LDH, MDH and GDH, try to determine how much LDH is in your set-aside 1 ml sample of "whole homogenate". First Tritonise it (add 10 – 20 µl of the 3% stock Triton) and stir it up a bit by mixing but don't froth it up – that tends to denature enzymes. The activity of LDH will be high, so you must use a small volume (10 µl) of the homogenate for the assay.

Carry out the following LDH assay measurement for the whole homogenate sample and repeat for the other samples and other two enzymes:

Kinetic measurements of enzyme activity on the spectrophotometer

Start-off by turning on the Computer and connected spectrophotometer. Log-on to computer as user. The Password will be BCSS followed by the last four digits on serial number.

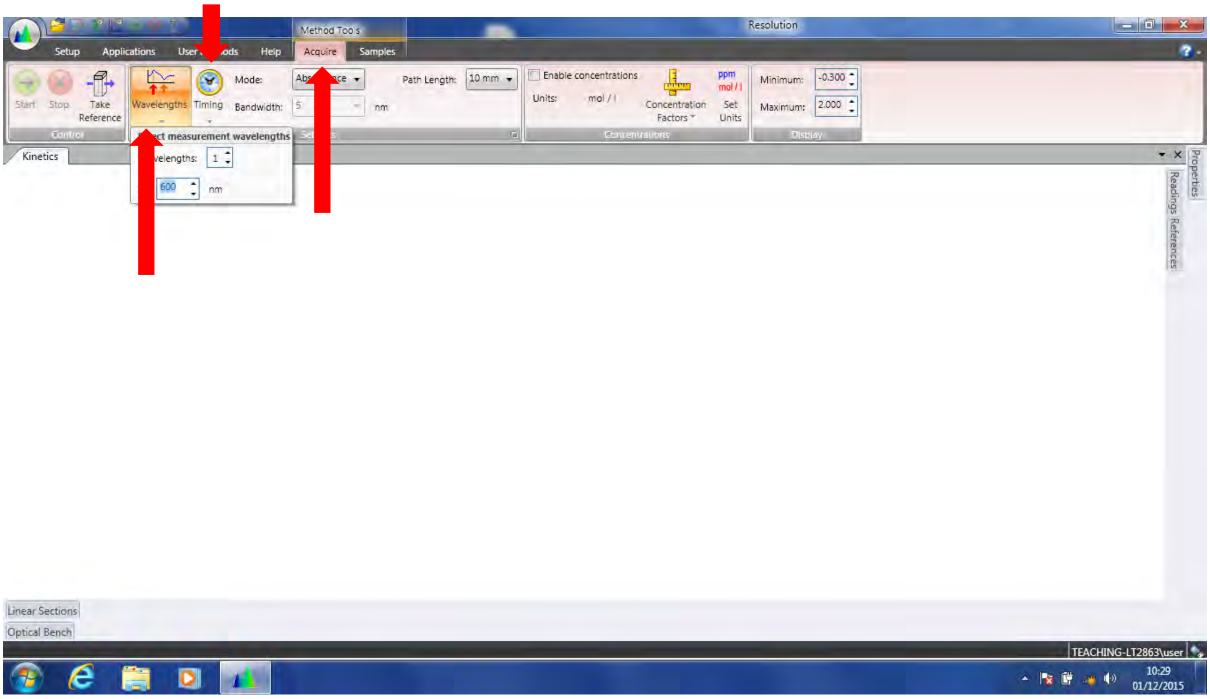
Wait until spectrophotometer has self-calibrated, then open the Resolution software, machine connects automatically and will display "Remote Terminal" on digital display.

Prepare a blank cuvette lacking NADH (3 ml of water in a cuvette will do). Put the cuvette in the cuvette holder, ensure orientation is correct.

Prepare for kinetic measurements and run the blank as follows:

APPLICATIONS – KINETICS

ACQUIRE (screenshot) – **WAVELENGTH** (screenshot) – enter 340 nM, **TIMING** (screenshot) – 300 sec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600 nm

TAKE REFERENCE – this will set the blank Absorbance to 0.

The green **START** button will now be active.

Add 3 ml of LDH/MDH cocktail to a cuvette.

START – the progress curve should proceed over time at a steady absorbance of about 0.8 Au. Monitor for 30 sec.

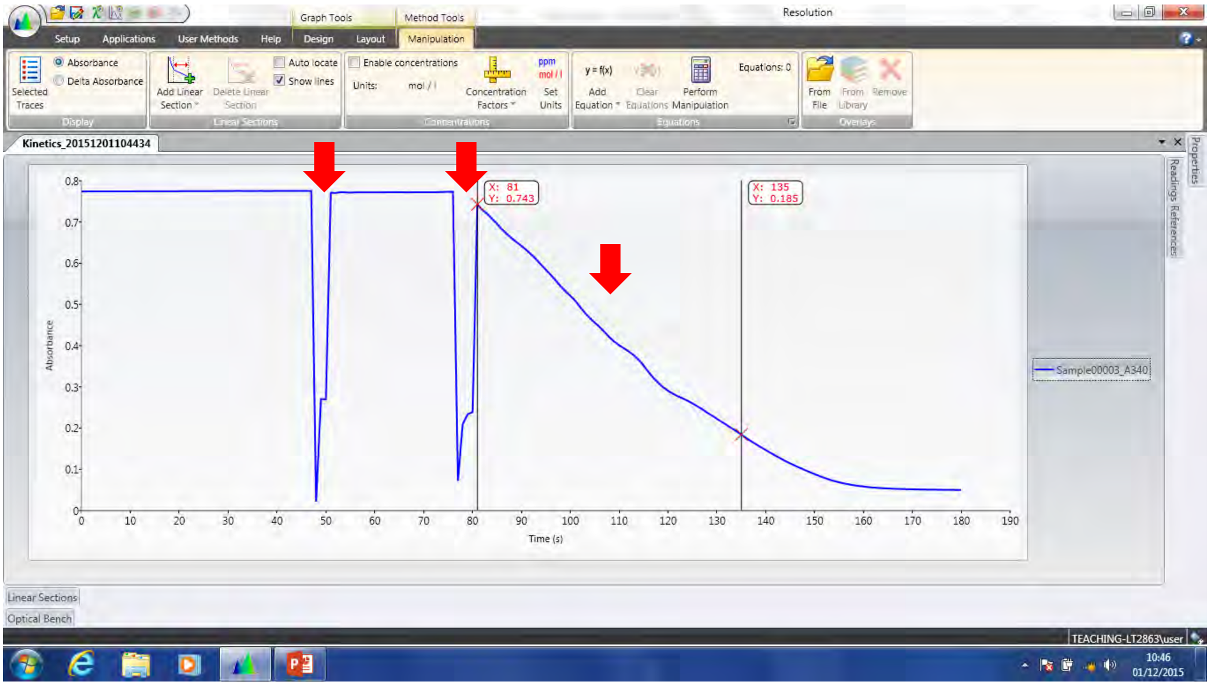
Add 10 μl of a sample (50 μl if you are using testes) to the cuvette, mix in by 3-4 strokes of the stirrer, and follow the progress curve (absorbance change) for about 30 sec – *does the progress curve remain stable?*

Now start the reaction by adding and mixing in 0.05 ml (50 μl) of 60 mM pyruvate solution, and resume measurement of the absorbance – *does the progress curve show a steady reduction in NADH absorbance?*

Adjust the amount of sample added in a new run:

- **Example of too much enzyme –**

  1st arrow is addition of the enzyme, 2nd arrow is addition of the pyruvate, 3rd arrow is the progress curve showing rapid reduction of NADH. Note, post-run the guide arrows appear allow you to dictate what part of the curve you will be calculating the rate from.


Graph Tools
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Resolution
Setup
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User Methods
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Absorbance
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Auto locate
Show lines
Linear Sections
Enable concentrations
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Concentrations
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Clear Equations
Perform Manipulation
Equations: 0
Equations
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Remove
Overlays
Kinetics_20151201104434
X: 81
Y: 0.743
X: 135
Y: 0.185
Absorbance
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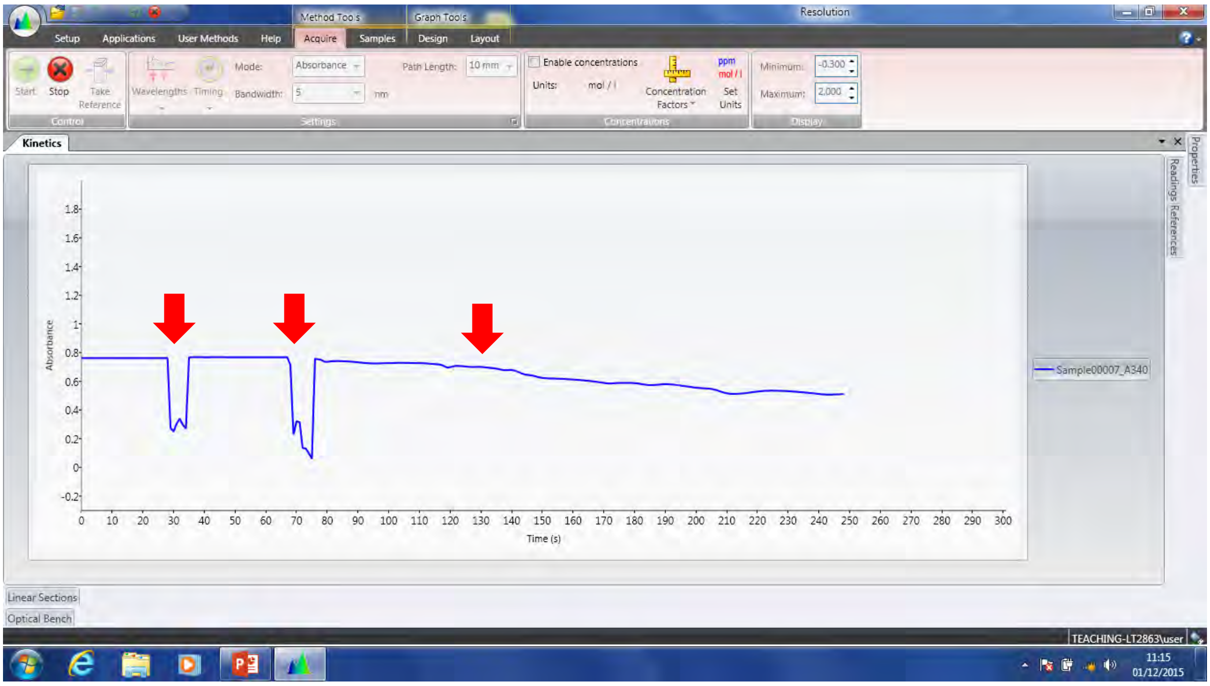
- **Example of too little enzyme –**

  1st arrow is addition of the enzyme, 2nd arrow is addition of the pyruvate, 3rd arrow is the progress curve showing slow, unsteady reduction of NADH.


Method Tools
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Set Units
Concentrations
Minimum: -0.300
Maximum: 2.000
Display
Kinetics
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0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
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40
50
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70
80
90
100
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Time (s)
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When run is done, (or press STOP), click yes to save results. Adjust guidelines to get the linear part of the kinetic run.

Obtain a slope of the line as follows: ADD LINEAR SECTION – LINEAR FIT – OK.

EXPORT each sample run as an Excel file to the "student data" folder on the desktop. Opening the file with Excel will show you all of the data and the slope, which corresponds to the rate of the progress curve in: change in Absorbance per Second.

Proceed to the next measurement: for every new run – APPLICATIONS – KINETICS – START.

Repeat the whole experiment with a sample of the other fraction. To determine whether there is any latency in the mitochondrial and supernatant fractions, also carry out assays in the presence of Triton. The most convenient way to do this is to add enough Triton, to an assay that is running but has not gone too far, to give a final concentration of 0.05% Triton (i.e. add 50 µl of a 3% stock Triton solution, and stir). "Tritonise" the assay in the case of both fractions.

b) Malate dehydrogenase

The method is exactly the same as for lactate dehydrogenase, except that a solution of 18 mM oxaloacetate is used instead of the pyruvate.

c) Glutamate dehydrogenase

The method is similar to those above, **but a different cocktail is used**. The "GDH cocktail" contains 0.05 M Tris buffer pH 8.2, 0.1 mM NADH, 0.05 M ammonium acetate and 1 mM ADP (which activates the enzyme). Following the outlines of the lactate dehydrogenase instructions (above) use 3.0 ml of "GDH cocktail", then a sample of a fraction (try 20 µl), then the substrate, this time 0.05 ml of 0.4 M 2-oxoglutarate. Don't worry if the absorbance jumps up when you add the oxoglutarate – it absorbs at 340 nm to a small extent.

N.B. For the testis extracts (less active) use 50 µl for all enzyme assays.

3. Calculation of enzyme content per organ from assay data

The slope $\triangle A / \triangle t$ obtained from the Excel file has units of A sec^{-1}. You know the volume of the sample (mitochondria or supernatant) that you added to the cuvette (typically 10 µl), and also the total volume of the fraction (typically 40 ml). The task is to use the raw data to calculate the total amount of the three enzymes in the tissue that you used. Use the rates measured in the presence of Triton. We can split the calculation up into logical steps.

(a) Convert from rate of change of Absorbance to rate of change of concentration of NADH.

Refer to Appendix 3 on "Spectrophotometry" and recollect that Absorbance (A) is proportional to concentration (c), with the molar absorption coefficient (ε_M) as the constant of proportionality. (The Beer-Lambert law.)

$$A = \varepsilon_M \cdot c \cdot l$$

Divide ΔA by the molar absorption coefficient of NADH (ε_M = 6220 M^{-1} for a 1 cm cell, so $l = 1$) to turn absorbance change into change in substrate concentration.

Rate of change of [NADH] in the cuvette = $\dfrac{\Delta A/\Delta t}{6220}$ M s^{-1}

(b) Convert from rate of change of concentration to rate of change of amount of NADH.

To go from concentration (moles per litre) to amount (moles), we need to multiply by the volume of the assay mixture, **v** (typically 3.06 ml i.e. 3.06×10^{-3} l).

Rate of change of NADH in the cuvette = $\dfrac{(\Delta A/\Delta t) \times v}{6220}$ moles s^{-1} or katals

The amount of enzyme that converts 1 mole of substrate per second corresponds to a unit called the katal. Thus we now have the number of katals in the cuvette as a measure of enzyme activity.

(c) Scale up from the volume of fraction used for the assay to the total volume of the fraction.

Suppose that sample of fraction, volume **y** ml, was added to the cuvette in the assay (typically 0.01 ml = 10 µl). Thus we can calculate the activity of the enzyme in katals per ml of fraction by dividing by **y**.

Activity of enzyme per ml of fraction = $\dfrac{(\Delta A/\Delta t) \times v}{6220 \times y}$ katals per ml

Finally, we multiply by the total volume of the fraction, **z** ml (typically 40 ml).

So, total acrivity of enzyme in fraction = $\dfrac{(\Delta A/\Delta t) \times V \times Z}{6220 \times Y}$ katals

Record this value in µkatal in the results table (for Q1 in the blue sheets).

(d) Estimate the recovery of Enzyme

Use the data from the LDH measurement obtained from the 1 ml sample that you kept back from the initial homogenate to calculate the total amount of LDH in the initial homogenate. Then compare that with the value calculated in step (d) above to estimate the percent recovery. In other words, what proportion of enzyme activity in the initial homogenate was still present in the fractions that you prepared?

$$\text{Percent recovery} = \frac{\text{activity in mitochondria + supernatant fraction}}{\text{activity in initial homogenate}} \times 100\%$$

Record this value in the results table (for Q1 in the blue sheets).

4. Calculate percent distribution of enzyme between mitochondrial and supernatant fractions prepared from an organ

By comparing the amount of the enzyme in the mitochondrial fraction with that in the supernatant, you can discover the percentage in each compartment.

Supernatant % =

$$\frac{\text{Amount in supernatant fraction}}{\text{Amount in (supernatant fraction} + \text{mitochondrial fraction)}} x100\%$$

and similarly for the mitochondrial fraction.

Record these values in the second results table (For Q3 in the blue sheets).
Look back at your results to see what effects Triton had. What information does this give you about the latency of the enzymes (latent: from the Latin for hidden)?

5. Electrophoresis of LDH isoenzymes

Safety Note: Acrylamide is toxic. Wear disposable gloves when handling gels.

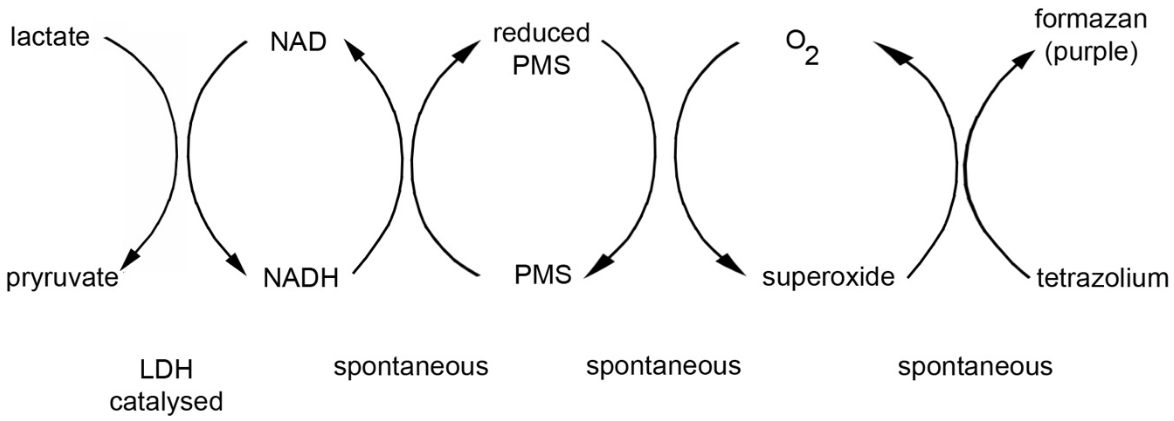
Make a polyacrylamide separating gel with a final acrylamide concentration of 7.5%. Each bench is provided with 10 ml of a "monomer mixture" in a conical flask made from 25 parts of 30% acrylamide + 0.3% *bis*acrylamide, 25 parts of 1.5 M Tris-HCl buffer pH 8.8, 47.9 parts of water and 0.1 parts of TEMED. This is enough for one gel. If you are not pouring the gel yourself, you should watch the pouring procedure. Add, when ready, all of the 10% ammonium persulphate (the polymerising agent) provided in the tube labelled AMPS (it holds 0.2 ml) and mix. Pour the mixture into the gel-holding frame; insert the plastic "comb" to form the sample “wells” and allow the whole to set (about 30 minutes). Put the flask containing remaining monomer onto the tray for toxic wastes (ask the demonstrator). After the gels have set in the frame, carefully remove the tubing around the edge of the frame (it is to be saved for re-use).

Half fill the bottom tank with tank buffer (0.05 M Tris/0.38 M glycine). Put the gel in its frame into the running apparatus. Place the gels by sliding them in at a left to right angle to minimise the trapping of air bubbles at the bottom of the gel. Make sure you put the gel frame into the tank the correct way round, with the shorter glass plate facing the top tank. Fill up the top tank with more tank buffer before removing the comb and after clamping the gel frame into place. Make sure the clamping is firm (otherwise the top tank will "leak"). Two benches (4 groups) can share the use of one gel.

Extracts of various rat organs will be provided (as well as liver, kidney and brain there will be heart and skeletal muscle); these extracts will have been made denser by being premixed with half their volume of a saturated sucrose solution containing bromophenol blue. Load samples (5 µl) into the slots ("wells") in the gel left by removal of the comb. You have to add the samples through the tank buffer: they will be sufficiently coloured to watch for successful loading. Load a couple of samples of "bromophenol blue + sucrose alone" into two vacant slots to use as "front markers". You may wish to run some of your own extracts if there is room on the gel; the "low-speed supernatant", the high-speed supernatant", and the mitochondria. Think about what you might expect. You will need to premix your material with and equal volume of sucrose + bromophenol blue (ask a Demonstrator). Run the electrophoresis at a current of 30 mA per gel. This should correspond to a PD of about 100 – 150 V. Watch for the movement of the bromophenol blue "front", and continue the electrophoresis until the dye gets near the bottom of the gel.

The electrophoresis takes about 1-1.5 hours.

Stop the electrophoresis, remove the gel (**remember gloves**) and stain it with an LDH assaying mixture, made of the following: 2.0 ml lactate (1.0 M); 2.0 ml NAD (5 mM); 4.0 ml Tris-HCl, pH 8.5; 4.0 ml nitroblue tetrazolium (2 mg/ml); 0.1 ml phenazine methosulphate (10 mg/ml); 13 ml water. Use the small square plastic trays for staining the gels. Place the enamel beaker over these trays to keep the incubation dark (phenazine methosulphate is light sensitive), but look at regular intervals for the precipitation of purple formazan bands at the sites of LDH activity. When ready wash away the staining solution into the "waste Stain" container. Then photograph the gel. The reason for the appearance of bands is that the following reaction pathway exists in the presence of LDH:



Notes

Subcellular Fractionation – Results and Assessment

Subcellular Fractionation

When you have completed (your part of) the Subcellular Fractionation practical, try to obtain other relevant data from the members of your team, and complete as much as you can of this sheet. Discuss the results with a demonstrator.

Q1. Total dehydrogenase activity in rat tissues

	Total µkatal			
	mitochondrial fraction	**supernatant fraction**	**µkatals of enzyme whole organ sample**	**% Recovery**
LIVER (HALF) Lactate DH Malate DH Glutamate DH				
2 KIDNEYS Lactate DH Malate DH Glutamate DH				
BRAIN Lactate DH Malate DH Glutamate DH				
2 TESTES Lactate DH Malate DH Glutamate DH				

Q2. What is the effect of adding the detergent Triton to the assays?

Q3. Percent distribution of enzyme activity in rat organs

Using the optimum experiments, and assuming that the sum of the activity in the mitochondrial and supernatant fractions equal 100%, complete the following table:

	Percent of recovered enzyme activity	
	mitochondrial fraction	**supernatant fraction**
LIVER Lactate DH Malate DH Glutamate DH		
2 KIDNEYS Lactate DH Malate DH Glutamate DH		
BRAIN Lactate DH Malate DH Glutamate DH		
2 TESTES Lactate DH Malate DH Glutamate DH		

Q4. What can you conclude about these enzyme distributions?

Q5. What do you conclude from the spacing of the bands? What would have been the result if the polyacrylamide gel electrophoresis had been carried out in the presence of SDS?

Week 3: Metabolic Control Analysis Practical

Metabolic Control Analysis in a Nutshell:

Metabolic engineering is generally referred to as the targeted and purposeful alteration of metabolic pathways found in an organism in order to better understand and utilize cellular pathways for chemical transformation, energy transduction, and supra-molecular assembly (Lessard, 1996). Metabolic Control Analysis (MCA) is a theoretical framework for investigating and understanding the control and regulation of metabolic pathways. The aim of this practical is to study the control of a metabolic pathway quantitatively focusing on how different steps in the pathway exert different amounts of control over the flux through the pathway.

It is an oversimplification to identify a single rate-limiting step imposing control over the whole pathway. More realistically, control is often shared, though unequally, amongst all the steps in a pathway, although the extent of control exerted by many of these steps may be negligible. The flux control coefficient (C^J_{Vi}) of a particular enzyme quantifies how much control the enzyme exerts over the pathway flux at steady state. C^J_{Vi} is the fractional change in the overall pathway flux ($\Delta J/J$) brought about by a fractional change in the activity or concentration of the enzyme per unit time (i.e. velocity of the reaction step catalysed by that enzyme) ($\Delta V_i/V_i$) for a particular reaction step i.

$$C^J_{Vi} = \Delta J / J \Big/ \Delta Vi / Vi$$

The sum of all the flux control coefficients for a simple linear unbranched pathway is 1.0 indicating that the total control exerted over the flux through the pathway is shared among the enzymes acting along the pathway. This is defined as the Flux Control Summation Theorem.

$$(\sum_i C^J_{Vi} = 1)$$

Thus, if physiological circumstances change, and a particular step becomes a more important control point i.e. its flux control coefficient is increased, the other steps in the pathway are bound to become less important control points.

The elasticity coefficient (ε) describes the fractional change in the activity of an isolated enzyme per unit time ($\Delta V_i/V_i$) in response to a fractional change in the concentration of a substrate ($\Delta S/S$) or an effector ($\Delta X/X$) with all the other substrates and effectors of that enzyme being held constant at the values that they have in the cell. The assumption here is that these values (the concentrations of all substrates and effectors of an enzyme) are known exactly. Unlike the flux control coefficient, which is a system property, the elasticity coefficient is a property of an individual enzyme and is defined as:

$$\varepsilon^{Vi}_{S} = \Delta Vi/V \Big/ \Delta S/S$$

The induction or repression of the synthesis of an enzyme, for catalytically changing the amount of active enzyme and its activation or deactivation by covalent modification, are ways of controlling pathways by altering the availability of catalytically active enzyme. These mechanisms will produce alterations in the flux control coefficients of pathway enzymes. The kinetic characteristics of enzymes may also be modified via routes such as the introduction of allosteric effectors, which change an enzyme's affinity for its substrate. Response coefficients are used to explain such control mechanisms over pathways. A response coefficient (R) is defined as the response of the metabolic system to an effector (X):

$$\frac{\Delta J}{J} = R_X^J = C_{Vi}^J \varepsilon_X^{Vi}$$

Getting to Know the Kinetic Model Used in the Practical:

A recent comprehensive kinetic model of yeast glycolysis revealed the existence of widely distributed control over the part of the metabolic network, which was most directly involved in glycolysis in yeast (Smallbone *et al.*, 2013). In this practical, you will be using a simpler kinetic model of the glycolytic pathway (Teusink *et al.*, 2000) (Figure 1). The substrate and the effector concentrations, as well as the kinetic constants of the enzymes in this model, were experimentally determined in yeast cells. The model was developed to answer the question of whether the behaviour of a complex metabolic pathway could be calculated from the known kinetic properties of its constituent enzymes. The answer to this question, as the research article suggests, is yes, but only to a limited extent.

The following set of ordinary differential equations may be used to describe the time dependence of the metabolite concentrations:

$$d[Glc_{in}]/dt = v_{GLT} - v_{HK}$$

$$d[G6P]/dt = v_{GLK} - v_{PGI} - 2v_{trehalose} - v_{glycogen}$$

$$d[F6P]/dt = v_{PGI} - v_{PFK}$$

$$d[F1,6bP2]/dt = v_{PFK} - v_{ALD}$$

$$d[DHAP+GAP]/dt = 2v_{ALD} - v_{GAPDH} - v_{G3PDH}$$

$$d[BPG]/dt = v_{GAPDH} - v_{PGK}$$

$$d[3PGA]/dt = v_{PGK} - v_{PGM}$$

$$d[2PGA]/dt = v_{PGM} - v_{ENO}$$

$$d[PEP]/dt = v_{ENO} - v_{PYK}$$

$$d[PYR]/dt = v_{PYK} - v_{PDC}$$

$$d[AcAld]/dt = v_{PDC} - v_{ADH} - 2v_{succinate}$$

$$d[ATP]/dt = -v_{GLK} - v_{PFK} + v_{PGK} + v_{PYK} - v_{ATPase}$$
$$-v_{trehalose} - v_{glycogen} - 4v_{succinate}$$

$$d[NADH]/dt = v_{GAPDH} - v_{ADH} - v_{glycerol} + 3v_{succinate}$$

$$d[NAD]/dt = - d[NADH]/dt$$

A unique rate equation representing the velocity of the reaction step is described in the model. In the case of PGI, PGM or ENO, one substrate-one product reversible Michaelis-Menten kinetics were used, such that:

$$v = \frac{V\left[\frac{a}{Ka}\left(1-\frac{\Gamma}{Keq}\right)\right]}{\left[1+\frac{a}{Ka}+\frac{p}{Kp}\right]}$$

where *a* and *p* represent the concentrations of the corresponding substrate and product, respectively. Γ is the mass action ratio (p/a) and K_{eq} is the equilibrium constant (p_{eq}/a_{eq}). K_a and K_p are the Michaelis-Menten constants for *a* and *p*, respectively.

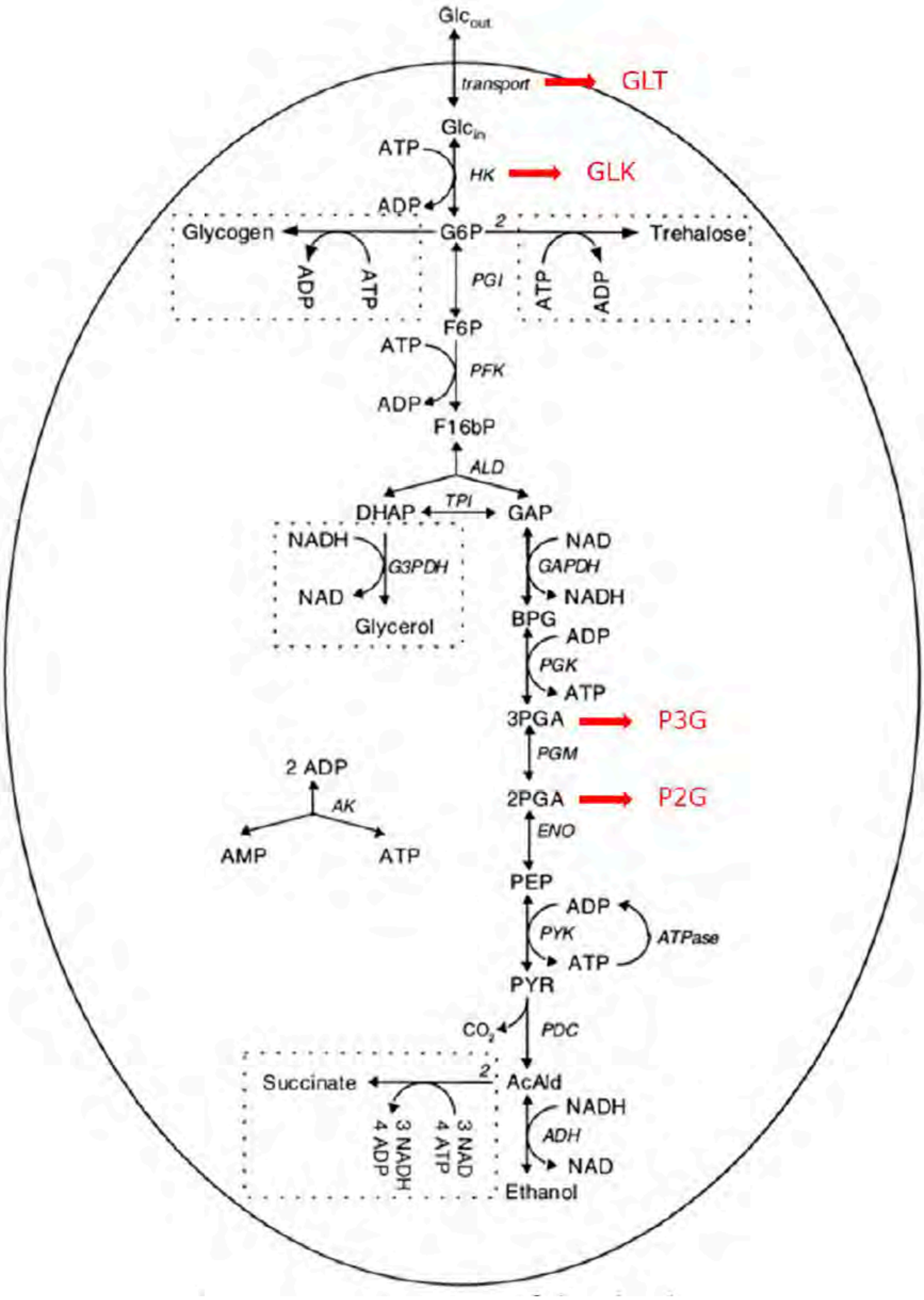

Glc$_{out}$
transport
GLT
Glc$_{in}$
ATP
HK
GLK
ADP
Glycogen
ADP
ATP
G6P
2
Trehalose
PGI
ATP
ADP
F6P
ATP
PFK
ADP
F16bP
ALD
DHAP
TPI
GAP
NADH
G3PDH
NAD
Glycerol
NAD
GAPDH
NADH
BPG
ADP
PGK
ATP
3PGA
P3G
PGM
2PGA
P2G
2 ADP
AK
AMP
ATP
ENO
PEP
ADP
PYK
ATPase
ATP
PYR
CO$_2$
PDC
Succinate
2
AcAld
4 ADP
3 NADH
4 ATP
3 NAD
NADH
ADH
NAD
Ethanol

Figure 1. A schematic representation of the glycolytic pathway. The alternative names for metabolites and enzymes given in "Red" indicate the corresponding nomenclature in COPASI.

COPASI as a Tool for Building and Investigating Kinetic Models:

COPASI (COmplex PAthway SImulator), is a user-friendly software environment that allows the construction, simulation and analysis of biochemical networks and their dynamics (www.copasi.org, Hoops *et al*., 2006). Any metabolic model, including the kinetic model presented above, can be integrated numerically into the software in order to be analysed.

COPASI can handle several analyses on metabolic pathways including, but not limited to: Metabolic Control Analysis, sensitivity analysis, elementary mode analysis, mass conservation analysis and time-scale separation analysis. To illustrate its use, we will run a Metabolic Control Analysis of the kinetic model of glycolysis in yeast in this practical session.

The Teusink model will be used to run some in silico experiments in COPASI. The effect of varying the enzyme concentration per unit time on the flux through the metabolic pathway will be investigated in the first instance. The concentration of 6-phosphofructo-1-kinase (PFK) per unit time will be varied and its effect on the glycolytic flux will be monitored by following the flux through the glucose transporter (GLT). This will then be used to estimate the flux control coefficient of that enzyme for glycolysis. The effect of varying the enzyme activity on metabolite levels will also be investigated in the glycolytic pathway. The activity of triosephosphate isomerase (TPI) will be varied and its effect on the concentration of dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) will be monitored. Finally, the metabolic regulation of a pathway via an external allosteric effector will be investigated. The concentration of fructose-2,6-bisphosphate (F26bP) will be varied and its effect on the flux through the glucose transporter (GLT) and on the concentration of fructose-6-phosphate (F6P) and fructose-1,6- bisphosphate (F16bP) per unit time will be investigated.

In the following practical, we will present the biological question that is being addressed in each step of the process. We will then introduce the mathematical formulation of this biological question within the framework of Metabolic Control Analysis. We then show how COPASI can be used to handle this question and finally, we will provide some tips on how to use COPASI more efficiently. For each question, you will be expected to run a set of simulations and record your results in a MS Excel Worksheet, which is provided along with other documentation in Course Share on the PC Desktops in the folder called ‘Metabolic Control in silico’. The cells you will be expected to fill in are highlighted in “Yellow” in the worksheet tabs. You will also be expected to interpret your results and use them to answer the biological question we have posed for each step of the analysis.

Getting to know the model: (20 min)

The kinetic model of yeast glycolysis has already been implemented in COPASI and is ready to be used. Double clicking 'Glycolysis model wt.cps' in the Folder called 'Metabolic Control in silico' under Course Share on the PC Desktop will open the model in COPASI. On the left-hand menu in the dialog, you will come across a tree for browsing and changing the model as well as running the simulations and saving the results from each successive step of the analysis. Once a model is loaded, the default node that is selected is 'Model'.

The 'Biochemical' and the 'Mathematical' structure of the model can be retrieved from the 'Model' node. The information on the different metabolites that appear in the model ('Species'), the biochemical reactions constituting the model ('Reactions'), the sub-cellular compartments in which these reactions take place ('Compartments') and the numerical values of the parameters (initial concentrations of the metabolites as well as the kinetic and the thermodynamic parameters of the enzymes catalysing each reaction step) that are supplied in the model ('Parameter Overview') are provided under the 'Biochemical' sub-node.

Please note that the present model involves 19 reactions and some 25 unique metabolites and all reactions take place in a single compartment, the cytosol. Please click on any random metabolite under the 'Species' element and observe that the initial concentration and the reactions in which the metabolite is either produced or consumed are displayed on the right-hand side; the input to the model may be changed as needed. Similarly, clicking on any randomly selected enzyme under the 'Reactions' element will open up the details for that individual reaction on the right-hand side. Please observe that the rate law for the reaction, details of which can be accessed by clicking 'Edit Rate Law' in the right-hand window, along with the corresponding parameter values as well as the substrates and the products of the reaction are provided individually. Any parameter belonging to a species or a reaction may be modified by clicking on its name and making the required changes in the window on the right-hand side. Please make sure to click on the COMMIT button after making the changes to ensure that the changes are saved in the model.

The structure of the mathematical model for the pathway under investigation is provided in the 'Mathematical' node in the tree on the left-hand menu. The set of 'Differential Equations' constituting the kinetic model of the glycolytic pathway is automatically generated once parameter inputs are provided in the 'Biochemical' node. The 'Matrices' node provides the stoichiometric matrix of the pathway, which is mainly required for metabolic analyses that are conducted at steady state. The reaction stoichiometries are used to generate this matrix automatically.

Important things to note regarding the model:

- v_{max} is denoted as V in COPASI terminology.
- In figure 1, 3PGA is denoted as P3G and 2PGA as P2G in the COPASI model.
- COPASI assumes that the model simulations take place in a unit compartment volume (1L) and uses mmol/min as the unit of concentration per time. These values could safely be used to make comparisons with the values given in mM/min in this text.
- 'Transport' and 'HK' reactions in Figure 1 are denoted as 'GLT' and 'GLK' flux, respectively, in the COPASI model.

Getting ready to run simulations: (10 min)

This is a very straightforward procedure; therefore, we only provide details on how to set things up in COPASI.

- General information
  - The 'Tasks' node provides options for running the simulations. 'Time Course' simulations will be carried out during this practical using the initial conditions and kinetic parameters provided in the model.
  - The reaction rates and the metabolite concentrations will be calculated at every time step throughout the dynamic simulation. This will allow us to monitor how the concentrations of the species and the fluxes of the reactions change over time until they reach a steady state.
  - The output plots and reports need to be set up prior to running any simulations.
- Setting up the output formats
- Any existing plots and reports first need to be cleared. To do this, click on:
  - 'Output Specifications' (on the left-hand menu) → 'Plots' (on the left-hand menu) → 'Delete All' (bottom of the right-hand window) will appear executable (not as shadow) if there are any plots that need to be deleted. Clicking this button will clear out any existing plots.
  - 'Output Specifications' (on the left-hand menu) → 'Reports' (on the left-hand menu) → 'Delete All' (bottom of the right-hand window) will appear executable (not as shadow) if there are any reports that need to be deleted. Clicking this button will clear out any existing reports.
- For setting up a new set of plots and reports in which the new simulation results will be recorded, follow the steps:
- To generate a plot of the metabolite concentrations click on the following:
  - 'Tasks' (on the left-hand menu) → 'Time Course' (on the left-hand menu) → 'Output Assistant' (right hand window) → 'Concentrations, Volumes, Global Quantities' (**under Plots section**) → 'Create'
- To generate a plot of the reaction fluxes click on the following:
  - 'Tasks' (on the left-hand menu) → 'Time Course' (on the left-hand menu) → 'Output Assistant' (right hand window) → 'Reaction Fluxes' (**under Plots section**) → 'Create'
- To generate a report of the metabolite concentrations click on the following:
  - 'Tasks' (on the left-hand menu) → 'Time Course' (on the left-hand menu) → 'Output Assistant' (right hand window) → 'Time, Concentrations, Volumes, Global Quantities' (**under Reports section**) → 'Create'
  - Report (right hand window still at 'Time Course') → 'Open folder icon' → Choose where to save the data file → Name the file using an .xls extension → Select Any File(*) as the Save As Type → Save → Unclick Append → Confirm

Important:

- Reports and plots are created after the simulation has run.
- You only need to set up the plots and reports once; they will then be produced every time you click “run”.
- It is up to you what combination of plots and reports you use to collect data, but the most straightforward method is to create a report for metabolite concentrations and then record V_{GLT} flux from a plot of fluxes. If you need to know the flux with greater accuracy (as is required for Q11), use the ‘Save data’ command in the plot of the reaction fluxes.
- Report files need to be closed before each simulation in order for the new results to be overwritten on the same file.
    - Throughout this practical, whenever a parameter is changed in the simulations, all output results should change. Obtaining the exact same result as that of the previously run simulation would indicate either: Report files were left open prior to running the simulation and they could not be overwritten.

OR

-
    - A bug has occurred in Report files. A quick solution to this would be repeating the report saving procedure provided above or saving the results from the Plot windows by overwriting those reports manually by:
        - Save Data (in Plot window) → name/rename/select the existing report file → Save
- The time interval of the model should be set such that 1000 iterations would be run (number of intervals) for intervals of 2 minutes, thus 2000 minutes will be the total time to be dynamically simulated in the *in silico* experiments.

- Before proceeding any further, check that your plots and reports are set up correctly by running the simulation (clicking ‘Run’ in the ‘Time Course’ window on the right-hand side) – check that two different plots (only 2) and a single report are being generated in each run.
- It is useful to jot in as many significant digits as possible. Some perturbations may result in very subtle changes.
- The concentration and flux values may be read from the plots generated in COPASI or, alternatively, or by selecting ‘Time Course’ → ‘Result’ on the left-hand menu and looking for the steady state values presented in the Table on the main window.

Running simulations using the model: (15 min)

For the first simulation, you should run the glycolysis model (Glycolysis model wt.cps) without performing any modifications. In fact, you have already done so as the test-step in the previous task. When you run the model, the plot for 'Concentrations, Volumes and Global Quantity Values' will display how the dynamic profiles of the concentrations of all metabolites vary from their initial values until they attain a steady state. The plot for 'Reaction Fluxes' shows the transient fluxes of all reactions. The profiles for any of the metabolites can be hidden on the plot by clicking on their label at the bottom of the figure.

Two very short questions to answer before you proceed with the actual practical:

Q1. What is the steady state concentration of F6P?

Q2. The steady state flux through both the glucose transporter; V_{GLT} and the glucokinase; V_{GLK} are both 85.45614 mM/min. What is V_{PGI}? Why is it lower? Can we use 'V' or 'J' interchangeably for either one of the three?

***Hint**: The schematic representation of glycolysis (Figure 1) may be helpful.*

The effect of PFK concentration per unit time on the glycolytic flux – Determination of a flux control coefficient at steady state: (40 min)

This exercise aims to investigate how the glycolytic flux (J) represented by the glucose uptake rate (V_{GLT}) and the concentrations of the following glycolytic intermediates; G6P, F6P, F16BP and P3G are affected by variations in the velocity of the reaction step catalysed by PFK. For this purpose, you will vary the maximum rate of PFK (V_{max} – denoted as V in COPASI) to change the velocity of the individual reaction step. To vary the V_{max} for PFK, click on the following nodes in the left-hand menu: 'Model' → 'Biochemical' → 'Reactions' → 'PFK'. Please make sure that you click on the COMMIT button in order for the changes to take place. For different V_{max} values of PFK, you will record the steady-state concentrations of G6P, F6P, F16BP and P3G and the steady-state flux V_{GLT}. The data should be recorded in Tab A of the MS Excel Worksheet provided. Please make sure that you change the name of the file to your name as you will be handing that document in at the end of the practical. You will be simulating the model for 3 different values of V_{maxPFK} = 50, 50.1, and 60 mM/min. The simulation results for other values of V_{maxPFK} (up to 200 mM/min) are already provided in the worksheet for the sake of convenience.

Q3. Two different scales are used in the 'Metabolite Concentrations' plot provided in the MS Excel worksheet Tab A. Why do you think we need two different scales? ***Hint**: Check how the metabolites are positioned in the glycolytic pathway in Figure 1.*

Now try simulating the model at V_{maxPFK} = 40 mM/min.

Q4. What happens if V_{max} = 40 mM/min? Why do you think this happens? ***Hint**: Answer to Q3 might be helpful in explaining this phenomenon.*

Now you will try to investigate how much control the PFK enzyme exerts over the glycolytic flux. The flux control coefficient of PFK for glycolysis (C^J_{VPFK}) is given by:

$$C^J_{V_{PFK}} = {}^{\Delta J/J}\!\Big/_{\Delta V_{PFK}/V_{PFK}}$$

where $\Delta X/X=(X_{final}-X_{initial})/X_{initial}$

***Hint**: making use of all the flux and V data recorded on the MS Excel worksheet Tab A, calculate ΔJ, ΔJ/J, ΔV, ΔV/V and finally C values for each simulation. It is important to note that the final and the initial values should preferable be consecutive ones.*

Q5. What is the effect of changing the V_{max}PFK on glycolytic flux? Is the effect more evident at higher or lower values of V_{max}PFK?

The Flux Control Coefficient is the fractional change in flux following an infinitesimally small fractional change in the concentration or activity of an enzyme. Experimentally, this value is impossible to achieve since an infinitesimally small increase or decrease in flux cannot be measured. However, it is possible to conduct such an experiment *in silico* by decreasing the step size. This would be very similar to measuring the tangent to the slope of the graph J_{GLT} vs V_{PFK} at a specific point V_{PFK} = 80 mM/min. For the actual V_{max}PFK = 80 mM/min, the true value of the Flux Control Coefficient is 0.646.

Q6. Conduct one *in silico* experiment to get a more realistic value for the Flux Control Coefficient.

Q7. How does the glycolytic flux and the control of PFK over the glycolytic flux change when the velocity of the reaction step catalysed by PFK is varied? A major portion of the remaining control exerted on this pathway would be shared between the glucose transport (GLT) and hexokinase (GLK) activities. What do you think happens as the control of PFK over the glycolytic flux changes?

Q8. The Flux Control Coefficient of PFK at low PFK concentrations (e.g. PFK V_{max}PFK = 50) is greater than 1.0. Why is this value so high? Why is it greater than 1.0?

The effect of TPI enzyme activity on the glycolytic flux – Determination of a Flux Control Coefficient at steady state: (25 min)

In the next exercise, you will work with an enzyme that is conventionally thought to have a very low Flux Control Coefficient for glycolysis, triosephosphate isomerase (TPI) catalysing the interconversion of dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). You will investigate how varying the activity of TPI affects its control over the glycolytic flux. The V_{max} for PFK should first be set back to 80 mM/min ('Model' → 'Biochemical' → 'Reactions' → PFK' (all in the left -hand menu)). Re-running the simulation, note down the values for the steady state concentrations of GAP, DHAP as well as V_{GLT} in Tab B of your MS Excel worksheet. The next step would be to vary the kinetic constants for TPI in the cell. This enzyme catalyses a reversible reaction occurring in the cell. The kinetic constants need to be varied together in order to maintain the thermodynamic equilibrium constant (K = (k_1/k_2) = 22.2) as constant at all times. In order to achieve a considerable change in enzyme activity, both kinetic constants should be increased or decreased by several orders of magnitude (keeping K constant at all times). The worksheet Tab B suggests a reduction of 10^5 in magnitude. After committing this change and re- running the simulation, you should record the GAP and DHAP concentrations as well as V_{GLT}.

Q9. What happens to the glycolytic flux in response to changing the enzyme activity of TPI? What happens to DHAP/GAP concentration ratio? Is the reaction catalysed by TPI still at equilibrium in the cell? What does this mean thermodynamically? Why do you think we encounter such an outcome?

Now you will see the effect of this change that you have artificially created on the control of TPI over the glycolytic flux. For each case, we suggest making a small change of 1% in k1, and calculating the change that needs to be made in k2 accordingly, keeping K = 22.2. This time, only VGLT needs to be recorded in the simulations. Either k1 or k2 can be used to calculate the Flux Control Coefficient for TPI over the glycolytic flux following a similar procedure as the one you have carried out for PFK.

Q10. Does TPI have high flux control? Explain why the control of TPI over the glycolytic flux is affected by the displacement of the reaction catalysed by TPI from equilibrium.

F26bP as a regulator of the glycolytic flux: (35 min)

Fructose-2,6-bisphosphate (F26bP) is perhaps the most important regulator of the glycolytic flux. F26bP affects the reaction step catalysed by PFK in glycolysis. This exercise is designed to explore this relationship, which is described by the elasticity coefficient of PFK for F26bP:

$$\varepsilon_{F26bP}^{V_{PFK}}$$

As elasticity coefficients are challenging to obtain directly, you will make use of the concept of response coefficient, which establishes the relationship between the glycolytic flux and both the enzyme's elasticity and its Flux Control Coefficient, as previously introduced. The response of the glycolytic pathway to F26bP could be formulated as:

$$\frac{\Delta J}{J} = R_{F26bP}^{J} = C_{V_{PFK}}^{J}\,\varepsilon_{F26bP}^{V_{PFK}}$$

where $C_{V_{PFK}}^{J}$ is the flux control coefficient of PFK for the glycolytic flux and $\varepsilon_{F26bP}^{V_{PFK}}$ is the elasticity coefficient of PFK for F26bP.

You will also investigate how the upstream substrate, fructose-6-phosphate (F6P), and the downstream product, fructose-1,6-biphosphate (F16bP), of the reaction step catalysed by PFK is affected by varying the concentration of the effector metabolite, F26bP. This analysis will further be extended to monitor the same effect at different reaction velocities of PFK (i.e. for different values of V_{max} for PFK).

The previous exercises demonstrated that PFK would have a relatively high Flux Control Coefficient for glycolysis in yeast at low concentrations. However, the true V_{max} for PFK is close to 200 mM/min producing a very low flux coefficient for the enzyme over the glycolytic flux ($C^{J}_{V_{PFK}} = 0.00151$) (Davies & Brindle, 1992).

As an initial step, the k1 and k2 settings for TPI need to be reverted back to the original values from the previous exercise. To access the relevant window on the right-hand side, click on the nodes in the left-hand menu in the dialog in the following order: 'Model' → 'Biochemical' → 'Reactions' → 'TPI' and set k1 = 2.2222e+07 and k2 = 1e+06 on the right-hand window. In order to implement the current exercise in COPASI, the V_{max} for PFK (represented as V in COPASI) needs to be varied. To access the relevant window on the right-hand side, click on the nodes in the left-hand menu in the dialog in the following order: 'Model' → 'Biochemical' → 'Reactions' → 'PFK'. The required settings are provided in Tab C of the MS Excel Worksheet. Please note that as you have already simulated the model for V=80 and V=200 for PFK (as given in COPASI), in the first exercise, these values are already included in the worksheet. The glucose uptake rate V_{GLT} needs to be recorded with a precision of 5 decimal places as well as the concentrations of F6P and F16bP.

To determine the effect of F26bP on flux, the simulations need to be carried out at a different concentration of F26bP = 0.2 mM. To make this change, please follow the nodes in the given order on the left-hand menu: 'Model' → 'Biochemical' → 'Species' → 'F26bP'. The glucose uptake rate V_{GLT} as well as the concentrations of F6P and F16bP again need to be recorded with a precision of 5 decimal places. These values will then be used to calculate the response coefficient (R^{J}_{F26bP}), which is the fractional change in V_{GLT}, and to determine the elasticity coefficient of PFK for F26bP using the relationship provided above.

IMPORTANT: Please note that incremental changes created in F26bP concentration will be used to calculate the response coefficient for a fixed value of V_{maxPFK}. The incremental changes created in V_{maxPFK} will be used to calculate FCC for a fixed value of F26bP concentration.

Q11. What does the elasticity tell us about the response of PFK to F26bP? How is substrate F6P and product F16bP concentrations are affected by changes in F26bP concentration at a constant reaction velocity for PFK? How do these results relate to PFK having high or low control over the glycolytic flux at V_{max} values of 80 or 200 mM/min?

Congratulations, you have completed the MCA practical!

BONUS for Math Gurus: If you are interested in the topic and would like to construct your own kinetic model from scratch, please inquire 😀😀

Notes

Metabolic Control Analysis – Questions Addressed in the Practical

Q1. What is the steady state concentration of F6P?

Q2. What is J(PGI)? Why is it lower? **Hint**: *The schematic representation of glycolysis on the second page of the green sheets may be helpful.*

Q3. What happens if the Vmax for PFK is lowered below 50?

Q4. What is the effect of changing the PFK V_{max} on glycolytic flux? Is the effect more evident at higher or lower values of V_{max}?

Q5. Is there a cross-over in metabolite concentrations as the PFK activity is changed?

Q6. What is the range of values of the PFK flux control coefficient? How does that compare with the effect of enzyme concentration on the glycolytic flux (as in Q4)?

Q7. Why is flux control coefficient of PFK very high and why is it greater than 1.0 at low PFK concentrations (e.g. PFK V_{max} = 50)

Q8. What happens to the flux in response to the changing the rate constants of TPI? Is the reaction catalysed by TPI still at equilibrium in the cell? Does it have a high flux control coefficient? How does this relate to any simplistic notions we might have regarding control – would you expect an enzyme catalysing a reaction that is displaced from equilibrium to have a high flux control coefficient?

Q9. What does the elasticity tell us about the response of PFK to F26P? Is this consistent with the changes in F6P and F16P concentrations?

Q10. If we calculated the elasticity coefficient of PFK for F26P with the V_{max} of PFK set to 80, we would find it is very low, around 3.08e-03. Why might this be? Remember that PFK has a relatively high flux control coefficient under these conditions.

Week 4 (Lent) – Mitochondrial Oxidative Phosphorylation

Objectives

This practical is intended to complement the lectures on Bioenergetics (Energy transduction in bacteria, mitochondria and chloroplasts). You will use mitochondria to assess the coupling of respiration to ATP synthesis, by measuring the P/O ratio and the respiratory control ratio. You will explore the characteristics of chemiosmotic energy coupling using uncouplers and inhibitors, and do a variety of calculations based on the results.

At the end of this practical you should be able to perform the following tasks:

- Use an oxygen electrode to assess how well respiration is coupled to phosphorylation in a preparation of mitochondria by measuring the respiratory control ratio.
- Measure P/O ratios for respiratory substrates.
- Follow and devise protocols to study the action of inhibitors and uncouplers on mitochondrial energetics.

Introduction and Background

In this practical you will use the oxygen electrode to demonstrate the effects of ADP and a selection of inhibitors (oligomycin, antimycin) and uncoupler (FCCP) on oxygen consumption by mitochondria isolated from rat liver. This part of the practical will be structured and is designed to illustrate the basic properties of isolated mitochondria.

To get the most out of the practical you should try to predict what each addition will do to the rate you are measuring, and then (if your predictions fail) try to explain the rate changes that you see as the experiment is running (or at least, before you run the next incubation). If you can't, talk to a demonstrator before proceeding.

Oxygen uptake by mitochondria can be studied using an oxygen electrode. The electrode (located at the bottom of the incubation vessel) is covered by a Teflon membrane (separating it from the incubation medium), which is permeable to gases only. As soon as oxygen diffuses across this membrane from the medium it is reduced at the surface of the platinum electrode (held at –0.6 volts), causing a current to flow that is proportional to the oxygen concentration in the medium.

Mitochondria contain the enzymes necessary to oxidise substrates, particularly those of the citric acid cycle, and to generate ATP from ADP and phosphate. The two processes of oxidation and phosphorylation are fairly tightly coupled together.

The phosphate:oxygen (P:O) ratio is defined as the number of molecules of phosphate incorporated into ATP per atom of oxygen consumed by a mitochondrial preparation oxidizing a substrate. Maximum values for the P:O ratio will be given by the number of H^+ ejected per oxygen consumed during electron transport divided by the number of H^+ used to synthesize each ATP (P:O ratio ≤ H^+:O ratio / H^+:P ratio). If it is assumed that all the ADP utilized by mitochondria is converted to ATP and there is a negligible rate of ATP hydrolysis, then the P:O ratio may be accurately calculated from the amount of oxygen consumed by mitochondria supplied with a limiting amount of ADP in the presence of an excess of the substrate and P_i.

The respiratory control ratio is the rate of respiration of mitochondria in the presence of substrate, P_i and ADP (state 3) divided by the rate of respiration of the mitochondria in the presence of substrate, and ATP (state 4). This indicates how ‘tightly-coupled’ or intact the mitochondrial preparation is.

The experiments to be carried out include a study of the P:O ratios achieved by mitochondria in oxidising various substrates, and an investigation of the effects of some typical inhibitors on mitochondrial respiration.

How many protons?

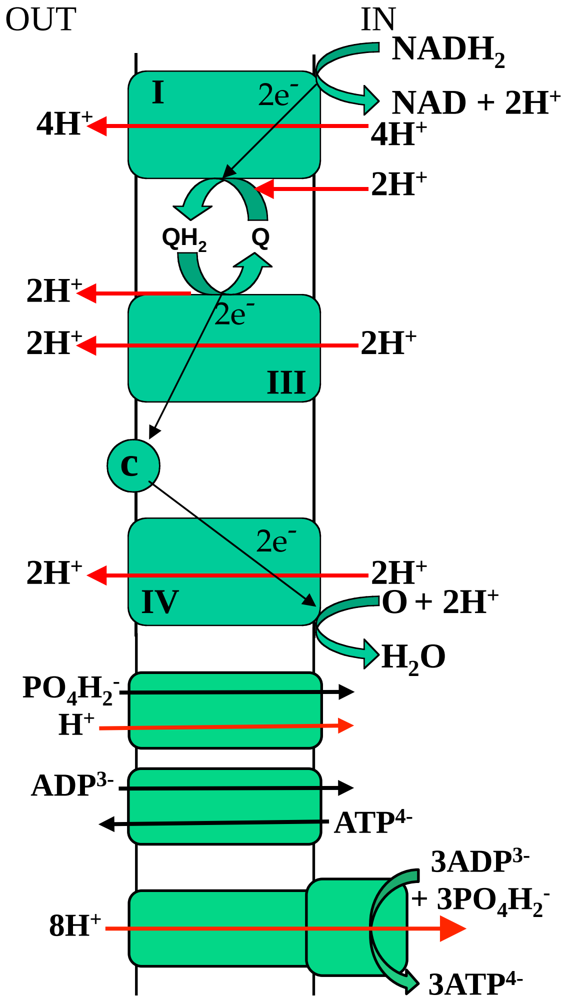
The number of protons transported by the mitochondrial respiratory chain is indicated on the left **PER 2 ELECTRONS**.

Complex I transports 4 H^+.
Complex II does not transport any protons.
Complex III transports 2 H^+ across the membrane plus releases 2 extra H^+ outside.
Complex IV transports 2 H^+ across the membrane, moves 2 extra – charges inside, and takes up 2 extra H^+ inside.

So for NADH-linked substrates (e.g. glutamate and malate) the total number of protons transported is 10 H^+ per 2 e^- moving from NADH to oxygen, and for succinate is 6 H^+ per 2 e^-.

ATP synthesis requires 8 H^+ per 3 ATP made, but transport of ATP, ADP and Pi requires the equivalent of another proton per ATP.

The P/O ratio = H^+/O ratio / H^+/P ratio
Thus for NADH linked substrates:
P/O = 10/3.67 ≈ 2.7
For succinate:
P/O = 6/3.67 ≈ 1.6



Oxidative Phosphorylation – Experimental

1 Mitochondrial respiration and coupling to ATP production

The mitochondria provided will have been isolated earlier in the day from rat liver. For maximum coupling and respiratory rates, the mitochondrial suspensions must be kept cold and must not be contaminated by ice or by pipettes that might have come into contact with inhibitors or uncouplers.

A. Rates of respiration and P:O ratios with succinate

This experiment is designed to measure the respiration rates and P:O ratio of mitochondria respiring on succinate.

- Shake your flask of 'medium A' (110 mM KCl, 10 mM KH_2PO_4, 5 mM NTA [nitrilotriacetic acid], 2 mM $MgCl_2$, pH 7.4) to ensure air saturation.
- Remove the lid from the oxygen electrode assembly and suck out the contents using the suction tube attached to the vacuum line. Wash with water, then use a P5000 Gilson pipette to add 1.9 ml of medium into the electrode vessel. Make sure that the stirrer is on. Check that the recorder pen is roughly 90% over to the right of the paper. If unsure ask a demonstrator – don't fiddle with the knobs.
- Check that the chart motor is on and the pen is making a mark on the paper. Make sure the chart is moving at 10 mm/min.
- Now add 30 µl (or other recommended volume) of mitochondrial suspension (using Gilson P200 pipette and yellow tip).
- Place the lid on the electrode, **and make sure no air bubbles are trapped.** Do **not switch off the stirrer** while you do this.

  ***NOTE*: It is ESSENTIAL that no air bubbles are trapped. To remove air bubbles, push the lid steadily down so that the air exits through the capillary "chimney" in the lid and a bubble-free column of liquid enters the chimney. Don't push so far that too much liquid is pushed up and forms a pond in the top part of the lid. Don't push down on the lid with your finger over the top of the lid (that will prevent air leaving). Check with a demonstrator if in doubt.**
- Check that a reasonably steady trace is obtained on the recorder (1 minute will tell). There may now be a slow rate of oxygen consumption (why?).
- Add 50 µl of 0.5 M sodium succinate, as respiratory substrate, through the "chimney" in the lid using a white Gel-tip with capillary extension attached to a Gilson P200 (**check that the end of the tip is in the vessel**). You can insert the Gel-tip so that the shank rests on the lip of the lid, but don't press so hard that you make an air-tight seal. Press the plunger of the Gilson as far as the resistance point (and no further) to expel the liquid and then remove Gilson (with tip attached) before releasing the plunger. Press the "Event Marker" on the top panel of the recorder to mark the addition on the chart paper, for this and subsequent events, and write what you added on the chart paper.
- Watch for an increased rate of oxygen consumption (give it 1 – 2 minutes) and then:
- Add exactly 20 µl 10 mM ADP using a white Gel-tip attached to a P20 Gilson. This should take the mitochondria into state 3 with a faster rate of respiration, but after a

minute or two (when all the ADP added has been converted to ATP) the rate should slow down again (this is state 4). When the mitochondria have been in state 4 for about 2 minutes:

- Followed by 5 µl of FCCP.

Preferably allow your experiment to reach anaerobiosis; this should be close to zero on the chart (consult a demonstrator if in doubt). However, these may be a slow process: if so, you can establish the zero by pressing the "Zero Button" on the recorder and noting the position of the pen. When finished, suck out the contents of the vessel and wash it and its top thoroughly with ethanol (supplied in a wash bottle) and then with distilled water. This is to remove all traces of the FCCP before the next experiment. If you are not going to do the next run immediately, put about 2 ml of distilled water in the vessel to stop the membrane drying out.

You can convert recorder readings into values of oxygen concentration because the concentration of oxygen in air-saturated medium at 22 °C is known to be 500 nmol O atoms per ml (0.5 mM O atoms). Thus, the reaction vessel (containing say 2.0 ml liquid) will contain a total of 2.0 x 500 = 1000 nmol O at the beginning of the experiment, and this amount of oxygen would be represented by the usable span across the recorder chart (e.g. 170 small scale divisions if the starting reading was 180 and 'zero' reading was 10). Note that oxygen concentrations have been expressed here in terms of O (atoms); in reality of course the oxygen is present as O_2 (at a concentration of 0.25 mM, or 250 nmol O_2 per ml), but, since each O atom accounts for a pair of electrons, it is convenient to use O concentrations.

If this experiment has worked well, you should be able to determine the respiratory control ratio, the P:O ratio and the state 4 rate for succinate. If not, repeat the experiment in order to determine these values.

The respiratory control ratio (RCR) is calculated by dividing the state 3 rate of respiration by the state 4 rate, and this indicates how intact the mitochondria are. The P:O ratio is calculated by dividing the amount of ADP (nmol ADP) added by the amount of oxygen (nmol O) consumed in state 3. This indicates the ratio of ATP produced to oxygen consumed by the mitochondria, and varies with different substrates. Put your calculated P:O ratio and RCR values on the board.

B. Substrates and inhibitors of the mitochondrial respiratory chain

The aim of the following experiment is to illustrate the sites of action of various substrate and inhibitors of the mitochondrial respiratory chain.

If you have not already done so, wash out the vessel and rinse its top thoroughly with ethanol and then with distilled water to remove all traces of FCCP. Make additions to the reaction vessel in the following sequence (and wait a minute or two between additions to see the effects):

- 1.90 ml medium A.
- 0.05 ml (50 µl) mitochondrial suspension (**now put the top on**)
- 50 µl glutamate + malate substrates (produce NADH for oxidation by Complex I)
- 200 µl of 10 mM ADP (this quantity will keep the mitochondria in state 3); then add
- 10 µl rotenone (an inhibitor of Complex I)

- 50 µl succinate (a substrate for Complex II)
- 10 µl antimycin (an inhibitor of Complex III)
- 50 µl ascorbate + TMPD (non-physiological substrate for Complex IV)
- 10 µl cyanide (an inhibitor of Complex IV).

Wash out the vessel at the end of the experiment and leave with water in it.

Note. Rotenone is dissolved in DMSO and antimycin is dissolved in ethanol.
The concentrations of inhibitors provided are rotenone, 500 µg/ml; antimycin, 500 µg/ml; cyanide, 0.1 M; oligomycin, 1.25 mg/ml.

Safety Note: These poisons are poisonous!

The concentrations of substrates provided are: 0.16 M glutamate and 40 mM malate; 0.5 M succinate; 0.4 M ascorbate and 20 mM TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine).

C. Control of respiration by ATP production and consumption

This experiment is designed to examine how the rate of mitochondrial respiration is controlled by ATP production and consumption. Hexokinase is an enzyme of glycolysis that catalyses the following reaction:

glucose + ATP → glucose-6-P + ADP

If you have not already done so, wash out the vessel and rinse its top thoroughly with ethanol and then with distilled water to remove all traces of inhibitors. Make additions to the reaction vessel in the following sequence (and wait a minute or two between additions to see the effects):

- 1.90 ml medium A.
- 0.05 ml (50 µl) mitochondrial suspension (now put the top on)
- 50 µl glutamate + malate substrates (produce NADH for oxidation by Complex I)
- 20 µl of 10 mM ADP (and wait until rate has slowed down to state 4)
- 10 µl glucose (hexokinase substrate)
- 10 µl hexokinase (and wait 1 min)
- 10 µl hexokinase (and wait 1 min)
- 10 µl hexokinase (and wait 1 min)
- 10 µl hexokinase (and wait 1 min)
- 10 µl of oligomycin (an inhibitor of the ATP synthase, wait 1 min)
- 5 µl of FCCP (and wait 1 min).

Wash out the vessel at the end of the experiment and leave with water in it.

Try to think why hexokinase stimulates oxygen consumption, and what oligomycin is doing.

Calculate the RCR and P:O ratio on glutamate and malate. Put the values on the board.

Calculate the rate of respiration (in arbitary units) after each addition of hexokinase. Plot the rate of oxygen consumption (J, in arbitrary units) against the amount of hexokinase ([H] in arbitrary units) on a piece of graph paper (with origin 0 & 0). Fit a curve to these points by eye. Try to think why the graph has the shape it does.

The flux control coefficient (C) of hexokinase over the oxygen consumption rate at any particular value of H can be calculated as:

$C = (dJ/dH) . (H/J)$

i.e. the gradient of your curve multiplied by H/J at that point. This is the extent to which hexokinase controls the rate of oxygen consumption (see Introduction to next practical).

Calculate C at your lowest and highest values of H.

D. Design an experiment to test how a toxin works on mitochondria

This section is intended to complement the theoretical "experimental design" sessions you will be attending, and challenges you to play the detective to discover how a mystery poison works. We have provided a sample of a toxin (labelled T). The toxin interferes with oxidative phosphorylation, and we want you to narrow down its site of action. Design one or more experiments to answer the following question, making selective use of the materials provided for experiments A, B & C.

You may wish to discuss your experiment with a demonstrator before you start.

Is substance T (a) an uncoupler, (b) an inhibitor of the electron transport chain or (c) an inhibitor that affects ATP formation by mitochondria?

Start with an incubation mixture like this, as a basis for further additions:

1.90 ml Medium A
50 µl mitochondria
50 µl glutamate + malate

Use 10 µl of substance T when you want to add it to the incubations.

NB. The rate of oxygen consumption in the absence of ADP varies with different substrates, so if you wish to study the effects of T on the different complexes of the electron transport chain (option (b) above) it is best to rule out options (a) and (c) first, and add excess ADP.

Summarise your design, and note your conclusion in the blue sheets. You may like to suggest further steps that would be needed to get more exact information about the site of action of substance T.

Notes

Notes

Oxidative Phosphorylation – Results

A. Rates of respiration and P:O ratio with succinate

a. What was the value of the P:O ratio?
Is this the expected ratio?

b. What was the value of the Respiratory Control Ratio?
Is this the expected ratio?

c. What was the value of the state 4 respiration rate?

d. Why, in a sentence, did ADP stimulate the rate?

e. Why did FCCP stimulate the rate?

B. Substrates and inhibitors of the mitochondrial respiratory chain

a. Why did rotenone have the effect they did?

b. Why did antimycin have the effect they did?

c. Why did cyanide have the effect they did?

d. How did glutamate and malate supply electrons to the respiratory chain?

e. How did succinate supply electrons to the respiratory chain?

f. How did ascorbate and TMPD supply electrons to the respiratory chain?

C. Control of respiration by ATP production and consumption

a) What was your value of the P:O ratio on glutamate and malate?

Is this the expected ratio?

b) Open the internet browser on your phone or computer, go to: www.graphpad.com/quickcalcs/ttest1.cfm, and enter the class results for the P/O ratio on succinate and glutamate/malate into the data table, and do an unpaired t-test. What were the means and standard deviations of the P:O ratios on succinate and glutamate+malate?

Why might different students get different results for the same experiment?

c) What was the two-tailed P value of the t-test?

What is the P value the probability of?

Was the difference between the P:O ratios significant?

Does the significance tell you whether there are real differences between the P/O ratios?

d) Why did hexokinase + glucose stimulate the rate?

e) Why did further hexokinase stimulate the rate less?

f) What were the values of the flux control coefficient of hexokinase over respiration rate at the lowest and highest amounts of hexokinase?

g) Why did oligomycin inhibit the rate of oxygen consumption?

D. Design an experiment to test how a toxin works on mitochondria

a. Which experiments did you do to test how toxin T acts on oxidative phosphorylation?

b. How does toxin T act on oxidative phosphorylation?

c. Did you use the optimum protocol to test how toxin T acts on oxidative phosphorylation, and if not, what would have been the best protocol to test this?

Cell Signalling 1: Tyrosine Phosphorylation in Platelets

Tyrosine phosphorylation is a feature of many signaling pathways. This practical aims to use this feature to explore some of the cell signaling pathways in human platelets, many of which are concerned with platelet activation, which leads to aggregation. You will investigate the action of various agonists and antagonists to modify tyrosine phosphorylation, which you will detect using a Western blot.

Objectives

This practical is intended to demonstrate the technique of Western blotting (immuno-blotting) in the context of cell signalling. You will explore tyrosine phosphorylation in platelet proteins in response to physiologically important and synthetic agonists and antagonists.

Introduction

Platelets

Human platelets are a useful experimental system because they express a variety of signal pathways and are very robust in comparison to other cell types. They are easily available from the National Blood Transfusion Service. Platelets are small, membrane-bound structures (not cells, but fragments of much larger precursors, megakaryocytes) that are responsible for haemostasis (prevention of blood loss) after injury.

Agonists

An agonist is a substance which initiates a physiological response. Those used in this practical are described below.

Collagen: Collagen is the most abundant human protein. One location of collagen is below the endothelial cells that line blood vessels, which can become exposed as a result of injury. Collagen binds to specific receptors on platelets, causing them to adhere to sites of vascular damage. The signalling pathways activated by these receptors may then promote platelet aggregation, thereby plugging leaks in the vasculature. The amino acid sequence in collagen is generally a repeating tripeptide unit, Gly-Pro-4-hydroxyproline. Synthetic peptides comprising repeats of this sequence also activate platelets *via* the glycoprotein VI receptor (GpVI). This practical uses one such peptide, $(G\text{-}P\text{-}O)_{10}$, known as collagen-related peptide (CRP).

Thrombin: Thrombin, the proteolytic product of the zymogen prothrombin, functions in blood clotting to promote the proteolysis of fibrinogen to form fibrin, the insoluble fibrous protein that holds blood clots together. However, thrombin also activates platelets and promotes their aggregation by interacting with protease activated receptors (PARs). Human platelets express PAR1 and PAR4, both of which are G-protein coupled receptors activated when thrombin cleaves a fragment from the N-terminus permitting the new N-terminus to bind to the receptor of which it is a part and activate intracellular signalling. Thus the agonist is an integral part of the receptor and is referred to as a tethered ligand.

A23187: A23187 is an ionophore that forms stable complexes with divalent cations. It is produced at fermentation by *Streptomyces chartreusensis*. Its lipid solubility permits the equilibration of ions across membranes. A23187 will rapidly increase the intracellular concentration of Ca^{2+} as a consequence of the high concentration gradient of this ion across the plasma membrane (~10,000-fold).

Antagonists

An antagonist blocks the action of an agonist. Those used in this practical are briefly described below.

BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'tetraacetic acid tetrakis(acetomethoxyl ester)

BAPTA is derived from EGTA and is a strong Ca^{2+} chelator. In the acetomethoxyl ester form the four carboxylate groups of EGTA are esterified, permitting diffusion of these otherwise negatively charged groups across the plasma membrane. Once inside the cell the ester bonds are hydrolysed by endogenous esterases to generate the chelator BAPTA. BAPTA is therefore used to chelate Ca^{2+}, which increases in concentration during platelet activation.

PP1 (4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]- pyrimidine)

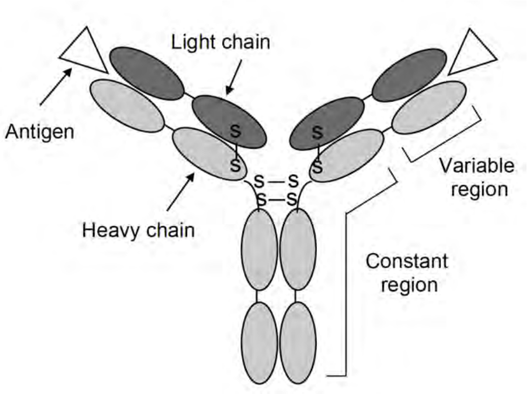
PP1 is a cell permeable drug and a potent inhibitor of tyrosine kinases (*e.g.* Src, Lyn, Fyn, *etc.*). It acts as a competitive inhibitor of ATP binding.

Phenylarsine oxide *(PAO:* C_6H_5AsO*)*

PAO is a tyrosine phosphatase inhibitor.

Some background about antibodies

Antibody proteins contain immunoglobulin (Ig) domains and are produced by B lymphocytes (B cells). They are Y-shaped molecules comprising four chains, two light and two heavy. The light chain contains one pair of Ig domains and the heavy chain contains two pairs of Ig domains connected by a flexible linker or hinge. The chains are linked by disulphide bonds, as illustrated for the IgG class of immunoglobulin (the most common class).



The binding sites for antigens are at the tips of the arm (hypervariable regions) and in a single antibody both arms will bind to the same antigenic component. All of the antibody molecules produced by a single B cell have the same antigen binding site. Since the immune system can respond to very many antigens, the organism must be able to produce a corresponding diversity of B cells that make antibodies. The antigen binding sites are produced during B cell maturation. An array of different V (variable), D (diversity) and J (joining) genes exists in the heavy chain locus (in the light chain locus only V and J are used) and from these a single V-D-J (or V-J) DNA sequence is created by a process called somatic recombination. Expression of these sequences forms the variable region on the arms of the Y, which are combined with the stalk of the Y (the constant region) as a result of RNA splicing. The enormous number of possible V, D and J DNA sequence combinations, along with different light and heavy chain combinations means that antibody diversity in terms of specific, high affinity binding to the antigen is almost limitless.

Antibodies are produced for research and diagnostics by injecting an antigen into an animal, often a mouse or a rabbit. An antigenic molecule, such as a protein, will contain many antigenic determinants, known as epitopes. Individual B cells display a surface immunoglobulin that can recognise the antigen and go on to differentiate and multiply (with the help of other cells), producing and secreting antibodies. Each B cell progenitor produces a different type of antibody with specificity for one epitope on the antigen. Thus the blood from the immunized animal will contain a mixture of antibodies to the antigen and this mixture is known as a polyclonal antibody.

To produce a monoclonal antibody (meaning a unique antibody made by a population of identical B cells derived from a single progenitor, in other words a clone), B cells are removed from the spleen of an immunized animal and are fused with cells from a myeloma (a B cell tumour) to produce hybrid cells that grow indefinitely like cancer cells but produce large amounts of antibody. The antibodies produced by single cells are tested for antigen binding. The cell that produces the tightest binding antibody can then be selected and used to generate a cell-line that manufactures large amounts of the antibody.

Antibodies can be used as molecular probes for the presence of antigen but this requires a system to detect the presence of antibody. One method is to use a secondary antibody (an “antibody to an antibody”). Secondary antibodies are raised against the constant region of the primary antibody and so recognise all IgG from an organism (*e.g.* mouse). For example, if the primary antibody is from mouse, the secondary antibody may be from rabbit. Use of secondary antibodies amplifies the signal from the primary antibody, due to several secondary antibody molecules binding to a single primary antibody molecule. Secondary antibodies can be conjugated to an enzyme, such as horseradish peroxidase, that is used for production of a coloured or luminescent product, or the secondary antibodies can be directly attached to a fluorescent dye.

Some examples of uses of antibodies that you may encounter:

- Detection of antigen in a lysate by western blotting.
- Detection of modified antigen by using an antibody raised against (say) a phosphorylated peptide.
- Quantitation of antigen in a lysate/serum/urine sample by ELISA. Identification of the localisation of antigen in fixed cells by immunostaining. Purification of antigen from a lysate by immunoprecipitation.
- Identification of protein ligands of an antigen by co-immunopurification.
- Exploiting bivalency of IgG in cross-linking studies (*e.g.* tyrosine kinase receptors).
- ‘Panning” in phage display.
- Neutralisation (*i.e.* inhibition of) protein function.
- Medical applications: “humanised” antibodies. Studies of transition state stabilisation in enzymes. Tethering of flagellar protein components.

Cell Signalling I – online version (2020-21)

Presentation on SDS PAGE & Western blots:

Activity: Prepare the polyacrylamide gel

Here you will add the necessary chemicals to prepare both a stacking and a separating gel. You will calculate the correct proportions of acrylamide to obtain the desired % gel, and see what happens when this is not appropriate.

Presentation on platelets: pathways and agonist/antagonists

Activity: Preparing the samples, running the gel, blotting

Here you will choose an agonist and an antagonist from the list provided ,and set up the experiment as you would in the lab. This will be followed by a demonstration of the blotting procedure.

Activity: Ponceau stain, Antibody stain

Here you will choose the correct solution, and appropriate washing conditions to stain your gel and observe the result. You will then add antibodies and test the various detection methods available.

Presentation on Ponceau staining, antibodies, and detection methods

Lecture theatre follow up discussion: Quick refresher of procedure and then determining the size of the bands revealed in the Western blot (semi log plot) and which platelet proteins these might represent.

Cell Signalling I – Experimental procedure (lab version) – Week 1

1. Prepare the polyacrylamide gel

The gel apparatus has been assembled for you. It consists of two glass plates, one with raised pieces on each side, like ears. Plastic tubing prevents leaks while the gel sets. Before pouring your gel make sure the apparatus is level. There are two stages: a "separating gel" is poured first and allowed to set, then a smaller "stacking gel" is poured on top of the separating gel. A plastic comb is used to create wells in the stacking gel into which the samples will be loaded.

Materials

These are coded as follows:

A	Acrylamide (4.2 M); N, N´-Methylene bisacrylamide (52 mM)
B1	Tris-HCl buffer, pH 8.8 (1.5 M)
B2	Tris-HCl buffer, pH 6.8 (0.5 M)
C	SDS (sodium dodecyl sulphate, 10% weight for volume)
AMPS	Ammonium persulphate (polymerising agent, 10% weight for volume)
T	N,N,N´,N´-tetramethylenediamine (TEMED: cross-linking reagent)

Preparation of the separating gel (8%)

Mix as follows, in the order below. Be careful to measure accurate volumes:

A	5.4 ml
B1	5.0 ml
C	0.2 ml
AMPS	170 µl
Water	9.2 ml
T (TEMED)	30 µl

Make sure that the materials are well mixed by swirling; do not shake as that may introduce air into the mixture that will slow down the setting process. Using a 5 ml Gilson, carefully pipette the gel mix between the plates to fill the space up to 1 cm below the bottom of the teeth of the comb. Then add 1 ml ethanol on top to ensure a flat level surface for the next gel. Leave it to set (about 30 minutes). YOU CAN START TO LABEL YOUR TUBES (see **Section 2**) WHILE YOU ARE WAITING FOR THE GEL TO SET. Tip off the ethanol and rinse between the plates with distilled water.

Preparation of the stacking gel (4%)

Mix as follows, in the order below. Be careful to measure accurate volumes:

A	0.65 ml
B2	1.25 ml
C	0.1 ml
AMPS	85 µl
Water	2.9 ml
T (TEMED)	15 µl

Pipette the stacking gel mix on top of the separating gel. Then insert the comb making sure that there are no tiny air bubbles trapped between the comb and the solution. Leave to set (about 15 minutes).

2. Platelet assays

You are provided with:

1. A platelet preparation at 5 x 10^8/ml.

   IMPORTANT: Do not put this on ice. Leave it at room temperature.
2. Laemmli buffer (0.26 M Tris-HCl, pH 6.8, 10% SDS, 40% glycerol, 5% β-mercaptoethanol, 0.05% bromophenol blue). Glycerol increases the density of the solution so that it collects at the bottom of the well when applied to the gel. Bromophenol blue is a dye that runs at the "front" of the gel.
3. Molecular weight markers dissolved in Laemmli buffer.

Each pair will set up FOUR pre-incubations in screw-cap, plastic microfuge tubes using one of the antagonists: EITHER BAPTA or PP1 or PAO: these are dissolved in DMSO, so DMSO as a control must be added to the other tubes.

Start the incubations by adding 40 µl of platelet suspension to each of the four tubes at 30 second intervals. After 10 minutes, add the agonist at intervals of 30 seconds as indicated in the table.

Replace the tubes in the 30 °C heat block and stop each incubation after 5 minutes, at staggered 30 second intervals, by adding 50 µl of Laemmli buffer. Tighten the lids and put the tubes in the 95 °C heat block for 5 minutes. The molecular weight markers **do not** need to be heated. Place the tubes on ice for 2 minutes to cool.

Experimental design for platelet assays:

			TUBE No. (screw-cap)			
STEP	**Agent added**	**Vol.**	**1**	**2**	**3**	**4**
1	Antagonist	5 µl	DMSO (1%)	DMSO (1%)	BAPTA or PP1 or PAO	BAPTA or PP1 or PAO
2	Platelets	40 µl	Add to each tube			
			~10 min @ 30 °C (heating block)			
3	Agonist	5 µl	H_2O	Thrombin or CRP or A23187	H_2O	Thrombin or CRP or A23187
			5 min @ 30 °C			
4	Laemmli buffer	50 µl	Add to each tube			
5			Transfer to heating block (5 min)			
6			Transfer to ice (2 min)			
7			Load gel			
8			Run @ 60 mA/gel (~1 hr)			
9			Remove gel: place in blotting buffer			
10			Transfer gel to blotter			

Final concentrations: Thrombin 10 U/ml, CRP 250µg/ml, A23187 10 µM.

3. Gel loading

Carefully remove the bulldog clips and then the plastic tubing from around the sides of the plates. Mount the plates in the apparatus, making sure the plate with 'ears' is to the rear, adjacent to the upper reservoir. Secure them with the larger bulldog clip on the left and the smaller on the right. Fill the top and bottom tanks of the gel apparatus with **running buffer** (25 mM Tris-HCl, pH 8.3, 200 mM glycine, 0.5% SDS) making sure that the level in the top tank flows onto the top of the gel to establish electrical contact. Mark the positions of the wells with your marker pen and then remove the comb. Using a P20 Gilson load the molecular weight markers (20 µl). Load 20 µl from each incubation tube into other wells, going from left to right. Use adjacent central wells, unless you have bubbles that need avoiding. Plug in to the power pack, taking care to get the positive (RED) and negative (BLACK) terminals correct**.** Switch on and set the current to 60 mA. The gel will take about an hour to run.

4. Setting up the Western blot

After the gel has run switch off the power pack, remove the leads and carefully pour away the running buffer. Take the plates out of the apparatus and place in your tray on top of some paper towel. Use a large spatula gently to lever the top plate off. Apply the leverage BETWEEN the top and bottom ends of the plate so you do not break the 'ears' or the corners. Carefully remove the gel and place it in **blotting buffer** (25 mM Tris-HCl, 150 mM glycine, 5% v/v methanol, pH 8.8) in the square plastic box. Take it to the blotting apparatus where we will assemble the blot. This involves laying the gel over a piece of PVDF membrane and sandwiching it between layers of filter paper. Application of a current at right angles to the gel transfers the proteins from the gel to the nitrocellulose membrane. Assembly of the blot completes the first week's work.

We will take the blots off and store them ready for the detection step the following week.

Cell Signalling I – Experimental procedure (lab version) – Week 2

You will stain your blot for protein and then use a monoclonal antibody to detect phosphorylated tyrosines. Remember that the platelets were treated with various agonists and antagonists, and you want to identify any changes in the level of tyrosine phosphorylation caused by these agents. These should allow you to predict any change in platelet activation.

Ponceau staining

Collect your PVDF blot and pour off the blotting buffer. Wash the blot with distilled water. Then pour in enough Ponceau stain to just cover the blot. **Leave for 1 minute ONLY.** A longer exposure makes it difficult to remove the stain. Pour off the stain into the small beaker provided, and wash 2 – 3 times with distilled water. Dispose of these washes into the larger beaker. Add distilled water to cover the blot; do not let it dry out. You should be able to see some of the proteins that have been transferred from the gel to the PVDF membrane, and be able to check relative amounts of protein in each sample you loaded onto the gel.

Draw a rough sketch. The most important point to note is the intensities of the bands in each lane. In an ideal experiment they would all be the same. If they don't look equal then note this down. It will help with your final analysis.

Probing with Antibodies

1. Pour off the water and add the solution containing antibody PY20.
2. Incubate for 45 minutes in the shaker.
3. Tip off the PY20 solution and wash the membrane with PBS for 2 x 10 seconds.
4. Add the secondary antibody and incubate for 15 minutes on the shaker.
5. Tip off the secondary antibody solution
6. Wash the membrane for 2 x 10 seconds with PBS.

Detection

1. Prepare detection buffer (collect the detection reagents to add to the detection buffer on your bench from a demonstrator)
2. Add the detection reagent to your blot and allow the bands to develop (don't shake); this should take 5-10 minutes.
3. Try and identify any differences in the densities of similarly sized bands and see if they relate to your treatment of the sample. You can stop the blot darkening further if you need to by gently tipping off the solution and adding water
4. Photograph the result.

Notes

Notes

Cell Signalling I – Question Sheet

1. Sketch or attach the photo of your Ponceau stained membrane and your Western blot

2. **Indicate by a simple drawing how the immunodetection procedure works.**

3. Compared to the control (DMSO alone) what were the levels of changes to tyrosine phosphorylation elicited by

(a) thrombin?

(b) collagen?

(c) A23187?

4. What effects did the antagonists have on the levels of tyrosine phosphorylation?

i) BAPTA

ii) PP1

iii) PAO

5. What do you deduce about the effects of the agonists and antagonists on platelet activation?

6. What further experiments might you perform to test your theories?

Cell Signalling 2: The production of Thromboxane by human platelets

This, the second of the Cell Signalling practicals, will correlate with the appropriate signalling lectures. You will investigate the ability of platelets to synthesise thromboxane A_2, (TxA_2), which is unstable, so levels of its stable metabolite, thromboxane B_2 (TxB_2) are measured. As you will recall from the first Cell Signalling practical, platelets bind to collagens via specific receptors, such as integrin $\alpha 2\beta 1$ and glycoprotein VI (GpVI). Human platelets are stimulated by binding to collagen, which promotes arachidonic acid release from membrane phospholipids. Free arachidonic acid is then converted to thromboxanes. The experimental work will be completed in Week 1, and data will be analysed and discussed in Week 2.

Objectives

You will learn how to perform an ELISA (Enzyme-Linked Immunosorbant Assay), a technique that is used to measure a huge number of agents and metabolites in many areas of biochemistry.

The strategy is to use collagen or other known activators to stimulate human platelets and measure the production of thromboxane. You will also investigate the action of aspirin, which blocks this pathway, and examine the role of intracellular calcium in mediating the collagen response.

At the end of this practical you should be able to carry out the following tasks:

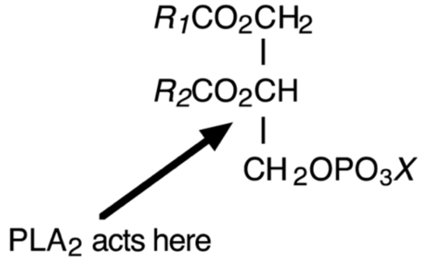
- Carry out timed protocols on activated platelets.
- Prepare samples for ELISA and use the 96-well plate reader.
- Prepare a standard curve and extract and analyse data.
- Plot concentration-response curves and determine the EC_{50} for the activator

Introduction and Background

Phospholipids in many different cell membranes are enriched at the R2 position (see figure below) in arachidonic acid. The enzyme that releases arachidonic acid from membrane phospholipids is known as **cytoplasmic phospholipase A_2** ($cPLA_2$). It is regulated either by ligands that bind to some G protein-linked receptors or by some tyrosine kinase-mediated signals, as you saw in the Cell Signalling I practical (Western blotting) last week. The regulation of $cPLA_2$ is not understood in detail, but several second messenger pathways interact to stimulate the activity of the enzyme.

Arachidonic acid is a C_{20} fatty acid with 4 double bonds (formally, 5:6, 8:9, 11:12, 14:15 eicosatetraenoic acid). It is the precursor of prostaglandins and some related paracrine or autocrine agents collectively known as eicosanoids. The eicosanoids are important mediators of inflammation and pain. In blood platelets, production of the thromboxane A_2 (TxA_2, a prostaglandin) amplifies the activation process via the paracrine activation of a 7-TM-R, TP2. This leads to platelet aggregation, an event vitally important to thrombosis and haemostasis (a topic included in the Cardiovascular Biology option of the Biochemistry Part II and Part III courses).

Schematic Phospholipid, Phosphatidyl- *X*

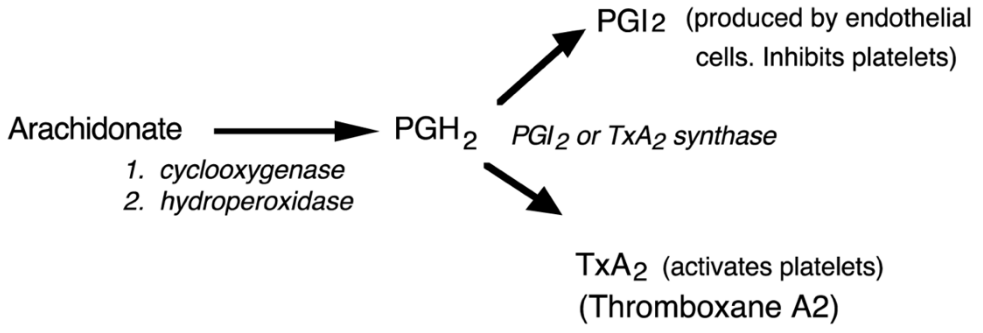

R1CO2CH2
R2CO2CH
CH2OPO3X
PLA2 acts here

X = Polar head group, e.g. choline or serine

R1 & *R2* = Fatty acid residues;

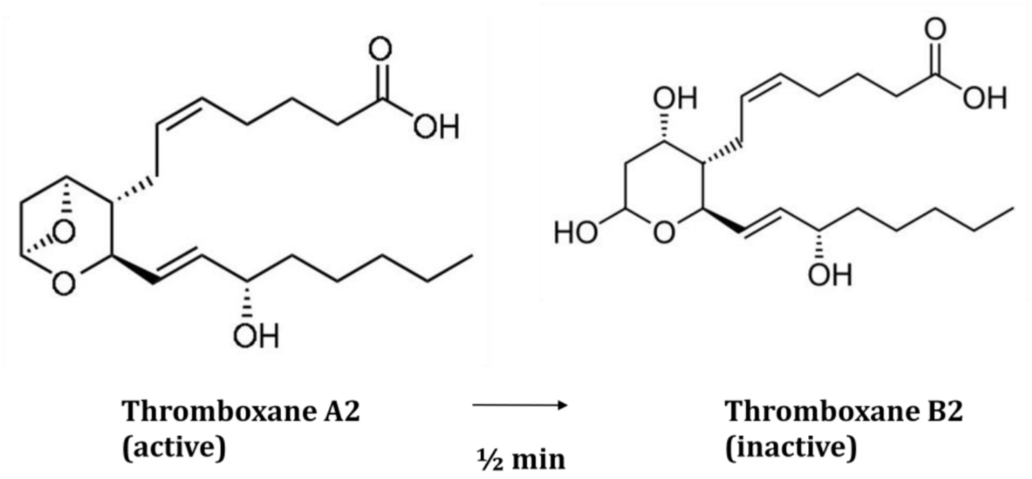
Higher proportions of arachidonate are located at R2 than at R1 in the phospholipid population

Subsequent Arachidonate Metabolism


PGI2 (produced by endothelial cells. Inhibits platelets)
Arachidonate
PGH2
1. cyclooxygenase
2. hydroperoxidase
PGI2 or TxA2 synthase
TxA2 (activates platelets)
(Thromboxane A2)

Platelet Thromboxane Production

You will be provided with suspensions of washed human platelets, obtained from National Blood Service. You will use the platelets untreated (the control) or treated with aspirin (acetyl salicylic acid) or BAPTA-AM to investigate the effects of aspirin and the role of intracellular calcium in the synthesis of TxA_2. TxA_2 is unstable and rapidly converts to the stable TxB_2, which is what you will measure in the Practical.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Thromboxane A2 (active) → ½ min **Thromboxane B2 (inactive)**

Collagens of the blood vessel wall become exposed after injury to the endothelial cell layer and are major physiological activators of circulating platelets. You will investigate thromboxane synthesis stimulated by collagen itself or one of the other known platelet activators.

You will produce concentration–response curves for novel synthetic collagens and you will discover whether you can stimulate TxB_2 production in **aspirin-**treated platelets. You will investigate the role of intracellular Ca^{2+} using **BAPTA-AM**, an intracellular Ca^{2+} chelator. The functions of these modulators are summarised below.

Platelet Thromboxane Production – additional notes:

Modulators of thromboxane synthesis

Aspirin is a specific inhibitor of the cyclo-oxygenases, COX1 and COX2, which convert arachidonic acid into prostaglandin H_2, from which prostanoids including TxA_2 are formed. It acts by acetylating the active site of the enzyme, leading to irreversible inhibition.

BAPTA (1,2bis[2-aminophenoxy]ethaneN,N,N',N'-tetraacetic acid) is a Ca^{2+} chelator, not unlike EGTA or EDTA, which blocks the Ca^{2+} rise that would otherwise occur as Ca^{2+} is mobilised from internal stores in response to collagen. (BAPTA itself is not membrane-permeant, so instead we use the acetoxymethyl ester, BAPTA-AM, which can readily pass through membranes. Intracellular esterases hydrolyse the AM group, trapping the active chelator in the cytoplasm. This trick is quite often used to load membrane-impermeant organic anions into cells.)

DMSO (dimethylsulphoxide) is the solvent used for all inhibitors. The final DMSO concentration in platelet suspensions with inhibitors is 0.1% (v/v) or 14 mM; after water and NaCl, it is the most abundant species in the pre-incubations. Therefore it is imperative that we add DMSO to the untreated platelet suspension as well.

Table 1. Concentration of TxA_2 synthase modulators

MODULATOR	Concentration in platelet suspension provided
None	0.1% (v/v) DMSO
Aspirin	100 µM, with 0.1% (v/v) DMSO
BAPTA-AM	20 µM, with 0.1% (v/v) DMSO

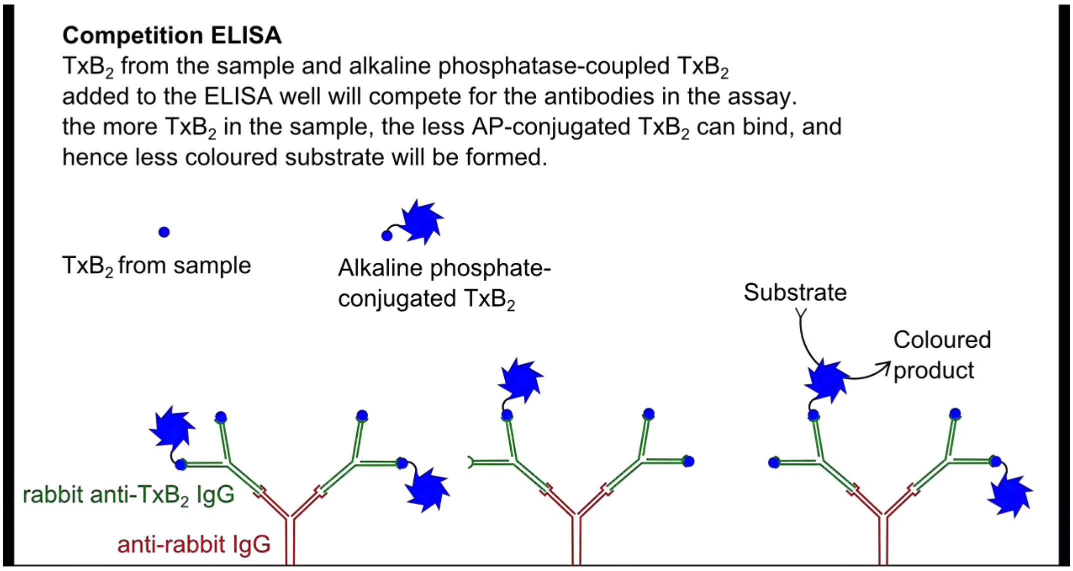
Technique

Thromboxane production assay

The platelets will be incubated with activator at 37 °C. The platelets will be centrifuged and the supernatant removed and diluted so that the [TxB_2] is within range for measurement by ELISA.

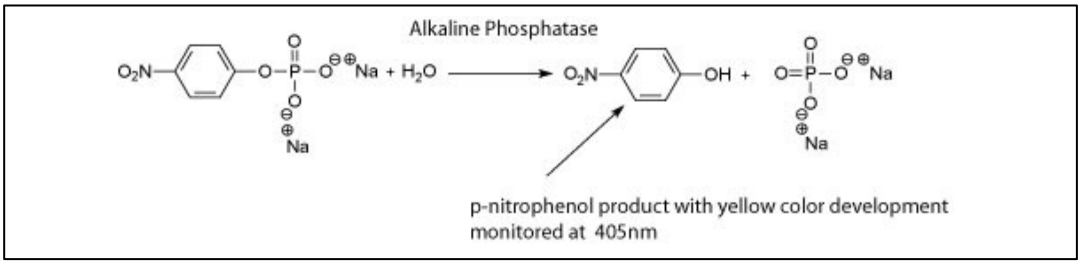
96-well plate competitive ELISA

Each well is coated with anti-rabbit Ig antibody (raised in goat). The principle of the assay is to mix TxB_2 from your samples with a fixed amount of TxB_2 to which alkaline phosphatase has been conjugated, in the presence of an anti-TxB_2 antibody (a rabbit polyclonal). The two forms (unconjugated and phosphatase-conjugated) of TxB_2 compete for binding to the antibody, and the whole complex is captured on the base of the well by the immobilised anti-rabbit Ig.


Competition ELISA

TxB_2 from the sample and alkaline phosphatase-coupled TxB_2 added to the ELISA well will compete for the antibodies in the assay. the more TxB_2 in the sample, the less AP-conjugated TxB_2 can bind, and hence less coloured substrate will be formed.

After washing off unbound proteins, a chromogenic substrate is added, which is converted by the phosphatase to a yellow-coloured product that can be detected spectrophotometrically.



p-nitrophenol product with yellow color development monitored at 405nm

The colour is then measured in a plate-reader at 405 nm. To compensate for scattering in the sample, the plate reader will also take a measurement at 595 nm (blue absorption) where the product will not absorb, but any scattering by particles in the sample or scratches or stains at the bottom of the plate are taken into account and subtracted from the reading at 405 nm. This is done automatically and you do not need to worry about this – you will receive the corrected absorbance readings. A standard curve will also be constructed by adding known quantities of TxB_2 to the conjugated TxB_2 and developing these samples in the same plate in which your samples are. You will use these data to draw a standard curve and use it to determine the TxB_2 concentrations in your samples during the data analysis session the week later.

Safety Notes

Human blood products

Platelets *must* be regarded as potentially pathogenic, though these platelets have been screened for viral contamination.

Thus the platelet suspensions present a relatively minor risk, but it is **essential** that you **wear lab coats and gloves throughout,** and **perform all manipulations over the trays provided.** This includes sampling of platelet supernatants, dilution, pipetting etc. Gloves should be discarded into the (floor) bins provided, and tips, tubes etc into the plastic- lined waste buckets on the benches.

Week 7 – Cell Signaling: The production of Thromboxane by Human Platelets – Experimental

Platelet Stimulation with Collagens (synthetic or native):

Each group of students will test **one ligand only** (i.e. native collagen fibre preparation or synthetic collagen peptide or thrombin or ionomycin) to produce a 7-point concentration-response curve, as well as examining the effects of aspirin and BAPTA-AM on stimulation by that ligand.

The assays are performed by incubating platelets with the activating ligand for a defined period of time, stopping the reaction, centrifuging the platelets, removing a supernatant sample and then diluting it to follow the synthesis of thromboxanes, measured as the amount of stable metabolite, TxB_2, described above.

Using the data generated in this experiment, during the data analysis session next week, we will produce a standard curve using authentic standards from the ELISA kit, prepare pooled dose–response curves for each ligand, compare the relative potencies of different ligands and discover whether TxA2 synthesis requires an elevation in intracellular calcium. We will also study the effect of aspirin in this process.

Materials

You should have on your own bench (or obtain from the demonstrator as needed):

1. Platelet suspension at 2 x 10^8 platelets/ml

Platelets have already been isolated from plasma by centrifugation and resuspended in an appropriate isotonic buffer (PB). Ensure that you have 2 ml of untreated platelets (PT) and 0.5 ml each of aspirin (ASP) – and BAPTA (BAP) – treated platelets.

2. Platelet Termination Reagent (TR)

This contains EDTA and indomethacin, which very quickly stops the synthesis of TxA_2, acting like aspirin to block COX1 and COX2. The reagent will be ready on your bench.

3. Activators

You are provided with 60 µl of a stock solution of activator ligand in buffer (HEPES-buffered isotonic saline + 0.5 mM EGTA), ready to use at the highest concentration condition A7). A1 is a control containing the buffer alone. You are provided with 20 ml of this buffer, labeled **PB** (platelet buffer). The lower concentrations of activator, A2 to A6, are made by diluting a small volume of the higher concentrations – see the dilution scheme below. For A2-A5, you need to prepare sufficient for 2 x 10 µl aliquots for the incubations. A6 is also used for the BAPTA- and aspirin-treated platelets.

Details of the activators to be used will be provided by the demonstrators. Make a note which activator you are using and its original concentration:

4. Snap-top microfuge tubes for incubations and dilutions

These are available on the bench or from the demonstrators.

5. ELISA reagents

Each group will use 22 wells of a 96-well plate, 11 wells in each of two rows. The last position in each row will be used for TxB_2 concentration standards, added by the demonstrators when the rest of the plate has been filled. Thus each plate will be shared between four groups.

Make sure to write your name in the form next to the plates to indicate which rows contain your samples!

Cell Signalling 2: Experimental Procedure

1. Dilution of the ligand (activator)

Your first task is to dilute the ligand to produce 6 concentrations (the 7th is the negative control, with no ligand) that should cover its active range. The final concentrations in both stocks and the assay will depend on **which ligand you are using**, so make sure you understand how this will be done, and which ligand you are testing.

Begin by labelling snap-top Eppendorf tubes for the ligand dilutions i.e. A1-A7 and add to these tubes PB buffer as shown in Table 2 (below). Starting with the activator **stock solution**, dilute the activator by adding the indicated volume of the sample into the next tube. Mix the new sample well with whirlimixer. Using a new tip, dilute this new solution further, until all samples are prepared (i.e. A7 to A2). Condition A1 is your negative control with no activator, just PB buffer.

Tick the box when done: ☐

Table 2. Dilution of activators

Condition	Stock volume	Add to PB volume	Final volume	[Stock] μg/ml	[Assay] μg/ml
A7	60 μl A7	0	60 μl (less 30 μl)	1000	100
A6	30 μl A7	70 μl PB	100 μl (less 30 μl)	300	30
A5	30 μl A6	60 μl PB	90 μl (less 30 μl)	100	10
A4	30 μl A5	70 μl PB	100 μl (less 30 μl)	30	3
A3	30 μl A4	60 μl PB	90 μl (less 30 μl)	10	1
A2	30 μl A3	70 μl PB	100 μl	3	0.3
A1 (use PB as is)	0	70 μl PB	70 μl	0	0

These same dilutions apply to all activators. It is important that, as you prepare each dilution, the tube is thoroughly whirlimixed to ensure the correct amount of activator is transferred to the next dilution.

Done: ☐

2. Dispensing ligand and setting up assay tubes

You will use 1.5 ml snap-cap Eppendorf tubes for your assays. Set up the assay tubes in duplicate, labelling them 1a-11a and 1b-11b (22 tubes in total), with the conditions as in **Table 3**.

Tubes 1-7 will include 10 μl from one of A1-A7. Tubes 8 and 9 will have 10 μl of A1 and A6 but the platelets used will contain aspirin; tubes 10 and 11 will also have 10 μl of A1 and A6 but the platelets used will contain BAPTA-AM.

Done: ☐

You will need to label a second set of tubes (D1a-D11a and D1b-D11b) which you will use to dilute the supernatant from the experiment before pipetting the sample onto the ELISA plate.

You should thus have a total of 44 labelled tubes. You could use a different colour marker for the different sets, to ensure they do not get mixed.

Done: ☐

Take the diluted activators (A1-A7) and dispense them as appropriate in 10 μl aliquots to your 22 assay tubes (Table 3) and transfer the rack of tubes to the heat block. To ensure you can easily lift the tubes from the block during the experiment, place them staggered in the block, leaving an empty place between the tubes.

Done: ☐

Table 3. Assay conditions for tubes 1a-11a and 1b-11b.

Tube IDs	1a 1b	2a 2b	3a 3b	4a 4b	5a 5b	6a 6b	7a 7b	8a 8b	9a 9b	10a 10b	11a 11b
Agonist dilution	**A1** 10 μl	**A2** 10 μl	**A3** 10 μl	**A4** 10 μl	**A5** 10 μl	**A6** 10 μl	**A7** 10 μl	**A1** 10 μl	**A6** 10 μl	**A1** 10 μl	**A6** 10 μl

Do not add the platelets into the tubes yet!!!

3. Pre-incubation

You should have platelets in your rack, ready for use, in total 2 ml of untreated platelets (at 2 x 10^8 platelets/ml, containing 0.1% DMSO) and 0.5 ml platelets to which **modulators** (aspirin or BAPTA, with 0.1% DMSO as solvent) have already been added. The modulators require some time to equilibrate with their sites of action, so the platelet suspension plus modulators have been incubated at room temperature for at least 25 min before you receive them.
Pre-incubate all the platelet tubes (untreated, aspirin- and BAPTA- treated) for 5 min in the 37 °C heat block before use. ***Do not mix the platelets and activators yet!***

Done: ☐

4. Incubation with activator

The assays will be started by transferring appropriate 90 μl of platelets (untreated, aspirin- or BAPTA-treated) into the pre-warmed tubes (1-11) in the heat block containing the **activator** (10 μl) at seven concentrations, **each in duplicate,** as per table 4 below**.** For all of the incubations, after 10 min exactly, the reaction will be stopped by the addition of **termination reagent (TR)**.

It is important to time these 10 min incubations with activator precisely so that the results between the different groups will be comparable. Timings are shown in **Table 5** below. In each case, 90 μl platelets will be added to the tubes. All assays will be performed in duplicate. Working in pairs, one student should call the times and the other one do all the pipetting. The better organized you are, the more punctual you can be with the timings and the better your duplicate samples will match each other. Make sure tips are close by, you know the order of the tubes, the waste container is close to your pipette hand etc. If you have a problem in the middle of the experiment, do not panic, but make a note when the next experiment starts and simply shift the schedule to ensure all samples are in the experiment for 10 minutes exactly.

Before starting the experiment, invert the three platelet tubes once, to ensure you have an even suspension of platelets, as they could have settled towards the bottom of the tube.

Table 4.

Tube IDs	1a 1b	2a 2b	3a 3b	4a 4b	5a 5b	6a 6b	7a 7b	8a 8b	9a 9b	10a 10b	11a 11b
Platelet condition	**PT** 90 µl	**PT** 90 µl	**PT** 90 µl	**PT** 90 µl	**PT** 90 µl	**PT** 90 µl	**PT** 90 µl	**ASP** 90 µl	**ASP** 90 µl	**BAP** 90 µl	**BAP** 90 µl

Table 5. TIMINGS of Additions

Platelets	**Tube ID**	**Start** Incubation. Add 90 µl platelets to duplicate tubes containing 10 µl ligand	**Stop** Incubation. Add 100 µl termination reagent	**Tick box** Mark the box when started	
Untreated platelets (PT)	1a, 1b	0.00 min, 0.15 min	10.00 min, 10.15 min	1a	1b
	2a, 2b	0.30 min, 0.45 min	10.30 min, 10.45 min	2a	2b
	3a, 3b	1.00 min, 1.15 min	11.00 min, 11.15 min	3a	3b
	4a, 4b	1.30 min, 1.45 min	11.30 min, 11.45 min	4a	4b
	5a, 5b	2.00 min, 2.15 min	12.00 min, 12.15 min	5a	5b
	6a, 6b	2.30 min, 2.45 min	12.30 min, 12.45 min	6a	6b
	7a, 7b	3.00 min, 3.15min	13.00 min, 13.15 min	7a	7b
Aspirin-treated (ASP)	8a, 8b	3.30 min, 3.45 min	13.30 min, 13.45 min	8a	8b
	9a, 9b	4.00 min, 4.15 min	14.00 min, 14.15 min	9a	9b
BAPTA-treated (BAP)	10a, 10b	4.30 min, 4.45 min	14.30 min, 14.45 min	10a	10b
	11a, 11b	5.00 min, 5.15 min	15.00 min, 15.15 min	11a	11b

Start the assay by adding 90 µl of control platelet suspension (containing 0.1% DMSO) to your assay tube labelled 1a and then15 sec later to tube 1b,15 sec later to 2a and so on. Whirlimix or flick the tube **gently** and return them to the heat block. Change the pipette tip before starting assays with aspirin-treated platelets and again before starting assays with BAPTA-treated platelets. Try to keep the timing between duplicates constant.

Done: ☐

After ***exactly*** 10 min incubation, add 100 µl of **termination reagent (TR)**, cap and whirlimix, maintaining the timing intervals as in **Table 5.** Transfer the stopped tubes to ice.

Done: ☐

When all the incubations are complete, microfuge the tubes for 1 min at full speed. After centrifuging, handle the tubes gently to avoid disturbing the platelet pellet, and ***carefully* remove** a 20 µl sample of the supernatant (avoiding the pellet) and transfer it to the second set of labelled tubes (D1-D11, in duplicate). Add 580 µl of Assay buffer (from the classroom staff) to each of these dilution tubes. Cap and whirlimix the tubes well.

Done: ☐

5. ELISA

To your two rows of the 96-well ELISA plate, add 100 µl of sample from the dilution tubes, D1-D11, in duplicate (see Table 6). It is important that exactly the same volume is added to each well, and therefore it is advisable that only one person does the pipetting and uses the same pipette without adjusting the volumes, to minimize problems with variable calibration between pipettors. Make note of the plate number and the rows you are using and write your names and the agonist you used on the sheet by the ELISA plates.

Done: ☐

A range of seven standards (10,000 to 1 pg TxB$_2$/ml) is added to the 12th column of the plate ***by the classroom staff****. The last well, H12, is a background or "zero" well without TxB$_2$.*

Once all the samples are on the plate 50 µl of alkaline phosphatase-conjugated TxB$_2$ (blue solution) will be added to each well, followed by 50 µl of anti-TxB$_2$ antibody solution (yellow).

The classroom staff will complete this step, using a multichannel pipettor.

Table 6: ELISA layout.
Each group uses two rows, columns 1-11, inside dark lines.

	1	2	3	4	5	6	7	8	9	10	11	12
A	D1a	D2a	D3a	D4a	D5a	D6a	D7a	D8a	D9a	D10a	D11a	Std 1
B	D1b	D2b	D3b	D4b	D5b	D6b	D7b	D8b	D9b	D10b	D11b	Std 2
C				*Same for Second group*								Std 3
D												Std 4
E				*Same for Third group*								Std 5
F												Std 6
G				*Same for Fourth group*								Std 7
H												zero

My plate number: ___________________________ My rows:_____

Once the ELISA plate is full, it is incubated for **2 hours** at room temperature on the shaker.

*****Break for lunch (plan for discussion at approx. 2pm)*****

Wash each well three times with 400 µl washing buffer (Tris-buffered saline plus detergents, provided by the kit manufacturer). Volunteers from the class will do this with guidance from the classroom staff using multichannel pipettes.

Add 200 µl of the phosphatase substrate (provided by the kit manufacturer) to each well. Incubate 45 min, room temperature, without shaking.

Finally, add 50 µl of tri-sodium phosphate to terminate the reaction. (This reagent is provided by the kit manufacturer.)

Read absorbance at 405 nm (ABS$_{405}$) using the plate-reader. This is usually done by demonstrators after the practical, but the use of plate reader will be demonstrated during the class.

Notes

Data Handling and Interpretation – Week 2

Drawing the standard curve

Use the absorbance results from the standards on the plate your samples were to plot a standard curve for the TxB_2 assay on four-cycle semilog paper with standard concentration on the X-axis (logarithmic) and absorbance values (ABS_{405}) on the Y-axis. The standard wells contained: 0, 1, 10, 30, 100, 300, 1000 and 10000 pg/ml of TxB_2.

For this assay the standard curve is not linear but takes the form of an inverse rectangular hyperbola. On a logarithmic scale, this appears as an upside-down sigmoidal curve (as in the computer derived example below). At high and low TxB_2 concentrations the curve tends towards an asymptote. This means that the assay is most accurate for those concentrations of TxB_2 in the middle of the standard curve, i.e., 10 – 1000 pg/ml.

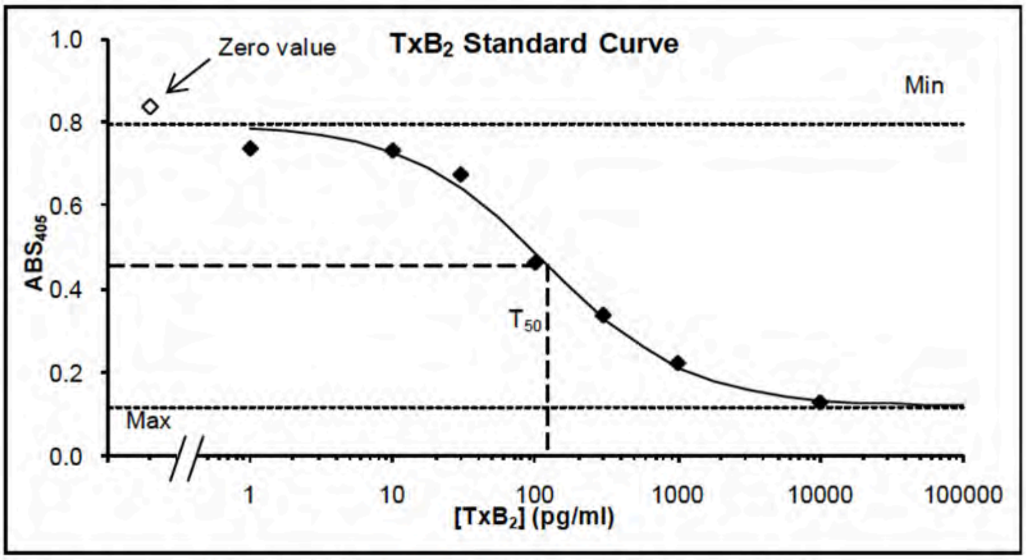
Remember that there is no zero on logarithmic scale, so place the 0 ug TxB_2 standard outside the graph, assuming it is in infinity.

If data analysis is done computationally, the standard concentration data can be fitted to a four parameter logistic equation (see below). Once the parameters are known, it is possible to calculate TxB_2 levels from individual absorbance values.

$$ABS_{405} = \frac{(Min - Max)}{\left(1 + \left(\frac{TxB_2}{T_{50}}\right)^{n_H}\right)} + Max \qquad TxB_2 = T_{50}\left(\frac{Min - ABS_{405}}{ABS_{405} - Max}\right)^{1/n_H}$$

Variables: ABS_{405} = Measured absorbance value (dependent variable)
TxB_2 = Concentration of TxB_2 standard

Parameters: $Max = ABS_{405}$ when $[TxB_2] = \infty$
$Min = ABS_{405}$ when $[TxB_2] = 0$
$T_{50} = [TxB_2]$ which gives an ABS_{405} value of $(Max + Min)/2$
n_H = Hill coefficient (relates to steepness of slope)



In this case: $Min = 0.80$, $Max = 0.12$, $T_{50} = 122.7$, $n_{HJ} = 0.88$

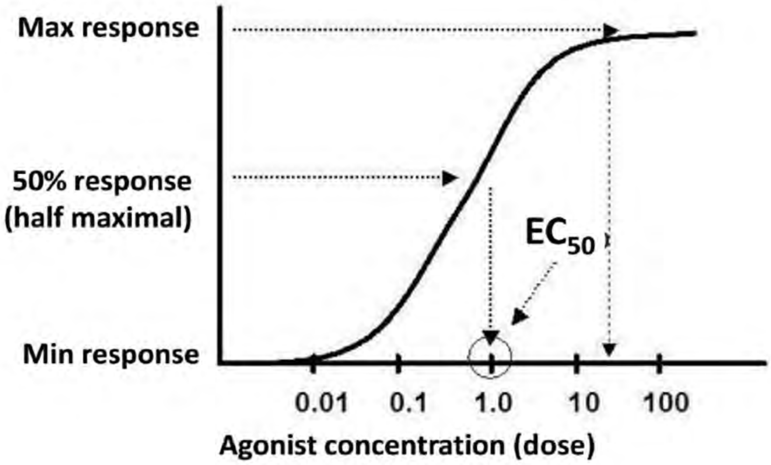
Determining the amount of TxB_2 in your samples

Standard curve can be used to determine the amount of TxB_2 in each of your sample wells. Use the table on the next page to first calculate average absorbance for your duplicate samples (D1-D11) and use this average value to read the corresponding TxB_2 concentration from the standard curve you have drawn.

As the curve becomes flat at both ends, the reliable part of the curve is in the middle, where there is a significant slope and differences in the absorbance can be correlated with differences in TxB_2 concentrations.

Dose response curve

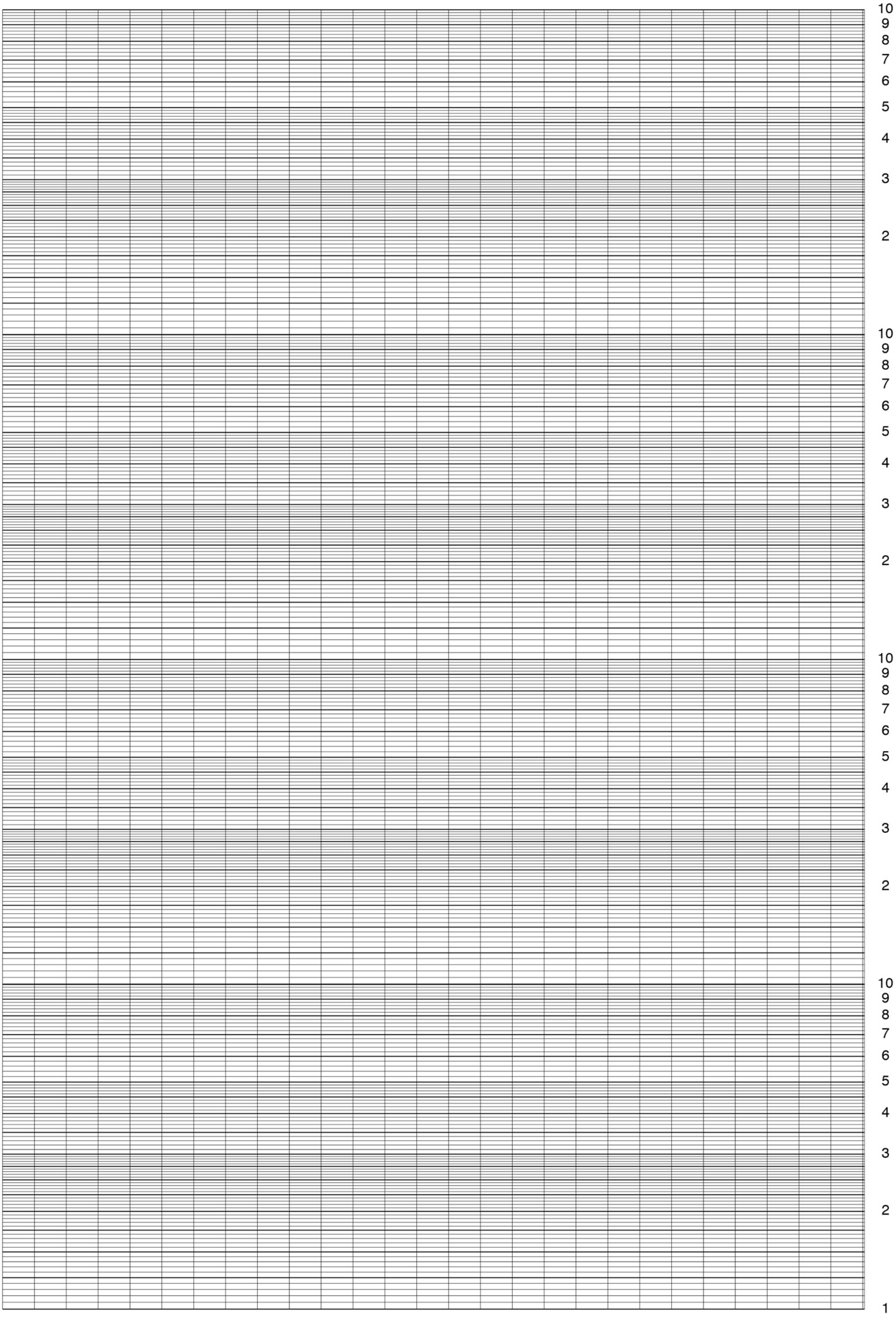
To evaluate the potency of your activator, you need to construct a concentration-response curve from your data and estimate the EC_{50} value (concentration at which the ligand induces half maximal, 50%, response in the cells) of the activating ligand that you used. This is done by plotting the amount of TxB_2 as a function of agonist concentration using four-cycle semi-log paper and determining the agonist concentration from the point in the X-axis where the effect is half of the maximum. Enter your EC_{50} value into the table at the front of the class room, and an average value will be determined from all the experiments over the week of practicals.



Converting the concentrations to biologically meaningful values

The values obtained from the standard curve are expressed as pg/ml and so is your EC_{50} value. But in order for this to be useful and comparable with data obtained with different amounts of platelets (or cell types), this value needs to be converted to a scale that reflects the experimental conditions, in this case the number of cells used in the experiments. This ensures that data done on different dates, using different concentrations of cells can be compared directly.

Taking into account all the steps in the experiment (various sample volumes, additions of reagents and dilutions), you need to derive a conversion factor to change the TxB_2 concentrations (pg/ml) to pg of TxB_2 / 10^8 platelets. There is only one conversion factor to be determined as all the samples were treated the same way. Check from the demonstrators that the conversion factor is correct before doing the conversions in the table below.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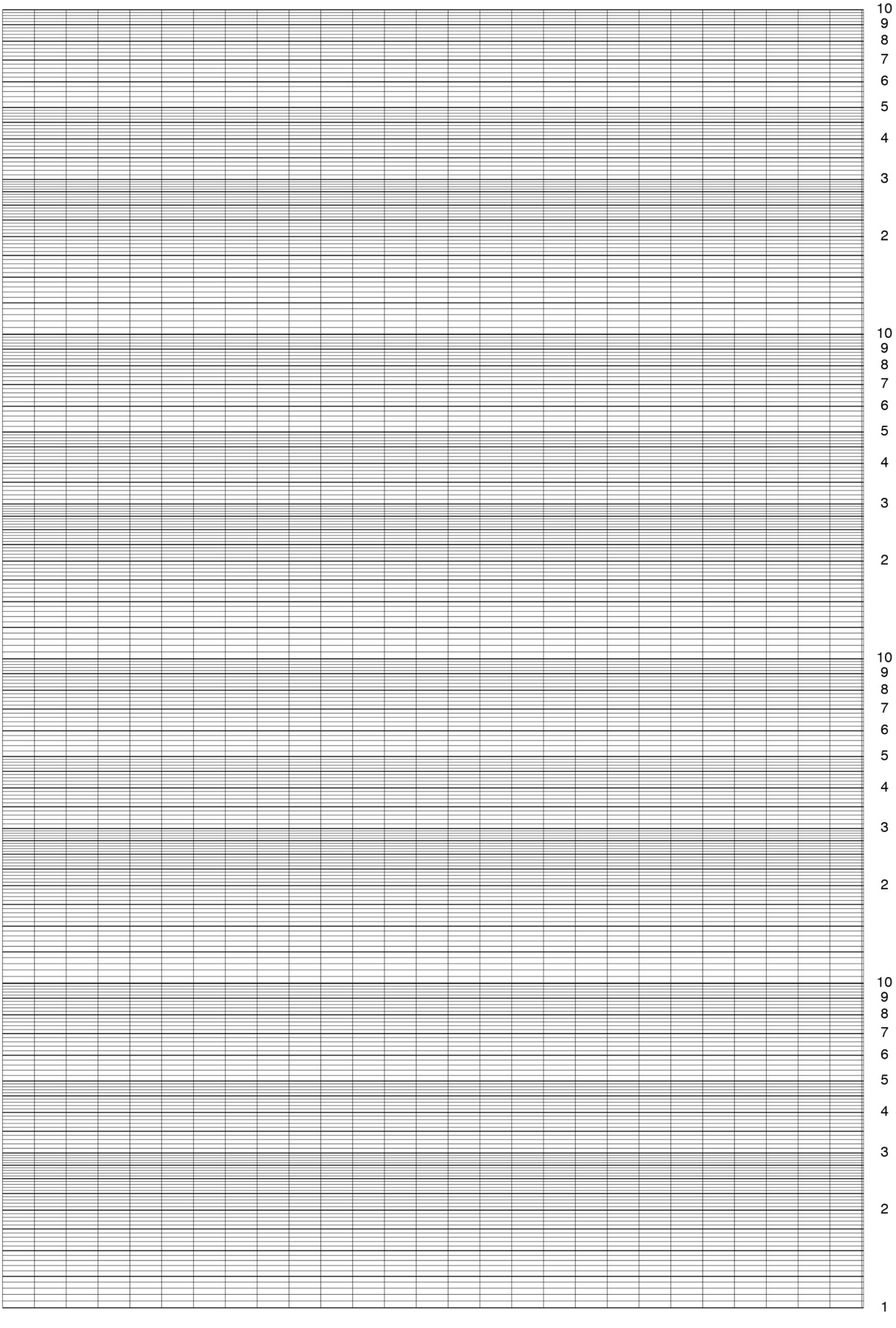
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Cell Signalling 2 – Results and Questions

Name: **Day & Date:** **Activator tested:**

TxB_2 Stds (pg/ml)	**0**	**1**	**10**	**30**	**100**	**300**	**1000**	**10000**
ABS405								
Samples	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	
ABS405 (a)								
ABS405 (b)								
Mean ABS405								
[TxB_2] (pg/ml)								
TxB_2 (pg/10^8 platelets)								
Samples	**Aspirin samples**:		**D8**	**D9**	**BAPTA samples**:		**D10**	**D11**
ABS405 (1)								
ABS405 (2)								
Mean ABS405								
[TxB_2] (pg/ml)								
TxB_2 (pg/10^8 platelets)								

What is the EC_{50} of the ligand? (Assume the maximum dose that you have used causes maximal TxB_2 production, in the case your curve does not reach plateau.)

Compare the TxB_2 production of the aspirin- and BAPTA-treated samples with corresponding non-treated platelets. Plot this as a bar chart showing the data for all relevant samples. What are the effects of aspirin and BAPTA?

Linear graph paper

Linear graph paper