Luisi Lab

Protocol Book

Contents

Chapter 1

Generally useful things

1.1 Transformation

1. Remove cells from –80^{0}C and let thaw on ice. **WARNING:** When handling frozen competent cells, it is important to **KEEP IT COOL**! Frozen cells are very sensitive to being warm and will not work as well if not kept on ice as much as possible.
2. Add 1-3 µL or less plasmid DNA (depending on the concentration of the plasmid; the volume of DNA solution should not exceed 10% of the volume of the competent cells). You don't need very much plasmid to get transformed colonies. Generally around 20ng (nanograms) is enough. If you are transforming ligation, use maximal volume of the ligation mixture.
3. Gently swirl tube(s) for a few seconds to mix.
4. Then incubate tube on ice for 5-15 minutes (or less depending on your patience level, but it is better to wait a bit; for ligation transformation, it is better to incubate it longer).
5. Heat shock tube(s) by placing in 42°C water bath for ~45 seconds (45 seconds is a good time for 50 µl of cells, for larger volumes use longer times – I double the time for 100 µl).
6. Replace tube(s) on ice for 1-5 minutes.
7. Add 300 µL of LB.
8. Gently shake and incubate tube(s) at for 30-60 minutes at 37°C. For ampicillin shorter incubation times are fine, for kanamycin or when using more than one antibiotic, use longer incubation times.
9. Spread on LB agar plates containing appropriate antibiotic (e.g., 100 µg/mL ampicillin/carbenicillin; 50 µg/mL kanamycin; 34 µg/mL chloramphenicol).
10. Place in the 37°C incubator with the agar side up and the lid side down. Incubate overnight and remove to fridge (4°C) the next day. For longer storage wrap the plate with parafilm.

1.2 Competent cell preparation - using rubidium chloride

This procedure is from the Promega Protocols and Applications Guide (3rd edition), p. 45-46.

1. 1. Plate the appropriate strain on LB-agar and grow o/n at 37°C. Make sure we have unopened bag of 1.5 ml eppendorfs, or autoclave some.
2. Inoculate a single colony from an LB plate in LB medium. Incubate overnight at 37°C with shaking (220 rpm).
3. On the following day inoculate 1:100 of LB medium containing 20 mM MgSO4 (use the volume appropriate for the amount of cells you wish to obtain – for large batches of communal competent cells use 250 ml). Grow the cells in a baffled flask at 37°C until the OD600 reaches 0.4–0.6 (typically 2–3 hours).

4. Pellet the cells by centrifugation at 5000 rpm for 5 minutes at 4°C.
5. Gently resuspend the cell pellets in 0.4 volume (based on the original culture volume) of ice-cold TFB1 (for a 250 ml culture, use 100 ml TFB1). For the remaining steps, keep the cells on ice and chill all pipets, tubes, buffers and flasks.
6. Incubate the resuspended cells on ice for 5 minutes.
7. Pellet the cells by centrifugation at 5000 rpm for 5 minutes at 4°C.
8. Gently resuspend the cells in 1/25 of the original culture volume of ice-cold TFB2 (for a 250 ml subculture, use 10 ml of TFB2) Note: Treat the competent cells gently as they are highly sensitive to handling and elevated temperature.
9. Incubate the cells on ice for 15–60 minutes and aliquot into 1.5 ml tubes (typically we prepare 50 ul aliquots)
10. Quick-freeze the tubes in liquid N2 and store at -80°C.

TFB1	250ml
30 mM potassium acetate	0.74g
10 mM CaCl2	1.25ml of 2M
50 mM MnCl2	2.47g
100 mM RbCl	3.023g
15% glycerol	37.5ml

Adjust pH to 5.8 with 1M acetic acid (glacial acetic acid ~17.4N). Filter-sterilize (0.2 µm). **Note:** *take care when titrating this solution; if you overshoot and try to bring the pH back up with hydroxide, the manganese will fall out of solution*

TFB2	100ml
10 mM MOPS or PIPES	0.335g
75 mM CaCl2	3.75ml of 2M
10 mM RbCl	0.12g
15% glycerol	15ml

Adjust pH to 6.5 with 1M KOH. Filter-sterilize (0.2 µm).

TFB1 and TFB2 can be stored at room temp or 4°C. They should be ice-cold for the procedure.

1.3 Voltages to run gels

Protein Gels

Depends on the buffer system used. MES: 150-200V (for 200V use fresh buffer and do not reuse it); MOPS: 150-180 V; SIGMA gels – 150-180V.

RNA gels

About 200-220 V for small gels (you may get smiling at higher voltages; lower voltages can be used as well)
Not more than 20 W for 20x20 cm gels, not more than 40 W for sequencing gels

Native gels

150 V for 1.5 h (for small gels) or 3 h (for 20 cm x20 cm gels)

DNA gels

100-130 V for 30-45 minutes

1.4 Buffer recipes

20X MES Running Buffer (500 mL)

1M MES	107.0 g
1M Tris Base	60.5 g
2% SDS	10.0 g
20 mM EDTA	3.7 g

pH should be approximately 7.3

20X MOPS Running Buffer (500 mL)

1M MOPS	104.6 g
1M Tris Base	60.5 g
2% SDS	10.0 g
20 mM EDTA	3.7 g

Reducing agent for NuPage Loading buffer (4x) (for protein gels)

40 µl βME for 1 mL Loading buffer

20X Sigma TruPAGE Tris-MOPS SDS Express Running buffer (1L)

Tris	145.2 g
MOPS	125.6 g
SDS	20.0 g

Antioxidant for Sigma TruPAGE gels (add 250 µL to central running chamber)

4 M Sodium Bisulfite (4.2 g in 10 mL)

5X Native Buffer (1L)

Tris	15.1	g
Glycine	94.0	g

Native gel, 8% (15ml, enough for two small 1 mm-thick gels)

30% Acrylamide	4	mL
MilliQ H_2O	5	mL
50% Glycerol	3	mL
1 M Tris pH 8.5	3	mL
To polymerize		
TEMED	15	µl
10% APS	150	µl

Gels are ready to use in 1h. ***USE FRESH****. Do not pre-make and use the next day or (worse) later.*

1.5 Antibiotics and agar plates

Antibiotic stock solutions

Choramphenicol:	30 mg/ml (in EtOH)
Ampicillin/Carbenicillin:	100 mg/ml *(amp and carb are interchangeable, but carb is more stable)*
Kanamycin:	50 mg/ml
Tetracycline:	10 mg/ml
Spectinomycin:	50 mg/ml

To make one sleeve of agar plates (500 ml)

1. Prepare 500 ml of LB solution. A 1 litre flask makes for easier pouring and handling, but if you intend to store for later use, a screw cap flask is better.
2. Add 7.5 g Agar
3. Autoclave as usual. Allow to cool until the flask is warm to the touch, then add antibiotic if necessary, mix gently to avoid bubbles and pour plates. 500 ml should be enough for a whole sleeve of plates.

1.6 rNTP recipe

Final concentration of each: 100 mM
Final pH: 7.5-8.0

Add water to each rNTP solution, but don't top up to final volume (volume close to final one is advised – pH might change quite a lot if large volumes of water will be added after pH adjustment). pH using clean NaOH and RNase-free tips (do not add to much at once), checking the pH with pH strips and stop when the pH is in the 7.5-8.0 range.
Also, compare the pH of the rNTP's to some other solutions in lab that are at pH 8. The pH strips can be misleading.
Keep the rNTPs at room temperature for as brief a period of time as possible as they aren't very stable. Aliquot into 50-100 uL aliquots and immediately put into a –20 C. Label each tube with the nucleotide. Label the outside of the main "rNTP" box with the date you made them.

Chapter 2

Protein expression and purification protocols

2.1 Hfq purification protocol

Expression Plasmid (ampicillin resistant): pEH-10-(hfq)

Cell line: E. coli BL21(DE3) (*be aware that Hfq is very abundant in E. coli, so Hfq mutants require additional purification steps or expression from pBAD vector in Top10Δhfq strain*)

Use 2xYT, induce with 1 mM IPTG at 37°C for 3 hours.

Lysis Buffer
- 50 mM Tris-HCl pH 8.0
- 1.5 M NaCl
- 250 mM $MgCl_2$
- 1 mM EDTA
- Protease inhibitor cocktail tablet (Roche)

Butyl Sepharose Buffer A
- 50 mM Tris-HCl pH 8.0
- 1.5 M NaCl
- 1.5 M $(NH_4)_2SO_4$

Butyl Sepharose Buffer B
- 50 mM Tris-HCl pH 8.0

SEC and Storage Buffer
- 50 mM Tris-HCl pH 8.0
- 100 mM NaCl
- 100 mM KCl
- Protease inhibitor cocktail tablet (Roche)

CAREFUL! *While in high salt, this purification **cannot** be done below room temperature*

Thaw the cell suspension and keep on ice or at 4 C. Supplement with DNase (1 µg/ml) to digest DNA (optional). Lyse the cells with the Emulsiflex and centrifuge the lysate in the 12150 rotor at 37500xg for 30 min at 4 C. When the spin is finished, leave the centrifuge open and change the temperature to 20°C.

Decant the supernatant to new ultracentrifugation tubes and incubate at 85°C for 20 minutes. Centrifuge again for 30 minutes at 37500 g at 20°C. Add Ammonium Sulfate to the clarified lysate to a final concentration of

1.6M and mix until dissolved. May take a few minutes, but expect some cloudiness. The solution will not appear perfectly clear. Centrifuge the sample one last time at 20°C for 30 minutes at 37500 g. Clarify the lysate by filtering through a 0.45 um filter (yellow) to remove any debris before loading on a HiTrap Butyl-Sepharose (HP- never FF, and not But-S as this will have very bad resolution) column at room temperature equilibrated in buffer A. Elute with an isocratic gradient of buffer B and analyse on SDS–PAGE (Hfq elutes usually in the end of the gradient, so an initial 50%B wash can be done to remove contaminants and gradient started from here to 100%B – sometimes the protein goes off the column in the final 100%B wash).

If you want to use coldroom AKTA, do the 50%B wash on bench and transfer the column to the coldroom for further purification steps

Pool fractions containing Hfq and concentrate to 2 ml with a 15 ml Amicon Ultra 30,000 MWCO concentrator (Millipore) and load onto a Sephadex 75 gel filtration column (GE Healthcare) equilibrated with SEC buffer (50 mM Tris pH 8.0, 100 mM NaCl, 100 mM KCl and protease inhibitor cocktail tablet (Roche)). Analyse the eluted fractions by SDS-PAGE and use the nanodrop to determine the concentration and extent of RNA contamination. The λ280nm molar extinction coefficient is 4470 M-1cm-1 for the monomer. It is very difficult to get Hfq completely free of RNA contamination, but a great result is a 260/280 ratio between 0.6-0.7.

Hfq sticks very well to heparin, so when butyl-sepharose fractions don't seem clean, heparin step can be added.

2.2 RNase E NTD (1-529) purification protocol

For expression plasmid and cell line please refer to supervisor

Use 2xYT. This protocol is suitable for the WT and all mutant versions. If the protein after nickel column has a lot of protein/RNA contamination, add heparin column before S200 step.

Buffer A

- 20 mM Tris-HCl pH 7.9
- 500 mM NaCl
- 5 mM Imidazole
- Protease inhibitor cocktail tablet (Roche)

Buffer B

Buffer A + 1 M imidazole

SEC and Storage Buffer

- 20 mM Tris-HCl pH 7.9
- 500 mM NaCl
- 10 mM $MgSO_4$ or $MgCl_2$
- 0.5 mM EDTA
- 10 mM DTT
- 5% vol/vol glycerol
- Protease inhibitor cocktail tablet (Roche)

(very long, but has to be done all together if the protein is prepared for enzymatic assays – the activity drops substantially when kept out longer than for a day)

Thaw the cell suspension and keep on ice or at 4 C Lyse the cells with the Emulsiflex (Supplement with DNase (1 µg/ml) as needed) and centrifuge the lysate in the 12150 rotor at 37500 g for 30 min at 4 C. Filter the lysate before loading on the column using 0.45 um filter.

Elute the protein with a linear gradient of 0 to 60% B. Analyze the protein from the peak fractions on SDS-PAGE. Concentrate fractions containing RNase E catalytic domain to 2 ml using 15 ml Amicon Ultra 30,000 MWCO concentrator (Millipore) and load the concentrated protein to a Sephadex 200 gel filtration column (GE Healthcare) equilibrated with SEC/Storage Buffer.

Run standard protocol and analyze the protein from the peak fractions SDS-PAGE. For activity assays aliquot some of the protein into small (10 µl) fractions before flash freezing. The λ280nmmolar extinction coefficient is 28880 M-1cm-1for the monomer.

When using the sample in the future, bear in mind that RNase E loses catalytic activity very quickly, so use thawed samples on the same day and throw away the leftovers.

2.3 RNase Ch2E (1-762)/RhlB expression and purification

For expression plasmid and cell line please refer to supervisor

HisTrap Buffer A

- 50 mM Tris-HCl, pH 7.8
- 1 M NaCl
- 5 mM imidazole
- 5 mM MgSO4
- 5 mM β-mercaptoethanol
- 5% (v/v) glycerol
- 1 tablet/500 ml EDTA-free protease inhibitor cocktail (Roche)

HisTrap Buffer B

HisTrap Buffer A supplemented with 0.5 M imidazole

Heparin Buffer A

- 50 mM Na phosphate, pH 7.9,
- 250 mM NaCl,
- 10 mM DTT,
- 5% (v/v) glycerol

Heparin Buffer B

Heparin Buffer A supplemented with 2 M NaCl

Gel filtration/storage buffer

- 50 mM Tris-HCl, pH 7.9
- 0.5 M NaCl
- 50 mM KCl
- 1 mM MgCl2
- 5 mM DTT
- 5% (v/v) glycerol
- 1 tablet/1 L EDTA-free protease inhibitor cocktail (Roche)

RNase E (1-762)/RhlB was expressed with an N-terminal His-tag from the vector pRSF_rne762rhlB. BL21(DE3) or C43 cells transformed with the vector were grown in 2×YT media supplemented with 50 µg/ml kanamycin, in dimpled flasks at 37□C, and induced with 1 mM IPTG at mid-log phase. Use 0.5 l of media per flask. C43 cells give much higher yield of the protein.

Cells were harvested after 3 hours and lysed by passing several times through an EmulsiFlex. The lysate was clarified by centrifugation (4°C, 30 minutes, 37500 g) and the soluble fraction was filtered (0.45 um syringe filter) and loaded onto HiTrap Chelating HP column charged with nickel, washed with HisTrap buffer A and eluted with a gradient of HisTrap buffer B.

Fractions containing RNase E/helicase complex were pooled and diluted with heparin buffer A without salt to salt concentration of 250 mM and loaded on a 5 ml HiTrap Heparin column equilibrated with Heparin buffer A. RNase E/RhlB complex was eluted with Heparin buffer B. Enriched fractions were concentrated and fractionated on a S200 column. The protein was stored at -80□C after freezing in liquid nitrogen (ExCo of monomer 66240). For activity assays aliquot some of the protein into small (10 µl) fractions before flash freezing. When using the sample in the future, bear in mind that RNase E loses catalytic activity very quickly, so use thawed samples on the same day and throw away the leftovers.

RNase E contructs that have C-terminal parts included are very sensitive to degradation. Resuspend the cells in as small volume of lysis buffer as possible, keep the sample on ice and load on the first column as quickly as possible. The degradation also occurs when protein is left in the coldroom o/n, so ideally whole purification should be done in one day. Activity of the protein also drops significantly with time.

2.4 RNase ECh2 (1-850)/RhlB/Enolase purification

For expression plasmid and cell line please refer to supervisor

HisTrap Buffer A/Lysis buffer
- 50 mM Tris pH 7.5
- 1 M NaCl
- 40 mM KCl
- 5 mM MgCl2
- 0.02% w/v β-DDM (+ 1 protease inhibitor tablet for lysis buffer)

HisTrap Buffer B

HisTrap buffer A supplemented with 0.5 M imidazole

SP buffer A
- 50 mM Tris pH 8.0
- 50 mM NaCl
- 10 mM KCl
- 0.02% w/v β-DDM

SP buffer B

SP buffer A supplemented with 2 M NaCl

Gel filtration buffer
- 50 mM Bis-Tris pH 6
- 400 mM NaCl
- 100 mM KCl
- 1 mM TCEP
- 0.02% w/v β-DDM

RNase E (1-850)/RhlB was expressed with an N-terminal His-tag from the pRSF-Duet vector (Km), together with Enolase from pET vector (Amp) in the ENS134-10 strain. Grow cells transformed with both vectors in 2×YT media in a starter culture for 2-3 hours, than use the pre-culture to inoculate 500 ml 2xYT in dimpled 2l flasks. Grow the cells at 37□C and induce with 1 mM IPTG at OD 0.6, than express the protein at 18□C. Harvest cells after about 11 hours and resuspend in smallest possible volume of lysis buffer with 1 protease inhibitor. Add 1% Triton X-100 then lyse cells with Emulsiflex. Spin the lysate at 37500 g for 30 min at 4ºC and filter the supernatant (0.45 um syringe filter). Load onto nickel column, wash with HisTrap buffer A for 65 mL and apply 0-60% HisTrap buffer B gradient.
Pool the fractions enriched in RNase E, RhlB and enolase and dilute 1/3 with SP buffer A without salt. Load onto 1ml SP column and elute with linear gradient of 1-50% SP buffer B. Concentrate enriched fractions and load on a Superose 6 column equilibrated with gel filtration buffer.

2.5 PNPase expression and purification

Q Buffer A
- 20 mM Tris-HCl pH 8.5,
- 30 mM NaCl,
- 1 mM DTT,
- 10% v/v glycerol,
- EDTA-free protease inhibitors (Roche)

Q Buffer B
Buffer A+1M NaCl

Butyl-sepharose Buffer A
- 50 mM Na-phosphate,
- 1 M (NH4)2SO4

Butyl-sepharose Buffer B
50 mM Na-phosphate

Gel filtration/Storage buffer
- 20 mM Tris-HCl pH 8.0
- 150 mM NaCl
- 5 mM MgCl2
- 5% v/v glycerol
- 1 mM DTT

Full-length untagged PNPase was expressed in the pETDuet vector in BL21(DE3) E. coli. Transformed cells were cultured in 2xYT (100 µg/ml carbenicillin) at 37°C until OD600=0.4. Cells were then induced (0.5 mM IPTG, 25°C, 3-4 h), harvested by centrifugation (4200 g, 4°C, 25 min), resuspended in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 150 mM KCl, 5 mM MgCl2, EDTA-free protease inhibitors (Roche), and lysed by passage through Emulsiflex.

The lysate was supplemented with DNase (1 µg/ml) and centrifuged (37500 g, 4°C, 30 min). Proteins from the supernatant were precipitated with (NH4)2SO4 (51.3% saturation; 302g for 1L [2M], 4°C, until dissolved). Following centrifugation (37500 g, 4°C, 20 min), the pellet was resuspended in 150 ml Q Buffer A without salt and loaded on to a HiTrap Q column (GE Healthcare) equilibrated in Q Buffer A. Protein was eluted with a linear gradient of 0-60% Q Buffer B. The peak fractions were pooled and diluted to make a solution with final concentrations of 1 mM MgCl2, 50mM Na-phosphate pH 7.9, 0.9 M (NH4)2SO4 , 1 mM DTT and loaded onto a HiTrap Butyl-Sepharose column (GE Healthcare) equilibrated in Butyl-Sepharose Buffer A.

The protein was eluted with a linear gradient of 0-60% Butyl-Sepharose Buffer B. Peak fractions were pooled, concentrated to 2 ml using Amicon Ultra 100,000 MWCO filter units (Millipore) and loaded onto a Superdex 200 16/60 gel filtration column equilibrated in gel filtration buffer.

Fractions containing purified PNPase, as analysed by SDS-PAGE, were flash-frozen and stored at -80°C. The protein concentration was determined spectroscopically using a NanoDrop and an extinction coefficient of 30370 M-1cm-1 (monomer).
If protein is needed for activity assays, prepare some small (10 ul) aliquots and store in -20. After thawing throw away the leftovers of the used aliquot.

2.6 RapZ expression and purification

RapZ was expressed in Escherichia coli Rosetta (Invitrogen) cells in Luria Broth (LB) (10 g/L Bacto tryptone, 5 g/L Bacto yeast extract, 10 g/L NaCl) supplemented with 1% glucose, 30 µg/ml Chloramphenicol, and 100 µg/ml Carbenicillin. Overnight precultures from a single colony in LB were diluted 2-5% in fresh LB+Chloramphenicol+Carbenicillin. Cultures were incubated at 37°C, 220 rpm, until the turbidity of the culture reached OD_{600} ~0.8, at which point the flasks were placed for 10 min in an ice bath. Expression was finally induced with 1 mM IPTG at 18°C for 1 hr. Cells were then harvested by centrifugation at 4,200 × g for 20 min at 4°C. The pellet was then resuspended in lysis buffer (50 mM Tris, pH 8.5, 500 mM KCl, 1 mM EDTA, 5 mM BME, Protease Inhibitor Cocktail Tablet (Roche)) and passed three times through an Emulsiflex-05 cell disruptor (10-15 kbar; Avestin). The lysate was clarified with centrifugation 37,500 × g for 30 min at 4°C before being passed through a 0.45 µm filter (Millipore) and loaded on a Strep Trap HP column (GE Healthcare) equilibrated with Strep buffer A (50 mM Tris, pH 8.5, 500 mM KCl, 1 mM EDTA, 5 mM BME). Protein was eluted with 100% Strep buffer B (50 mM Tris, pH 8.5, 500 mM KCl, 1 mM EDTA, 5 mM BME, 2.5 mM desthiobiotin) and analysed by SDS-PAGE. The Strep tag was typically cleaved by TEV protease for 1 hr at room temperature.
Fractions containing RapZ were pooled and loaded onto a Heparin HiTrap HP column equilibrated with Heparin buffer A (50 mM Tris, pH 8.5, 200 mM KCl, 1 mM TCEP). Protein was eluted with an isocratic gradient of Heparin buffer B (50 mM Tris, pH 8.5, 1 M KCl, 1 mM TCEP). Fractions containing pure RapZ were pooled, concentrated to 0.5 ml with 15 ml Amicon Ultra 30,000 MWCO concentrator (Millipore) and loaded onto a Sephadex 200 Increase gel filtration column (GE Healthcare) equilibrated with buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, and 50 mM KCl, and 2 mM DTT. Fractions were analysed by SDS–PAGE and the concentration of pure RapZ in the fractions was determined spectroscopically using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and λ280nm extinction coefficient of 22920 $M^{-1}cm^{-1}$. Samples were either used immediately for crystallography and assays or supplemented with 10% glycerol, flash frozen, and stored at -80°C for other applications.

2.7 Rz154e Expression and Purification

Rz154e was expressed in Escherichia coli Rosetta (Invitrogen) cells in Luria Broth (LB) (10 g/L Bacto tryptone, 5 g/L Bacto yeast extract, 10 g/L NaCl) supplemented with 1% glucose, 30 µg/ml Chloramphenicol, and 100 µg/ml Carbenicillin. Overnight precultures from a single colony in LB were diluted 2-5% in fresh LB+Chloramphenicol+Carbenicillin. Cultures were incubated at 37°C, 220 rpm, until the turbidity of the culture reached OD600~0.8, at which point the flasks were placed for 10 min in an ice bath. Expression was finally induced with 1 mM IPTG at 18°C for 2 hr. Cells were then harvested by centrifugation at 4,200 g for 20 min at 4°C. The pellet was then resuspended in lysis buffer (50 mM Tris, pH 7.5, 500 mM KCl, 1 mM EDTA, 5 mM BME, Protease Inhibitor Cocktail Tablet (Roche)) and Benzonase (Sigma) was added to achieve a final concentration of 10 Units/ml. The final solution was passed three times through an Emulsiflex-05 cell disruptor (10-15 kbar; Avestin).
The lysate was clarified with centrifugation 37,500 × g for 30 min at 4°C before being passed through a 0.45 µm filter (Millipore) and loaded on a Strep Trap HP column (GE Healthcare) equilibrated with Strep buffer A (50 mM Tris, pH 7.5, 500 mM KCl, 1 mM EDTA, 5 mM BME). Protein was eluted with 100% Strep buffer B (50 mM Tris, pH 7.5, 100 mM KCl, 1 mM EDTA, 5 mM BME, 2.5 mM desthiobiotin) and analysed by SDS-PAGE. The Strep tag was typically cleaved by TEV protease for 1 hr at room temperature. Fractions containing RapZ were pooled and loaded onto a MonoS (GE Healthcare) column equilibrated with MonoS buffer A (50 mM Tris, pH 8.5, 100 mM KCl, 1 mM TCEP). Protein was eluted with an isocratic gradient of Heparin buffer B (50 mM Tris, pH 7.5, 1 M KCl, 1 mM TCEP). Fractions containing pure Rz154e were pooled, concentrated to 0.5 ml with 15 ml Amicon Ultra 30,000 MWCO concentrator (Millipore) and loaded onto a Sephadex 200 Increase gel filtration column (GE Healthcare) equilibrated with buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, and 50 mM KCl, 1 mM DTT. Fractions were analysed by SDS–PAGE and the concentration of pure Rz154e in the fractions was determined spectroscopically using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and λ280nm extinction coefficient of 19940 $M^{-1}cm^{-1}$. Samples were either used immediately for crystallography and assays or supplemented with 10% glycerol, flash frozen, and stored at - 80°C for other applications.

2.8 ProQ expression and purification

ProQ was expressed in BL21(DE3) cells grown in 2XYT media (Formedium) supplemented with 100 µg/ml carbenicillin at 37□C. Overnight precultures from a single colony in 2XYT were diluted 2-5% in fresh 2XYT-Carbenicillin. Cultures were incubated at 37°C, 220 rpm, until the turbidity of the culture reached OD600~0.4 and induced with 1mM IPTG. After 2 hr cells were then harvested by centrifugation at 4,200 × g for 25 min at 4°C. The pellet was then resuspended in lysis buffer (20 mM Tris,

pH 7.5, and Protease Inhibitor Cocktail Tablet (Roche)) and passed three times through an Emulsiflex-05 cell disruptor (10-15 kbar; Avestin).
The lysate was clarified with centrifugation 37,500 × g for 30 min at 4°C and passed through a 0.45 µm filter (Millipore) before being loaded on an SP HP column (GE Healthcare) equilibrated with SP buffer A (20 mM Tris, pH 7.5). Protein was eluted by a gradient with 100% SP buffer B (20 mM Tris, pH 7.5, 1M NaCl) and analysed by SDS-PAGE. Fractions containing ProQ were pooled and diluted with SP Buffer A to achieve a final buffer composition of 20 mM Tris, pH 7.5, 100 mM NaCl. In order to remove contaminating nucleic acid, the sample was then loaded onto a Heparin HiTrap HP column (GE Healthcare) equilibrated in Heparin Buffer A (20 mM Tris, pH 7.5, 100 mM NaCl). Protein was eluted by a gradient of Heparin Buffer B (20 mM Tris, pH 7.5, 1M NaCl) and analysed by SDS-PAGE.
Fractions containing pure ProQ were pooled, concentrated to 2 ml with 15 ml Amicon Ultra 10,000 MWCO concentrator (Millipore) and loaded onto a Sephadex 75 gel filtration column (GE Healthcare) equilibrated with buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, and 100 mM KCl. Fractions were analysed by SDS–PAGE and the concentration of ProQ in the fractions was determined spectroscopically using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and λ280nm extinction coefficient of 9970 M-1cm-1 before they were flash frozen and stored at -80C.

2.9 ProQ truncations (including NTD and CTD) expression and purification

ProQ NTD (residues 1-119) and CTD (residues 180-232) with N-terminal hexahistidine tags were expressed and purified using the same procedure. The given construct was expressed in BL21(DE3) cells grown in 2XYT media (Formedium) supplemented with 100 µg/ml carbenicillin at 37□C. Overnight precultures from a single colony in 2XYT were diluted 2-5% in fresh 2XYT-Carbenicillin. Cultures were incubated at 37°C, 220 rpm, until the turbidity of the culture reached OD600~0.4 and induced with 1mM IPTG. After 2 hr cells were then harvested by centrifugation at 4,200 × g for 25 min at 4°C. The pellet was then resuspended in lysis buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 10 mM Imidazole, and Protease Inhibitor Cocktail Tablet (Roche)) and passed three times through an Emulsiflex-05 cell disruptor (10-15 kbar; Avestin).
The lysate was clarified with centrifugation 37,500 × g for 30 min at 4°C and passed through a 0.45 µm filter (Millipore) before being loaded on an HisTrap HP column (GE Healthcare) equilibrated with His buffer A (20 mM Tris, pH 7.5, 500 mM NaCl, 10 mM Imidazole). Protein was eluted by a gradient with 100% His buffer B (20 mM Tris, pH 7.5, 500 mM NaCl, 500 mM Imidazole) and analysed by SDS-PAGE. Fractions containing ProQ NTD or CTD were pooled and diluted with 20 mM Tris, pH 7.5 to achieve a final buffer composition approximating 20 mM Tris, pH 7.5, 100 mM NaCl. In order to remove contaminating nucleic acid, the sample was then loaded onto a Heparin HiTrap HP column (GE Healthcare) equilibrated in Heparin Buffer A (20 mM Tris, pH 7.5, 100 mM NaCl). Protein was eluted by a

gradient of Heparin Buffer B (20 mM Tris, pH 7.5, 1M NaCl) and analysed by SDS-PAGE.
Fractions containing pure ProQ NTD or CTD were then pooled, concentrated to 2 ml with 15 ml Amicon Ultra 10,000 MWCO concentrator (Millipore) and loaded onto a Sephadex 75 gel filtration column (GE Healthcare) equilibrated with buffer containing 20 mM Tris pH 7.5, 100 mM NaCl, and 100 mM KCl. Fractions were analysed by SDS–PAGE and the concentration of ProQ in the fractions was determined spectroscopically using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) before they were flash frozen and stored at -80□C.

2.10 Expression and purification of ProQ NTD (residues 1-119) for NMR

Uniformly 15N or 13C/15N labelled proteins were produced using nitrogen-deficient phosphate buffered media, wherein nitrogen was supplied by 15NH4Cl. Where necessary, glucose was replaced with 13C-incorporated glucose. Protein was expressed in BL21(DE3) cells at 25 °C for 20 hrs after induction with 1 mM IPTG. Cells were harvested by centrifugation at 4,200 × g for 25 min at 4°C. The pellet was then resuspended in lysis buffer (20 mM Sodium Phosphate, pH 6.0, 500 mM NaCl, 10 mM Imidazole, and Protease Inhibitor Cocktail Tablet (Roche)) and passed three times through an Emulsiflex-05 cell disruptor (10-15 kbar; Avestin).
The lysate was clarified with centrifugation 37,500 × g for 30 min at 4°C and passed through a 0.45 µm filter (Millipore) before being loaded on an HisTrap HP column (GE Healthcare) equilibrated with His buffer A (20 mM Sodium Phosphate, pH 6.0, 500 mM NaCl, and 10 mM Imidazole). Protein was eluted by a gradient with 100% SP buffer B (20 mM Sodium Phosphate, pH 6.0, 500 mM NaCl, and 500 mM Imidazole) and analysed by SDS-PAGE. Fractions containing ProQ were pooled and diluted with 20 mM Sodium Phosphate, pH 6.0 to achieve a final buffer composition approximating 20 mM Sodium Phosphate, pH 6.0, 100 mM NaCl. In order to remove contaminating nucleic acid, the sample was then loaded onto a Heparin HiTrap HP column (GE Healthcare) equilibrated in Heparin Buffer A (20 mM Sodium Phosphate, pH 6.0, 100 mM NaCl). Protein was eluted by a gradient of Heparin Buffer B (20 mM Sodium Phosphate, pH 6.0, 1M NaCl) and analysed by SDS-PAGE.
Fractions containing pure ProQ were pooled, concentrated to 2 ml with 15 ml Amicon Ultra 10,000 MWCO concentrator (Millipore) and loaded onto a Sephadex 75 gel filtration column (GE Healthcare) equilibrated with buffer containing 20 mM Sodium Phosphate, pH 6.0, 100 mM NaCl, 50 mM KCl, 1 mM TCEP, and Protease Inhibitor Cocktail Tablet (Roche). ProQ NTD was then concentrated to 0.70 mM and D2O was added to 10% for data collection and 3,3,3-trimethylsilylpropionate (TSP) solution was added to provide an internal chemical shift reference signal at 0 ppm.

2.11 Purification of His-tagged T7 RNA Polymerase

Use 2xYT and Carbenicillin

Lysis Buffer/Buffer A
- 50 mM HEPES Na pH 7.5
- 300 mM NaCl
- 10 mM Imidazole

Buffer B
- 50 mM HEPES Na pH 7.5
- 300 mM NaCl
- 250 mM Imidazole

Dialysis Buffer
- 10 mM Potassium Phosphate pH 7.4

  (0.5M Potassium Phosphate buffer stock is made by made by dissolving 27 g KOH and 68 g KH_2PO_4 in 1 L water [make sure that pH is approximately 7.5])
- 50 mM KCl

Storage Buffer
- 20 mM Potassium Phosphate pH 7.5
- 100 mM NaCl
- 1 mM EDTA
- 1 mM DTT
- 50% Glycerol
- 100 ul/ml BSA(optional)

Transform competent BL21 (NOT DE3) with pT7-911(Thomas Shrader) and plate it on a LB+Carb plate and incubate at 37 C
Collect colonies from plate and resuspend in 2xYT with carbenicillin/ampicillin and grow at 37 C in a shaking incubator for approximately 2 hrs. Place the 0.5 L flasks in the 37 C incubator to pre-warm the media (optional). Transfer the starter culture to each 0.5 L flask supplemented with carbenicillin/ampicillin and place the flasks in shaking incubator.
When the OD_{600} is 0.6-0.9 add IPTG to 0.4 mM and continue growing the culture for 3-4 hrs. Harvest the cells and resuspend in Buffer A, and store in -80ºC.
Thaw the cell suspension. All manipulations must be carried out on ice or at 4 C Lyse the cells with the Emulsiflex and centrifuge the lysate at 37500 g for 30 min at 4 C. Filter the lysate using 0.45 um syringe filter (yellow)
Load the lysate onto a nickel column and wash the column with Buffer A until the OD280 stabilizes.
Elute the protein with a linear gradient of Imidazole (0 - 100% B) in 25 column volumes. You should observe first a small peak and then a large

one, which contains the T7 RNAP. Analyze the protein from the peak fractions on a 4-12% Sigma gel.
Pool peak fractions and dialyze against two changes of chilled Dialysis Buffer (3 hrs each wash)
Connect a hydroxylapatite column to an Akta. If one is not alreay prepared, suspend 25 g of hydroxylapatite in Dialysis Buffer and pack it into 4 cm(diameter)x10 cm column (or something comparable). First wash the column with the same buffer before connecting to the fraction collector as fines often come off the column.
Apply the dialyzed protein sample to the hydroxylapatite column and wash the column with 100 mL Dialysis Buffer. Elute the protein with a linear gradient formed with 500 ml of Dialysis Buffer and 500 ml of Dialysis Buffer with 30g (NH4)2SO4 (6% w/v). Analyze the protein samples from peak fractions on a polyacrylamide gel. Dialyze against storage buffer, measure OD, and store at -80 C. You can expect up to 100 mg from 3 L culture.
You can also skip the hydroxyapatite column and do gel filtration instead. Purify the sample by S200 gel filtration using 20 mM Potassium Phosphate pH 7.5, 100 mM NaCl, 1 mM EDTA. Then dialyze into storage buffer or just mix directly in.
Molar Extinction Coefficient 1.4 x 10 5 M -1
Note: OD = 1 corresponds to approx. 0.7 mg/ml
MolecularWeight Approximately 100 kDa
Remember to use BL21 strain as a host, at minimum, since it is OmpT-. Do not use BL21(DE3), which is commonly used for protein expression with T7 pol vectors. OmpT is an outer membrane protease known to cleave and inactivate T7 RNA polymerase. Unless you use BL21, a large fraction of the T7 RNA Polymerase will be cleaved and inactive.

Chapter 3
RNA-related

3.1 In vitro transcription

DNA template must have T7 promoter sequence

Either
5' – GAA ATT AAT ACG ACT CAC TATA – 3'
if RNA sequence starts with **G**
or
5' – GAA ATT AAT ACG ACT CAC TATT – 3'
if RNA sequence starts with **A**

10X In vitro Transcription Buffer
- 400 mM Tris, pH 8.0
- 250 mM MgCl2
- 0.01% Triton
- 100 mM DTT
- Spermidine - *[liquid at RT (d = 0.91 g/ml), may be solid at 4 C – 32 µl (= 29mg)]*

Make up to 10 mL, but add the water first! Freeze aliquots.

FUD = 2x formamide loading dye
- Formamide 50 mL
- Bromophenol blue 50 mg
- Xylene cyanol 50 mg
- 1 mM EDTA (optional)

Reaction:

Test for best DNA template amount to use for reactions. It should be within 1000-4000 ng per 200 ul reaction (usually 3000 ng works well). rNTP concentrations can also be optimized, but this is really tedious and the standard usually works fine. Optimization of T7 Polymerase can be worth the effort. Do not think more is better. Too much T7 Polymerase in the reaction can be inhibitory to the reaction. 0.15 mg/ml T7 final concentration is a safe bet.

Reaction recipe (200 µl)
- DNA Template
- 10X T7 Buffer 20 µl
- 100 mM DTT 20 µl
- 100 mM rNTPs 10 µl each (40 µl mix)
- T7 Polymerase between 0.1 and 0.2 mg/ml final concentration
- 0.5 U/µl RNase out 2.5 µl (optional)
- ddH2O bring up to 200 µl final volume

Incubate at 37C for 4 hrs without shaking. Digest DNA template with Turbo DNase1 (6-8 µl) for a further 30 min at 37C. Finally quench reactions with 20 µl 500 mM EDTA per 200 µl reaction (this should result in removal of the white precipitate). From here you can purify as you prefer: you can proceed to ethanol precipitation before running on a gel and excising bands, but depending on the volume, you can load to a gel directly.

Visualise bands containing RNA by UV–shadowing and excise. RNA is recovered from gel slices by overnight electroelution at 100 V in TBE buffer using an EluTrap System

3.2 RNase E degradation assay protocol

RNase E Reaction Buffer (1X)	*[For 1 mL]*
· 25 mM Tris-HCL pH 7.5	25 µl 1 M Tris-HCl pH 7.5
· 50 mM NaCl	10 µl 5 M NaCl
· 50 mM KCl	25 µl 2 M KCl
· 10 mM MgCl2 *(for gel shifts, replace with CaCl2)*	20 µl 0.5M MgCl2
· 1 mM DTT *(must be fresh)*	10 µl 100 mM DTT
· 0.5 U/µl RNaseOUT *(Invitrogen)*	12.5 µl Stock (40 U/µl)
· 897.5 µl H2O	

For setting up reactions it is useful to prepare more concentrated buffer, e.g. 2x.

Proteinase K Buffer (2X) – mix 1:1 with sample to stop reaction	*[For 1 mL]*
· 200 mM Tris-HCl pH 7.5	200 µl 1 M Tris-HCl pH 7.5
· 25 mM EDTA	50 µl 0.5M EDTA
· 300 mM NaC	60 µl 5 M NaCl
· 2% wt/v SDS	200 µl 10% wt/v SDS
· 490 µl H2O	

- Add Proteinase K (dilute 10 µl of 10 mg/ml stock into 190 µl Proteinase K Buffer) – mix 1:1 w/reaction
- Use 0.2 µM RNA or 0.3 µM RNA oligo, double for 10% PAA high resolution non- radioactive gels; 0.05 µM RNase E (1-762)/RhlB or 0.15-0.2 µM RNase E (1-529)
- Final reaction volume is 10 µl.

Protocol:

1. Anneal the RNA (in H2O) – usually 2 minutes at 50°C is sufficient. Slowly cool to room temperature (approximately 3 min)
2. Assemble the reaction (buffer, RNA, water, relevant binding partner e.g. Hfq) and incubate for 5-10 min at 37°C, if needed.
3. Initiate degradation assay by adding RNase E (1-529)

4. Collect time course reactions. Stop reactions at 0, 1, 2, 3, 5, 15, and 30 min by incubation with 1:1 2X Proteinase K Solution in 50°C for 15-30 min. The initial 0 time point is taken from the reaction mixture immediately after RNase E is added and corresponds, more accurately, to 10-15 seconds.
5. After Proteinase K treatment, add RNA Loading Dye (Fermentas) and denature the samples for 3 min at 95°C.
6. Load samples on Urea PAGE
7. Stain gels with SYBR Gold (Invitrogen) and visualize RNA by UV imaging or using Typhoon.

3.3 RNA snap Protocol (for RNA extraction from cells)

Neccesary Materials:

- RNA Extraction Solution
- 18 mM EDTA
- 0.025% SDS
- 1% 2-mecaptoethanol
- 95% formamide (RNA grade)
- M Sodium Acetate, pH 5.2
- 75% Ethanol (EtOH)
- 100% Ethanol (EtOH)
- RNase-free Water
- 1.5 ml and 2 ml Eppendorf tubes
- Heating block
- High-speed table-top centrifuge

Procedure

RNA Isolation

1. Centrifuge one millilitre of bacterial culture (108cells) – or any desired volume, but it must fit in a vessel that can be spun at high speed (16,000 g) – at 16,000 g for 30s and remove the supernatant with a pipette tip, taking care not to disturb the pellet. If the pellet will not be used immediately, store in dry ice until ready for extraction.
2. Resuspend the pellet in 100 µl of RNA extraction solution [18 mM EDTA, 0.025% SDS, 1% 2-mecaptoethanol, 95% formamide (RNA grade)] by vortexing vigorously. Alternatively, if working with a different starting volume, resuspend in 1/10 the starting volume of RNA extraction solution.
3. Lyse the cells by incubating the sample at 95°C for 7 min.
4. Pellet the cell debris by centrifuging the warm sample at 16,000 g for 5 min at room temperature.
5. Carefully transfer the supernatant to a fresh tube without disturbing the clear gelatinous pellet.

Sodium acetate/ethanol precipitation

6. Dilute the sample with four volumes of water

7. Add 1/10 of 3 M Sodium Acetate, pH 5.2 and mix by pipetting.
8. Add three volumes of 100% Ethanol and mix the sample by vortexing briefly.
9. Incubate the sample for at least 60 min at -80°C to precipitate the RNA.
10. Pellet the RNA by centrifuging the sample for 30 min at 16,000 g at 4°C.
11. Carefully remove the supernatant with a pipette.
12. Wash the pellet with 250 µl of 75% Ethanol and centrifuge at 8,000 g for 5 min at 4°C.
13. Carefully remove the supernatant with a pipette.
14. Briefly centrifuge again and remove any remaining ethanol with a pipette.
15. Once the ethanol has been removed from the sample, air dry the pellet for approximately 5 min in the fume hood. Take care not to over-dry the pellet, as this will make it difficult to redissolve.
16. Resuspend the RNA pellet in water.

3.4 Protocol for 4sU-tagging, thiol-specific biotinylation and separation of total RNA into nascent and pre-existing RNA (using Biotin-HPDP)

Adapted from the protocol completed 29.11.2010 by Lars Dölken (Doelken@mvp.uni-muenchen.de mailto:Doelken@mvp.uni-muenchen.de) and Zeiner et al. in Methods in Molecular Biology.

Very Important:

Ensure all materials and reagents are RNAse free!

Material to order and prepare:

- 15 ml Polypropylene Tubes SuperClear™ Gatefree™ (tolerate up to 15,000 g) (VWR International, Order no. 525-0153) (or any 15,000 g tolerant Falcon tube)
- 4-thiouridine (Sigma, T4509 4-thiouridine, 100mg)
  - Dissolve in sterile PBS (or water)
  - Stock concentration: 50 mM
  - Store in small aliquots at -20°C, thaw only once
- 5 M NaCl
- RNA precipitation buffer
  - 0.8 M NaCl
  - 1.2 M NaCitrate
- Biotin-HPDP (Pierce, 50mg EZ-Link Biotin-HPDP, Cat. Nr. 21341)
  - Stock concentration: 1 mg/ml dissolved in Dimethylformamide (DMF)
  - Gentle warming will ensure complete solubilisation
  - Store at 4°C
- RNAse free 10x Biotinylation Buffer (BB)
  - 100 mM Tris pH 7.4
  - 10 mM EDTA
  - store in aliquots of 1 – 1.5 ml at 4°C
- Phase Lock Gel Heavy Tubes (2.0 ml), Eppendorf (Order No. 0032 005.152)

- µMacs Streptavidin Kit (Miltenyi, Order No. 130-074-101)
  - use only the beads, but not the supplied buffers
- 1x Washing Buffer (WB)
  - 100 mM Tris pH 7.5
  - 10 mM EDTA
  - 1 M NaCl
  - 0.1% Tween20
- Dithiothreitol (DTT)
  - Always prepare fresh: 100 mM DTT in H2O
- Magnetic Stand
  - (2 - 3 required, one stand holds 4 columns) has to fit the small columns of the µMacs Streptavidin Kit
- Chloroform/Isoamylalcohol (24:1)
- Isopropanol
- Ethanol

Labelling Cellular RNA with 4-thiouracil/uridine

Needed Materials:

- 50 mM stock solution of 4-thiouracil and 4-thiouradine
- LB-Agar Plates
- LB media
- Antibiotics (as appropriate)

1. Prepare a 100 mM stock solution of 4-thiouracil (MW = 128.15) and 4-thiouridine (MW = 260.27) in ddH2O. Although it is also possible to aliquot and store at -20°C, it is best to prepare fresh whenever possible.
2. Grow target cells on separate LB plates with appropriate antibiotic (e.g., Salmonella against Tetracycline) at 37°C overnight. After 16-20 h choose a single colony from each plate and inoculate a 5 mL LB culture and let it grow overnight. From this pre-culture, take 10 µL and inoculate a fresh culture of 10 mL LB and antibiotic. Allow these to grow for approximately 2-2.5 hr to desired OD (2 hr for cultures in baffled flasks; 2.5 hrs for those in 50 ml conical Falcons). If working with Salmonella, ensure that only plastic materials and containers are used in order to minimize the risk of accidental exposure.
3. At mid-log phase (approx. OD 0.5-0.6) introduce the following into the cultures: 1, nothing (negative control); 2, 4-thiouracil; 3, 4-thiouradine. The final concentration of 4-thiouracil and 4-thiouridine should be 5 mM. If using the above stock solutions, this should require 1 mL.
4. Allow for the cells to grow for the necessary time period (e.g., 10 min).
5. Prepare for RNA isolation via RNA Snap.

3.5 Biotinylation Assay

3.5.1 Biotinylation Assay

Needed Materials:

- RNAse free 10x Biotinylation Buffer (BB)
  - 100 mM Tris pH 7.4
  - 10 mM EDTA
  - *store in aliquots of 1 – 1.5 ml at 4°C*
- RNAse free water
- Biotin-HPDP (Pierce, 50mg EZ-Link Biotin-HPDP, Cat. Nr. 21341)
  - Stock concentration: 1 mg/ml dissolved in Dimethylformamide (DMF)
- Chloroform/Isoamylacohol (24:1)
- Phase Lock Gel Heavy Tubes

Labelling Reaction (possible 30 – 100 µg total RNA; 50 µg total RNA MAX per reaction if incorporation is high):

2 µl Biotin-HPDP (1mg / ml DMF) per 1 µg RNA
1 µl 10x Biotinylation Buffer per 1 µg RNA
7 µl RNAse free H2O per 1 µg RNA

Example Reaction

110 µl RNAse free H2O
30 µl 10x Biotinylation Buffer
50 µg/µl 4-sU RNA (if at a concentration of 1 µg/µl)
100 µl Biotin-HPDP (1mg / ml DMF)

1. Incubate at room temperature for 3 h with rotation in the dark (allowing the reaction to proceed for 3 h rather than the traditional 1.5 h appears to improve RNA biotinylation; raising the temperature to 60°C may also increase the efficiency of biotinylation by denaturing the RNA and providing improved access to the 4sU).
2. Add an equal volume of Chloroform/Isoamylacohol (24:1).
3. Mix vigorously. Incubate for 2 – 3 minutes until phases begin to separate and bubbles start to disappear.
4. Centrifuge at full speed (20,000 g) for 5 min.
5. Carefully transfer upper phase into new tubes.
6. Repeat steps 2-5 (This step can also be done using phase lock gel heavytubes to avoid loss of material, however biotinylated volume should not exceed 1 mL).

Comments:

The chloroform extraction is required to remove unincorporated biotin-HPDP. To reduce the loss due to the extraction procedure the initial volume should be at least 500 µl. Smaller volumes should be increased by the addition of water.

To further reduce RNA loss we perform the second chloroform extraction using Phase Lock Gel Heavy tubes (2.0 ml, Eppendorf) following the manufacturer's instructions.

In principle, a single chloroform extraction step is enough to remove virtually all unincorporated Biotin-HPDP. Still, we initially perform two rounds to ensure complete removal. Usually we only use the phase-lock tubes for the second round as 1 ml biotinylation volume is too much for these tubes.
After the initial chloroform extraction only about 80% of the volume remains as the DMF is also removed.

Precipitation of RNA

At this phase, the sample will contain both labelled and unlabelled RNA. You can choose either ethanol or isopropanol precipitation. Though ethanol precipitation does require more time, yield is typically higher and loss of sRNA is less likely.

Isopropanol Precipitation Needed Materials:

- 5 M NaCl
- Isopropanol
- 75% Ethanol
- 1xTE

1. Add 1/10 the reaction volume of 5 M NaCl
2. Add 1.1 volume of isopropanoland centrifuge at 20,000 g for 20 min.
3. Remove supernatant.
4. Add an equal volume of 75% ethanol
   Note: *For precipitation of RNAs that are 20-100 nucleotides long, use 3 vol of 95% ethanol in place of isopropanol. For RNAs that are 20-50 nucleotides long, do not wash the pellet in 75% ethanol at any step, as smaller RNAs are soluble in 75% ethanol and will be lost in the wash step.*
5. Centrifuge at 20,000 g for 10 min.
6. Resuspend RNA in 100 µl 1x TE

Sodium acetate/ethanol precipitation

Needed Materials:

- M Sodium Acetate, pH 5.2
- 100%Ethanol
- 75% Ethanol
- Water

1. Add 1/10 of 3 M Sodium Acetate, pH 5.2 and mix by pipetting.
2. Add three volumes of 100% Ethanol and mix the sample by vortexing briefly.
3. Incubate the sample for at least 60 min at -80°C to precipitate the RNA.
4. Pellet the RNA by centrifuging the sample for 30 min at 16,000 g at 4°C.
5. Carefully remove the supernatant with a pipette.
6. Wash the pellet with 250 µl of 75% Ethanol and centrifuge at 8,000 g for 5 min at 4°C.
7. Carefully remove the supernatant with a pipette.
8. Allow the pellets to dry and evaporate any remaining ethanol by placing the Eppendorf under the laminar flow fume hood for 15 min.

9. Resuspend the RNA in an appropriate volume of water, approximately 50 µl after biotin processing.

3.5.2. Dot Blot Assay

Dot blot analysis on biotinylated RNA samples was carried out to detect and quantify the amount of RNA-bound biotin residues within a given RNA sample.

Needed Materials

- Hybond N+ Membrane
- Streptavidin-HRP
- Detection Solutions 1 and 2
- SaranWrap
- Autoradiography Film
- Phosphate-Buffered Saline (PBS) pH 7.5
  - 11.5 g di-sodium hydrogen orthophosphate anhydrous (80 mM)
  - 2.96 g sodium dihydrogen orthophosphate (20 mM)
  - 5.84 sodium chloride (100 mM)
  - Dissolve in 800 mL of distilled water – dilute to final volume of 1L. Check pH.
- PBS/0.1% Tween-20 (PBS-T)
  - Add Tween-20 to PBS to give a concentration of 0.1% Tweem-20, e.g., 1 mL of Tween-20 added to 999 mL PBS.
  - *PBS-T should be stable for at least 3 months at room temperature, although storage in a refrigerator (2-8°C) may be necessary to avoid microbial growth. Sodium azide should not be used as a bacteriocide.*
- Blocking Solution (5% blocking agent/PBS-T)
  - *Blocking solution should be prepared fresh. Dissolve 2.5 g of blocking agent in 35 mL of PBS-T. Make the volume up to 50 mL with PBS-T.*

1. Cut a piece of Hybond N+ membrane according to the needed dimensions. Apply to the dry membrane 1µL of the biotinylated RNA sample. If possible, use a control biotinylated nucleic acidof known concentration to create a gradient standard. A good start would be to do 100 mM, 50 mM, 25 mM, 10 mM concentrations of the control and of the RNA sample. From the comparison, it will be possible to assess approximate efficiency.
2. Allow membrane to dry. Wait approximately 20 min.
3. Irradiate the sample for 2 min. at 120,000 J/cm2to UV crosslink the biotinylated RNA to the membrane.
   When using the UV box upstairs, make sure the screen is on the TIME setting as this will insure that it will run for the full 2 min as opposed to 30 sec, which may occur when on other settings.
4. Block the membrane. Non-specific binding sites are blocked by immersing the membrane in 5% blocking agent (non-fat dried milk)/PBS-T (Blocking Solution) for 1 h at room temperature on an orbital shaker.
5. Wash the membrane with PBS-0.1% Tween 20 (PBS-T). Briefly rinse the membrane twice with PBS-Tthen wash 3 times in PBS-T, once for 15 min

and twice for 5 min at room temperature with fresh changes of wash buffer on an orbital shaker.

6. Dilute the streptavidin-HRPconjugate 1/1000 in PBS-Tand incubate the membrane for 1 h at room temperature on an orbital shaker.
7. Briefly rinse the membrane twice with PBS-T then wash 3 times in PBS-T, once for 15 min and twice for 5 min at room temperature with fresh changes of wash buffer on an orbital shaker.
8. Mix an equal volume of detection solution 1 and detection solution 2 allowing sufficient total volume to cover the membrane. The final volume required is 0.125 ml/cm^2 membrane.
9. Drain the excess wash buffer from the washed membranes and place them, sample side up, on a sheet of SaranWrap. Pipette the mixed detection reagent on to the membrane. Incubate for 1 min at room temperature. Drain off excess detection reagent by the membrane gently with forceps and touching the edge against a tissue. Place the blots sample side down on to a fresh piece of SaranWrap, wrap up the blots and gently smooth out any air bubbles.
10. Place the wrapped blots, sample side up, in an X-ray film cassette.
11. Place a sheet of autoradiography film (e.g., Hyperfilm ECL) on top of the membrane. Close the cassette and expose for 15 sec.
12. Remove the film and replace with a second sheet of unexposed film. Develop the first piece of film immediately, and on the basis of its appearance estimate how long to continue the exposure of the second piece of film. Second exposures can vary from 1 min to 1 h.

3.5.3 Separation of labelled and unlabeled RNA using Streptavidin-coated magnetic beads

Needed Materials:

- Washing Buffer (50 ml):
  - 100 mM Tris pH 7.5
  - 10 mM EDTA
  - 1 M NaCl
  - 0.1% Tween20
  - Heat Washing Buffer to 65°C.
- Elution Buffer (always prepare fresh):
  - 100 mM Dithiothreitol (DTT) in RNAse free H2O
- Either Buffer RLT (Qiagen) or Binding Solution (Norgen)

Protocol:

1. Heat biotinylated RNA samples to 65°C for 10 min and immediately place on ice for 5 min.
2. Add up to 50 µg (max. 100 µl)(could go up to 100 µg biotinylated RNA, but you run the risk of overloading the column and not having complete binding/elution of sample) of biotinylated RNA to 100µl of streptavidin beads.
3. Incubate with rotation for 15 min.

4. Place µMacs columns into magnetic stand. Do not process more than 12 samples at a time (6-8 samples are optimal).
5. Add 0.9 ml of Washing Buffer to columns (pre-run and equilibrate)
   Note: *To initiate the flow through the column you can gently press on the top of the column with your finger. Once the flow through the column has started it drains rapidly.*
6. Apply beads (RNA) to the columns. Discard the flow-through.
7. Wash 3x with 0.9 ml 65°C Washing Buffer (pipet tips shrink when pipetting buffers at 65°C)
8. Wash 3x with 0.9 ml room temperature Washing Buffer
9. Pipet 700 µl Buffer RLT (RNeasy MinElute Cleanup Kit, Qiagen) or 500 µL Binding Solution (RNA Clean-Up and Concentration Micro Kit, Norgen) into new 2 ml Eppis.
10. Elute RNA directly into Buffer RLT/Binding Solution by placing the Eppis underneath the columns and adding 100 µl Elution Buffer (100 mM DTT) to the columns.
11. Perform a second elution round into the same tubes 3 min later.

3.5.4 Recovery of newly transcribed RNA

Continue with the RNease MinElute Cleanup Protocol (Qiagen)/ RNA Clean-Up and Concentration Micro Kit (Norgen) following the manufacturer's instructions (shown below – Norgen Protocol follows after).

Qiagen Protocol

1. Add 500 µl 96-100% ethanol to the diluted RNA and mix thoroughly by pipetting. Do not centrifuge.
2. Apply 700 µl of the sample to an RNAeasy MinElute Spin Column in a 2 ml collection tube. Close the tube gently and centrifuge for 15 s at >8000 g. Discard the flow-through.
3. Apply the remaining 700 µl and repeat the centrifugation. Discard the flow-through.
4. Transfer the spin column into a new 2 ml collection tube.
5. Pipet 500 µl Buffer RPE onto the spin column. Close the tube gently and centrifuge for 15 s at >8000 g to wash the column. Discard the flow-through.
6. Add 500 µl of 80% ethanol to the spin column. Close the tube gently and centrifuge for 2 min at >8000 g to dry the silica-gel membrane. Discard the flow-through and collection tube.
7. Transfer the spin column into a new 2 ml collection tube. Open the cap of the spin column and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.
8. To elute, transfer the spin column to a new 1.5 ml collection tube. Pipet 20 µl RNase free-water directly onto the center of the silica-gel membrane. Close the tube gently and centrifuge for 1 min at maximum speed to elute.

Norgen Protocol

1. Add one volume of 70% ethanol equal to the volume of sample following elution from the previous process. Mix by vortexing for 10 sec.
2. Assemble a column with one of the provided collection tubes.
3. Apply up to 600 µL of the RNA mixed with the ethanol onto the column and centrifuge for 1 min.
4. Discard the flow-through. Reassemble the spin column with its collection tube. Repeat as necessary until all sample has been passed through the column.
5. Apply 400 µL of Wash Solution to the column and centrifuge for 1 min. Ensure that all of the Wash Solution has passed through the column; otherwise spin for an additional min.
6. Discard the flow-through. Reassemble the spin column with its collection tube.
7. Repeat steps 5-6 for a total of 3 washes.
8. Spin the column for 2 minutes in order to thoroughly dry the resin. Discard the collection tube.
9. Place the column into a fresh 1.7 mL elution tube provided with the kit.
10. Add 50 µL of RNA Elution Solution to the column (for a higher concentration sample, the elution volume may be reduced, but should be at least 20 µL)
11. Centrifuge for 2 min at 200 g, followed by 1 min at 14,000 g. If the entire volume has not passed through the column, centrifuge once more at 13,000 g for an additional minute.

The RNA is now ready for further analysis.

3.5.5 Recovery of unlabeled, unbound RNA

In case the unbound RNA needs to be recovered, collect the flow-through and the first wash for subsequent precipitation (together these contain >90% of the unbound RNA). Combine the two fractions and recover the unbound RNA by isopropanol/ethanol precipitation as performed after the biotinylation reaction (no salt need to be added as the washing buffer already contains 1 M NaCl).

Comments:

Small differences in sample volume do not matter when adding the biotinylated RNA to 100 µl beads. In case RNA input concentrations vary by >2-fold simply add the required volume of 1x TE to the beads to equalize conditions.

Like many column based assays collection of newly transcribed RNA, using the RNAeasy minelute kit may leave something in the final sample which absorbs at 230-260 nm and may interfere with OD260 measurements. You

therefore cannot trust OD measurements at all if OD260/280 ratios are <1.7!!!

Recovery of newly transcribed RNA is highly quantitative. If you started with the same RNA concentration you can expect the same amounts of newly transcribed RNA. For down-stream analysis (microarrays) it is usually best to decide on one RNA concentration to be used for all samples.

In case you want to quantify 4sU incorporation you have to precipitate the eluted RNA with isopropanol/ethanol adding 20 - 40 µg glycogen. In this case you have to add 10% 5M NaCl as the eluate does not contain high salt concentrations. Quantifying 4sU concentration for RNA prepared with the RNeasy kit doesnot work!!!

3.6 Denaturing Gel

There are three solutions from SequaGel (Natinal Diagnostics):

1. Diluent
2. Concentrate
3. Buffer

- Mix all above solutions aiming for the desired gel concentration according to instruction on the leaflet
- add APS
- add TEMED
- pour the gel

1 mm gels require:

- ~9ml of final solution for a small gel,
- ~40ml of final solution for a medium gel
- ~70ml of final solution for a large gel

adjust volumes for different gel thicknesses!

3.7 Total RNA isolation from bacteria cultures – TRIZOL method

1. Mix 5 ODs of cultures with Stop solution (95% ethanol, 5% phenol) and immediately flash freeze in liquid nitrogen.
2. Thaw samples on ice, spin in a centrifuge (4°C, 11000 g, 20 minutes) and gently resuspend in 1 ml of TRIZOL (Invitrogen).
3. Transfer the samples to 2 ml heavy Phase lock tubes (5 Prime), mixe with 400 µl of chloroform and incubate at room temperature for 5 minutes. Spin down for 15 minutes at 20°C, precipitate the aqueous phase separated during centrifugation with isopropanol.
4. Resuspend the pellets in 17 µl of RNase free water and remove DNA by incubation with DNase I according to manufacturer's instructions.

Remove the enzyme by PCI extraction and precipitate RNA with ethanol in the presence of ammonium acetate (30:1).