The Coller Lab Protocol Book

Revised Thursday, April 22, 2021

Table of Contents

SOLUTIONS (GEL MIXES)

Acrylamide is used in the lab to make special types of electrophoretic gels which we can use to separate very small pieces of nucleic acids. Acrylamide will polymerize when combined with APS (Ammonium Persulfate) and another solution called TEMED. We add these two catalysts to acrylamide and pour the solution between two glass plates (gel plates). When the acrylamide polymerizes, if forms a gel with very small pores through which nucleic acids can travel. These are referred to as polyacrylamide gels or PAGE (polyacrylamide gel electrophoresis).

WARNING: Polyacrylamide is a neurotoxin when liquid, harmless when polymerized. Always wear gloves and avoid contact with skin. Safety glasses should also be worn.

20% Acrylamide Solution

	500 mL
10 X TBE	50 mL
Urea	210 g
Acryl/Bis solution	250 mL
dH_2O	35 mL

Mix until dissolved
Do not autoclave
Wrap bottle in foil (photoreactive)
Store in cooler

6 % Acrylamide Solution

	1 L
20 % Acrylamide Solution	300 mL
Dilutor A	700 mL

Mix until dissolved
Do not autoclave
Wrap bottle in foil (photoreactive)
Store in cooler

##Heat the 20% acrylamide soln first to dissolve the contents

10 % Ammonium Persulfate (APS)

APS is used to assist in the polymerization of polyacrylamide. APS acts as a catalyst in this reaction.

	50 mL
Ammonium Persulfate	5 g

Dissolve in water and bring to volume
Aliquot 500 µL into eppendorf tubes.
Use a different color to distinguish from older batch if possible.
Store at 4˚C

Make a fresh batch every month!

Ampicillin (100 mg/mL) Stock Solution

Ampicillin is a common laboratory antibiotic used to select for growth of bacteria which contain plasmids which provide resistance to ampicillin's effects.

	50 mL	10 mL
Dry Ampicillin	5 g	1 g

Dissolve in dH_2O and dilute to final volume.
Make 0.5 mL aliquots in unsilanized eppendorf tubes. Use a different color to distinguish from older batch if possible.
Label with an 'A' on the lid.
Freeze

Do not autoclave, it will destroy ampicillin.

BSA (10 MG/ML) STOCK SOLUTION

BSA = Bovine Serum Albumin. BSA is generally used in the lab to provide a non-reactive protein into a reaction or an experiment.

	50 mL	10 mL
Dry BSA	0.5 g	0.1 g

Dissolve in dH_2O and dilute to final volume.
Make 1.0 mL aliquots in unsilanized eppendorf tubes. Use a different color to distinguish from older batch if possible.
Label with an 'B' on the lid.

Freeze

Do not autoclave.

1 M CALCIUM CHLORIDE

(MW 147.02)

For 1 liter:

Dissolve 147.02 g of $CaCl_2 \cdot 2H_2O$ in 800 mL of dd water.
Adjust volume to 1 liter.
Dispense into 100 mL bottles
Autoclave on *liquid cycle* for 20 mins.

Quantity needed:

It may be sufficient to make 500 mL at a time dependent on demand.

CHRUCH BUFFER (MODIFIED)

For 500 ml:

16.75 g $Na_2HPO_4 * 7H_2O$
500 ul 85% Phosphoric Acid
1 ml 0.5M EDTA pH 8.0

Heat to dissolve

Add 35 g SDS

Heat to dissolve

Add 5 g BSA

Heat to dissolve, either microwave for short bursts or stir and heat for several hours.

Store at 65º

CHURCH WASH (MODIFIED)

For 1 L:

11.17 g $Na_2HPO_4 * 7H_2O$
333 ul 85% Phosphoric Acid
2 ml 0.5M EDTA pH 8.0

Heat to Dissolve

Add 50 g SDS

Heat to Dissolve

Store at 65º

COMPETENT CELL PROTOCOL

Media/Glassware

2L very clean flask which has not been exposed to detergents.
500 ml very clean flask which has not been exposed to detergents.
Sterile eppendorf tubes.
Sterile 200 ul Pipets.
1L plastic sterilization filter bottle.

TYE plate (or minimal media plate)

DMSO

KCl (250mM):
For 100 ml, add 1.86g KCl to ddH2O
Autoclave

MgCl2 (2M):
For 100 ml, add 19 g MgCl2 to ddH2O
Autoclave

SOB: In a 2L flask:
237 ml ddH2O
5 g Bacto-tryptone
1.25 g bacto-yeast extract
0.125 g NaCl
Dissolve by shaking.
Add 2.5 ml KCl (250mM)
PH to 7.0 with NaOH (5M)~ approx. 50 ul
Adjust volume to 250 ml
Autoclave 20 min

Add 1.25 sterile MgCl2 (2M) to sterile flask just before inoculation.

TB: For 500 ml
1.51g H-Pipes
1.10g CaCl2
9.32g KCl
pH to 6.7 with KOH. Then add 5.44 g MnCl2. Raise volume to 500 ml and filter sterilize into 1L bottle.

Procedure:

Day 1: Streak out sells from a frozen stock onto a TYE plate. Grow O/N @ 37ºC (If competent cells will later be used to transform phage, cells should grow on minimal medium instead).

Day 2: Inoculate 250 ml SOB with 10-12 good sized colonies. Grow O/N at 18º with vigorous shaking. (There is a cold shaker in the basement of Biochem.)

Day 3: Cells should be removed from shaker when OD600 is approximately 0.6. Place flask on ice for 10 min. Transfer culture to the 500ml centrifuge pot and spin 10 min., 4ºC at 2500 x g. Pour off supernatant. Resuspend pellet in 80 ml ICE-COLD TB. Incubate suspension on ice for 10 min. Spin suspension 10 min., 4ºC at 2500 x g. Pour off supernatant. Gently resuspend pellet in 20 ml ICE-COLD TB. Add DMSO to a final concentration of 7 %. Incubate suspension on ice for 10 min. Aliquot 200 ul cells into sterile eppendorf tubes. Quick freeze in liquid nitrogen. Store at –80ºC.

DENHARDT'S SOLUTION (100X)

	500 mL
Ficoll	10 g
polyvinylpyrrolidone	10 g
BSA	10 g

Dissolve in 300 mL of water, bring up to volume to 500 mL. Filter and dispense into 50 mL aliqouts. Store at -20ºC

DEPC Treated Water (for RNA work)

DEPC will destroy any RNAase present in a solution when autoclaved in its presence, therefore we use DepC water for any manipulations of RNA to prevent degradation.

WEAR GLOVES WHEN MAKING DEPC WATER (Two reasons)

1] DEPC water is used for RNA work and must be RNAase free
2] DEPC is a suspect carcinogen and contact with skin should be avoided

Protocol:

Treat 1 liter of double distilled water with 10 mL of 10% DEPC solution (in the refrigerator in the undergrad room) and stirr overnight.

The DEPC, which can be harmful to chemical reactions and modify RNA must then be destroyed by autoclaving the solution (**in 100 or 500 mL bottles: which have previously been baked such that they are RNAase free) <u>twice</u>** for thirty minutes. After autoclaving the water should no longer smell of DEPC. (DEPC smells like Juicy Fruit gum)

Quantity needed:

The lab should be stocked with DEPC water on a regular basis. We should at least have ten bottles of depc water at all times.

Special Note

Due to the extremely sensitive nature of RNA, please, please ensure that:
1. At no time in the procedure should the DEPC water touch any glassware which is not baked. 2. Nor should it touch any plastic ware with the exception of disposable plastic pipettes.
3. Do not leave the DEPC water sitting uncovered on the bench where it could become contaminated.

<u>Orange capped bottles</u>

Orange capped bottles will only be used for DepC water. The lids will be left on them when they are placed on the wash trolley. Please store them and wash then in batches separate from all other glass ware. This is so proteins or RNAses potentially present on other glassware do not contaminate these bottles. The best way to prevent RNAse contamination is to have no RNAses present rather than to attempt to "kill" the RNAses by baking. Once they are washed they should be baked (and the lids autoclaved) and stored in the assigned cupboard.

10% DEPC SOLUTION

100% DEPC is supplied in 50 ml bottles. Add one of these bottles to 450 ml of ethanol in the fume hood, mix throughly and store at 4 degrees in a 1 litre bottle. Leave the residual contents of the 50 ml bottle to evaporate in the fume hood before discarding the bottle.

DILUTOR A SOLUTION

Dilutor A is a convenient solution used to dilute high concentrations of acryamide for running lower percentage gel.

	500 mL	1 L
5 X TBE	100 mL	200 mL
Urea	210 g	420 g
dH_2O	235 mL	470 mL

Mix until dissolved
Don't autoclave
Wrap in foil
Store in cooler

DOUBLE DISTILLED H_2O (STERILE)

Double distilled water (dd H_20) should be collected in the carboys from the Rez lab next door. The distillation process removes impurities such as salts from the water, IT DOES NOT sterilise the water. In order to sterilse it it must be autoclaved as described below:

Decant this water into 100 mL and 500 mL bottles and label them.
Autoclave for 20 mins on *liquid cycle*.

Quantity needed:

10 bottles, 100 mL each should be sufficient to last the lab about four days. Round bottles preferred to the square bottles.

0.5 M EDTA pH 8 (ethylene diamine tetra-acetate)

MW 292.25g

EDTA is a chelator of divalent cations, which are necessary for many biological reactions. Therefore it can be used to stop enzymatic reactions from occurring, since they usually require these cations.

To make 1 liter:

1] Weigh out 146.13 g of EDTA,
2] Add 800 mL dd water.
3] Stir vigorously and then add approximately 20g (not more) of sodium hydroxide pellets.
4] Stir for a further 10 mins.
5] Adjust the pH to 8.0 with more pellets or using a 10N sodium hydroxide solution. The EDTA will not go into solution until almost pH 8.
6] Once the EDTA solution has become clear check the pH once more.
7] Adjust the volume to 1 liter.
8] Dispense into 100 mL bottles and autoclave on liquid cycle for 20 min.

Quantity needed:
It may be sufficient to make 500 mL at a time, dependent on demand.

1M Glucose

Glucose is also know as Dextrose, and D-Glucose.

To make 500 mL

Add 90.08 g of Anhydrous Dextrose
Dissolve in dH_2O
Dilute to final volume with dH_2O
Filter Sterilize

IPTG Stock Solution (0.12 g/mL)

IPTG is used to induce RNA production from the lac operator bacterial promoter

	25 mL	50 mL
IPTG	3 g	6 g

Dissolve in water and bring to volume
Filter sterilize
Aliquot 1 mL into unsilanized eppendorf tubes. Use a different color to distinguish from older batch if possible.
Label with an 'I' on the lid.

LB Media

LB is a nutrient rich media used to grow *E. coli*. We generally use LB to grow bacteria which contain a plasmid so we can make large quantities of DNA stocks for other applications.

	1L	500 mL
Tryptone	10 g	5 g
Yeast Extract	5 g	2.5 g
NaCl	5 g	2.5 g

Autoclave

Add 1 µL / 1 mL AMP (100 mg/ mL)

LB Media Plates

LB is a nutrient rich media used to grow *E. coli*. We generally use LB to grow bacteria which contain a plasmid so we can make large quantities of DNA stocks for other applications.

	1L	500 mL
Tryptone	10 g	5 g
Yeast Extract	5 g	2.5 g
NaCl	5 g	2.5 g
Bacto-Agar	20 g	10 g

Autoclave

Add 25 µL AMP (100 mg/ mL) / plate

M9 MEDIA

M9 media is used for growing E. coli cultures. M9 media minus casamino acids is used to grow E. coli when we want to label newly synthesized proteins with radio-label so we can monitor them. The radio-label we commonly use is the amino acid methionine (^{35}met) therefore we don't want any non-radiolabelled (or cold) methionine (which is a constituent of casamino acids) present.

For 1 liter: 100 mL 10x M9 salts
1 mL 1M $CaCl_2$
1 mL 0.1 M magnesium sulfate

Add dd water to 850 mL.

Dissolve 8g glucose in 50 mL of dd water then add this dropwise, stirring vigorously.

Increase the volume to 950 mL and autoclave.

Add 50 mL of 200g /liter casamino acids after autoclaving (for *M9 media minus casamino acids* omit this step and increase the volume to 1 liter before autoclaving).

M9 MINIMAL MEDIA PLATES

For 500 ml:

Add 10 g Difco Agar to 425 ml ddH_20

Autoclave 12 min.
Cool 15 min in water bath.

Add 50 ml 10xM9 salts
Add 5 ml 40 % Glucose

Add requested additives:

+Amp	300 ul for 500 ml
-L	2.5 ml Trp, 10 ml Ura
-U	2.5 ml Trp, 2.5 ml Leu
-T	2.5 ml Leu, 10 ml Ura

Stir on stir plate.

Pour plates as usual (500 ml makes 1 sleeve).

M9 PLATES

M9 media is used for growing E. coli cultures. M9 media minus casamino acids is used to grow E. coli when we want to label newly synthesized proteins with radiolabel so we can monitor them. The radiolabel we commonly use is the amino acid methionine (^{35}met) therefore we don't want any non-radiolabelled (or cold) methionine (which is a constituent of casamino acids) present.

For 1 liter: 100 mL 10x M9 salts
50 mL of 200g /liter casamino acids *
8 g glucose #
1 mL 1M $CaCl_2$
1 mL 0.1 M magnesium sulfate
20 g Agar

Add dd water to 850 mL.

Dissolve glucose in 50 mL of dd water then add this dropwise after the volume has been increased to 850 mL, stirring vigorously.

Increase the volume to 950 mL and autoclave.

* Add this after autoclaving, for M9 media minus casamino acids omit this step and increase the volume to 1 liter before autoclaving.

10 X M9 SALTS

M9 salts are a constituent of M9 media: see page 13

For 1 liter:
60g Na_2HPO_4 anhydrous (Sodium Phosphate anhydrous, MW: 141.96)
30g KH_2PO_4 anhydrous (Potassium Phosphate monobasic, MW: 136.09)
5g NaCl (sodium chloride, MW: 58.44)
10g NH_4Cl (ammonium chloride, MW: 53.49)

Dissolve in 900 mL of dd water and then adjust the volume to 1 liter.
Autoclave on *liquid cycle* for 20 mins.

1M MAGNESIUM CHLORIDE

(MW 203.3g)

For 1 liter:

Dissolve 203.3g of $MgCl_2 \cdot 6H_20$ in 800 mL of dd water.
Adjust volume to 1 liter.
Dispense into 100 mL bottles
Autoclave on *liquid cycle* for 20 mins.

Quantity needed:
It may be sufficient to make 500 mL at a time dependent on demand.

1M $MgCL_2$ (MAGNESIUM CHLORIDE), 1M $MgSO_4$ (MAGNESIUM SULFATE)

For 500 mL: 101.65g $MgCl_2 \cdot 6H_20$ (MW 203.30)
123.23g $MgS0_4 \cdot 7H_20$ (MW 246.47)

Should be dissolved in 450 mL dd water.
The volume should then be adjusted to 500 mL,
dispensed in 100 mL aliquots
Autoclaved for 20 min. on *liquid cycle*.

NEUTRALIZATION SOLUTION

For 500 ml:

147.2 g Potassium Acetate
57.5 ml Glacial Acetic Acid

No sterilization required.

10x MMR

To make 1 litre of 10 x MMR:

Dissolve 58.44g sodium chloride (MW: 58.44g)
1.49g pottasium chloride (MW: 74.56g)
11.92g HEPES (MW: 238.31g)

in 900 ml of dd water. Adjust the pH to 7.4 using 10M NaOH. Incresae the volume to 1 litre and filter sterilise.

This solution is only to be made on demand.

We use 1 x MMR (100 mM NaCL, 2 mM KCl, 5 mM HEPES) to bathe our frog oocytes in once they are removed from the frog. Oocytes can be used for micro-injection experiments or matured (to form an egg which is ready to undergo fertilisation) for about 24 hours after surgery when they are maintained in this buffer.

20x MOPS

For 1 Liter:

Dissolve in 500 ml H_2O

83.72 g MOPS
8.20 g Sodium Acetate
7.44 g EDTA

Adjust pH to 7.0 with 10M NaOH
Adjust to 1L
Autoclave (will turn yellow after being autoclaved).

PEN-STREP 1000 X STOCK SOLUTION

Pen-Strep = Penicillin-Streptomycin. These two antibiotics are used in our buffers we store frog oocytes in. The antibiotic keeps fungi, and bacteria from growing on the oocytes, thus keeping the oocytes relatively healthy.

To make add the following to dH_2O

10 mg/mL penicillin
10 mg/mL streptomycin

Filter sterilize and store in 1 mL aliquots at -20˚C. in unsilanized eppendorf tubes. Use a different color to distinguish from older batch if possible. Label with an ‘S’ on the lid.

PHOSPHATE BUFFER

Part 1:

Place 2160 ml ddH_2O in a 4 L Flask
Add 340.23 g KH_2PO_4

Part 2:

Place 620 ml ddH_2O in a 4 L Beaker
Add 130.64 K_2PO_4

Part 3:

Add Solution 1 to Solution 2 until pH 6 is reached.
Store in carboy
Dispense into 75 ml bottles for NGM pouring.

Make only on demand!!

1x PBS (Phosphate-Buffered Saline)

Dissolve in 800 ml ddH_20:

8.0 g NaCl
0.2 g KCl
1.44 g Na_2HPO_4
0.24 g KH_2PO_4

pH to 7.4 with HCl or NaOH
adjust volume to 1L with water and autoclave in 500 ml bottles.

10x PBS

Dissolve in 800 ml ddH_2O:

80 g NaCl
2.0 g KCl
14.4 g Na_2HPO_4
2.4 g KH_2PO_4

pH to 7.4 with HCl or NaOH
Adjust volume to 1 L with water and autoclave in 500 ml bottles.

PIPET CLEANING PROTOCOL

Glass pipets need to be cleaned very carefully to ensure that they are exceptionally free of all contaminants.

Dirty pipets are placed in bleach baths around the lab. They need to be emptied two - three times a week, pipets cleaned, and the water changed (water only needs to be changed if cloudy).

Protocol for Pipet Baths:

1] Fill each bath 3 inches above the top of pipets
2] Put approximately 2 cap fulls of bleach in each bath

To clean pipets we soak them in Sulfuric Acid with Nochromix added. The acid bath ensures that all contaminants are removed from the pipets, this includes, RNases.

Protocol for Acid Bath

You must wear your lab coat, thick rubber gloves, and safety glasses when washing pipets after this point. Sulfuric acid will burn your cloths as well as your skin!!!!

1] Add 1 packet of Nochromix to an entire jug (2.5 L) of Sulfuric Acid (H_2SO_4)
2] Pour the Sulfuric Acid/Nochromix solution into the acid bath until full

Make sure acid covers the pipets completely.

Washing Protocol:

1] Remove pipets from bleach baths and put into the washing container. Separate the 25 ml and 10 ml pipets into separate batches. Place 25 ml tip down and 10 ml tip up.
2] Rinse with distilled water in pipet washer in undergrad room for 15 mins
3] Drain
4] Lift out and put in acid bath in dish washing room down by dishwasher.
5] Soak in acid bath overnight. Flip 25 ml Pipets tip up so they drain well and soak over night again.
6] Drain well and be careful not to drip acid around the sink
7] Rinse any spilled acid down the drain.
8] Rinse pipets in the pipet cleaners in dish washing room by setting timers at 1 hour with tap H_2O then 1 hour with dH_2O. Only set timers, do not touch faucet
9] Put pipets into drying oven for 40 mins at setting 3.

Warning: Do not use a higher setting, as it will melt the plastic pipet holders

10] Separate pipets into 1, 10, and 25 mL and also in blow out vs. non-blow out
11] Put into metal canisters
12] Bake in oven at 400˚F for 4 hours.

1 M POTASSIUM CHLORIDE

(MW 74.56)

For 1 liter:
Dissolve 74.56g of KCl in 800 mL of dd water.
Adjust volume to 1 liter.
Dispense into 100 mL bottles
Autoclave on *liquid cycle* for 20 mins.

Quantity needed:
It may be sufficient to make 500 mL at a time dependent on demand.

PROTEINASE K (10 MG/ML)

Proteinase K is an enzyme which degrades proteins, and can be used to help separate DNA/RNA from proteins when purifying nucleic acids.

	10 mL
Proteinase K	100 mg

Dissolve in water and bring to volume
Aliquot 1 mL into eppendorf tubes. Use a different color to distinguish from older batch if possible. Label with an 'K' on the lid.
Incubate at 37˚C for 1 hour
Incubation allows proteinase K to digest any nucleases which might be in the solution.
Store at -20˚C

RNase A Stock Solution (10 mg/mL)

RNase A is normally the enemy in this lab (among many other RNases), however, many times we actually want to degrade RNA, such as when we wish to separate RNA from DNA, and then purify DNA. So we do need to have this around. You should always be conscious, however, that when you make RNaseA you are exposing the glassware and everything else you may touch while making RNaseA to an incredible concentration of RNases. It would be wise and appreciated if you made sure that you change your gloves after making RNaseA, so you don't spread it around the lab as you work.

	20 mL	50 mL
RNaseA	200 mg (0.2 g)	500 mg (0.5 g)
1 M Tris pH 7.5	200 µL	500 µL
NaCl	60 µL	150 µL

Make 1.0 mL aliquots in unsilanized eppendorf tubes. Use a different color to distinguish from older batch if possible. Label with an 'RN' on the lid. Heat to 100˚C in water bath for 15 mins (kills any contaminating DNases). Cool to room temperature. Store in -20˚C freezer in hot room

SILANIZED GLASS SLIDES

When we do micro-injections of materials into frog oocytes or eggs we draw very small volumes of concentrated materials up into the injection needle. To make it slightly easier to load the needle we normally silanise the glass slides onto which we pipette the material to be loaded. The silanising makes the drop of material bead nicely on the slide ie. it forms a concentrated drop rather than spreading out on the slide.

Protocol:

1. Dip slides into a large weighing boat containing 5% dimethly dichloro silane and 95% chloroform (bottle in the fume hood). This procedure should be performed in the fume hood to avoid fume inhalation. The slides should be dipped using forceps.

2. Dry the slide briefly by placing on a kimwipe. Make sure there are no spots, wipe slides with a kimwipe. If spots will not come off, wipe with ethanol on both sides until dry. Wrap two slides together in aluminium foil.

3. Place in a pyrex dish and bake at 400 degrees for 4 hours.

Quantity needed:

Silanise a box at a time.

Caution:
Work in the fume hood and wear gloves.

10% SDS (SODIUM DODECYL SULFATE)

(MW: 288.38)

SDS is a detergent which has many uses in molecular biology, such as lysing cells and denaturing protein

Percentage solutions are made weight/volume. i.e. a 10% solution contains 10g in 100 mL.

Weigh 50g and dissolve in 450 mL dd water.
It will be necessary to heat the solution to 68 degrees to assist dissolution.
Adjust the volume to 500 mL.
Filter sterilize, in 100 mL units, **do not autoclave.**

Wear a mask when weighing SDS it is harmful especially when inhaled. A lab coat and safety glasses will not hurt either.

SDS-PAGE RUNNING BUFFER (10X)

For 1 L:

30.3 g Tris Base
144.1 g Glycine
10.0 g SDS (Electro grade)

pH to 8.3 with HCl

Filter sterilize

SDS-PAGE SEPARATING BUFFER (4X)

This is used in combination with SDS-Page Stacking buffer for running gels and separating proteins.

For 250 mL
Dissolve in 250 mL water

Tris base	45.0g
SDS (electrophoresis grade)	1.0g

Adjust pH to 8.8 with Hcl (about 6 mL).
Filter sterilize and store at 4°C.
Quantity: 1

SDS-PAGE STACKING BUFFER (4X)

Used for running gels and separating proteins.

For 250mL

Dissolve in 250 mL water

Tris base	15.12g
SDA (electrophoresis grade)	1.0g

Adjust pH to 6.8 with HCl.
Filter sterilize and store at 4 C.

Quantity: 1

SDS-PAGE STAIN/DESTAIN

Stain

Create a solution that is:

- 0.25% Coomaissie Blue
- 50.0% MeOH
- 10.0% HOAc

Destain

Create a solution that is:

- 40.0% MeOH
- 10.0% HOAc

SDS GEL LOADING DYE (2X)

For 10 ml:

- 2.5 ml Stacking buffer (4X)
- 2.0 ml Glycerol
- 0.5 ml Bromophenol Blue (0.5%)
- 0.6 g SDS (electro grade)

20 x SSC (Sodium Chloride / Sodium Citrate)

For 8 Liters: 1402.4 g NaCl
705.6 g Sodium Citrate

Dissolve in 8 L of ddH_2O
No need to autoclave
May take time to get into solution

3M SODIUM ACETATE PH 5.2

(MW 82.03g)

For 500 mL:
Dissolve 123.05g of sodium acetate in 400 mL of dd water.
Adjust pH to 5.2 with glacial acetic acid.
Adjust volume to 500 mL.
Dispense into 100 mL bottles
Autoclave on *liquid cycle* for twenty minutes.

Quantity needed:

It may be sufficient to make 500 mL at a time dependent on demand.

5M SODIUM CHLORIDE

(MW 58.44g)

For 1 liter:
Dissolve 292.2g of NaCl in 950 mL of dd water.
Adjust volume to 1 liter.
Dispense into 100 mL bottles
Autoclave on *liquid cycle* for 20 mins.

Quantity needed:

It may be sufficient to make 500 mL at a time dependent on demand

For frogs:
Dispence NaCl into 1 liter plastic bottles, need not be sterile.
Place on shelf in upstairs frog room labled “5 M NaCl for Frogs”.

SOB MEDIA

SOBis used especially when performing transformations (making bacteria take up plasmid DNA). It is essentially the same as LB, except it contains KCl.

	500 mL
Tryptone	10 g
Yeast Extract	2.5 g
NaCl	0.292 g or 1 mL of 5 M NaCl
KCl	0.093 g or 1.25 mL of 1 M KCl

Dissolve reagents in dH_2O and dilute to final volume
Place 50 mL aliquots (be exact) into 100 mL glass bottles
Autoclave 20 mins on *liquid cycle*

For SOC media:

Add in sterile room:	50ml
1M MgCl2	0.5 ml
1 M MgSO4	0.5 ml
1M Glucose	1.0 ml

Be sure that all additives have been filter sterilized.

SOFT TOP AGAR

Soft Top Agar is used primarily to assay bacteriophage (bacterial viruses) grow. It is usually spread over the top of TYE plates.

	1 L	500L
Tryptone	10 g	5 g
Yeast Extract	5 g	2.5 g
NaCl	5 g	2.5g

Dissolve reagents in dH_2O and dilute to final volume
Place 100 mL aliquots into 100 mL glass bottles

Add 0.6 g Bacto-Agar to each bottle

Autoclave 20 mins on *liquid cycle*

25% SUCROSE, 50 MM TRIS PH 7.5

For 500 mL:
125 g sucrose (i.e. 25g per each 100 mL)
25 mL of a 1M stock solution of Tris pH 7.5 (i.e. 1M = 1000 mM, therefore we want to dilute this 1 in 20 to achieve a 50 mM solution)

Add 450 mL of dd water and stir until the sucrose is dissolved.
Adjust the volume to 500 mL with dd water
Dispense in 100 mL aliquots.
Autoclave on *liquid cycle* for 20 min.

1 M TRIS

(MW 121.1g)

Tris is one of the most commonly used buffers in molecular biology.

For 1 liter:

Dissolve 121.1g of Tris base (NOT TRIS-HCl) in 800 mL of dd water.
Adjust to the desired pH as follows by adding concentrated HCl.
pH 7.5: approx. 55 mL
pH 8.0: approx. 42 mL

The pH of Tris solutions is temperature dependent so ensure the solution is at room temperature before it is pHed, also ensure that the pH meter is set for room temperature.

Bring up the volume to 1 liter and make 100 mL aliquots.
Autoclave on *liquid cycle* for 20 mins.

Quantity needed:

500 mL may be sufficient at one time.

5 xTBE Buffer (Tris-Borate-EDTA Buffer)

TBE is used as a 1x or 0.5x stock as a buffer for running either agarose or polyacrylamide gels. We use agarose gels for separating fairly large pieces of DNA and polyacrylamide gels for separating smaller pieces of RNA or RNA/ protein complexes.

To make 1 liter: 54g Tris Base (Not Tris-HCl) MW 121.1
27.5g boric acid MW 61.83
20 mL 0.5 M EDTA pH 8.0

Autoclave in 1 liter bottles for 20 min on liquid cycle.

1 x TBE Buffer

	14L	8L	6L
Tris Base (Not Tris-HCl)	151.2g	86.4g	64.8g
Boric Acid	77 g	44g	33g
0.5M EDTA pH 8.0	56 ml	32 ml	24 ml

Make in carboy for the hot room. Do not sterilize.

10 X TBE

For 1 L:

108 g Tris
55 g Boric Acid
20 mL 0.5 M EDTA pH 8.0

Dilute to 1 L

Autoclave 20 min Liquid Cycle

10x TBS (Tris-Buffered Saline)

For 2 L:

48.4 g Tris Base
160.0 g NaCl

pH to 7.6 with HCL.
filter sterilize into 1L bottles.

Make 2L at a time.

TYE PLATES = TRYPTONE YEAST EXTRACT MEDIA

TYE plates are used in the lab to grow the gram negative bacteria *E. coli.* TYE plates are specifically used when growing bacteria for stocks.

	1L	500 mL
Tryptone	10 g	5 g
Yeast Extract	5 g	2.5 g
NaCl	5 g	2.5 g
Agar	15 g	7.5 g

Add to dH_2O, and dilute to final volume with water.
Autoclave 20 mins on *liquid cycle.*
Cool in a hot water bath, until cool enough to touch with thin gloves on, usually 50- 60˚C, usually takes 30 mins. Cooling the media prior to pouring reduces condensation on plates, as well as make it easier on the pourer.

Different additives are requested depending on their use, such as ampicillin to select only resistant bacteria. Common requests are TYE/ AMP and all XGAL requests are photolightsensitive (keep in the dark).

After cooling, add requested additives to media.

Chemical	1 L	500 mL
Ampicillin (100 mg/mL)	1 mL	0.5 mL
X-Gal (20 mg/mL)	2 mL	1 mL
IPTG	1 mL	0.5 mL
KAN (60 mg/ml)	1 mL	0.5 mL

500 mL is enough media for 1 sleeve of petri plates.
Pour plates in plate pouring room.

5X WESTERN TRANSFER BUFFER

For 5L:

362.5 g Glycine
72.5 g Tris Base

Prepare in a carboy and store in Hot Room.

X-GAL STOCK SOLUTION

Mix 0.4 g X-Gal in 20 ml DMF (In Hood).

Aliquot into 0.5 ml eppendorf tubes.

Store in –20º freezer door, wrapped in foil so no light can penetrate.

SD media Drop-Out Amino Acid Mix

Dropout mix is a combination of the following ingredient minus Uracil, Leucine, Histadine, and Tryptophan. It should be mixed very thoroughly by turning end-over-end for at least 15 mins: adding a couple of clean marbles helps.

Adenine	0.5 g
Alanine	2.0 g
Arginine	2.0 g
Asparagine	2.0 g
Aspartic Acid	2.0 g
Cysteine	2.0 g
Glutamine	2.0 g
Glutamic Acid	2.0 g
Glycine	2.0 g
Inositol	2.0 g
Isoleucine	2.0 g
Lysine	2.0 g
Methionine	2.0 g
para-Aminobenzoic acid	2.0 g
Phenylalanine	2.0 g
Proline	2.0 g
Serine	2.0 g
Threonine	2.0 g
Tyrosine	2.0 g
Valine	2.0 g

Add in various combinations the following

Leucine	10g
Uracil	2g
Histadine	2g
Tryptophan	2g

YEAST LIQUID MEDIA: SD, SG, OR SR MINIMAL MEDIA

	1L	500 ml
Bacto-Yeast Nitrogen Base w/o amino acid w/o ammonium sulfate	1.7 g	0.85 g
Ammonium Sulfate	5 g	2.5 g
Add one of the following depending on need: SD = Glucose (Dextrose) SG = Galactose SR = Raffinose	20 g	10 g
Dropout mix	2 g	1 g

pH to 6.5 with 10 N NaOH
Bring up in 950 mL or 450 mL water
Autoclave 15 mins

YEAST PLATES: SD, SG, OR SR MINIMAL MEDIA

	1L	500 ml
Bacto-Yeast Nitrogen Base w/o amino acid w/o ammonium sulfate	1.7 g	0.85 g
Ammonium Sulfate	5 g	2.5 g
Add one of the following depending on need: SD = Glucose (Dextrose) SG = Galactose SR = Raffinose	20 g	10 g
Bacto Agar	20 g	10 g
Dropout mix	2 g	1 g

pH to 6.5 with 10 N NaOH
Bring up in 950 mL or 450 mL water
Autoclave 15 mins

Pour

YPD Media = Yeast Peptone Dextrose Media

YPD is a rich nutrient media which is used to grow yeast. Care must be taken in making YPD, particularly when autoclaving. If autoclaved to long it will the sugar in the media will caramelize and turn a dark brown, thus making the yeasties grow slowly.

	500 mL
Peptone (Bacto-Peptone)	10 g
Dextrose (D-Glucose)	10 g
Yeast Extract	5 g

Dissolve all reagents in dH_2O, dilute with water to final volume.
Pour 50 mL into 500 mL flasks. The ratio of 50 mL culture to 500 mL flask (1:10 ratio) is important for proper airation (O_2) so the yeast can grow rapidly.
Autoclave 10 mins on *liquid cycle*.

Remove from autoclave immediately (when pressure is 0 psi). YPD media must be taken out of autoclave as soon as possible to avoid carmalization of Dextrose.

YPD Plates = Yeast Peptone Dextrose Plates

YPD is a rich nutrient media which is used to grow yeast. Care must be taken in making YPD, particularly when autoclaving. If autoclaved to long it will the sugar in the media will caramelize and turn a dark brown, thus making the yeasties beasties grow slowly.

	1L	500 mL
Peptone (Bacto-Peptone)	20 g	10 g
Dextrose (D-Glucose)	20 g	10 g
Yeast Extract	10 g	5 g
Agar	20 g	10 g

Add all reagents to dH_2O (Agar will not dissolve until autoclaved).
Dilute with water to final volume.
Autoclave 10 mins on *liquid cycle*.
Remove from autoclave immediately (when pressure is 0 psi).
YPD media must be taken out of autoclave as soon as possible to avoid carmalization of Dextrose.

(If *YPD +ADE* is requested, add 40 mg adenine per liter before pouring.)

Cool in a hot water bath, until cool enough to touch with thin gloves on, usually 50- 60˚C usually takes 30 mins. Cooling the media prior to pouring reduces condensation on plates, as well as make it easier on the pourer.

500 mL is enough media for 1 sleeve of petri plates.
Pour plates in plate pouring room.

SPORULATION MEDIA

Diploid cells will sporulate on this media after 18-24 hours without vegetative growth

	1L	500 mL
Postassium acetate	10 g	5 g
Drop-out mix	0.5 g	0.25 g

PREPARATION OF DNA MARKERS

Digest vector with restriction enzymes
Treat digested DNA with CIP
Kinase label with gamma ATP

pUC18 digested with HpaII

501
489
404
353
242
190
147
110
89
67
34
26

Lambda digested HindIII and EcoRI

21226
5144
4969
4264
3526
2023
1900
1705
1371
943
827
560

PREPARATION OF HEAT SHOCK COMPETENT CELLS (HB101)

1] Grow a 1mL overnight

2] Inoculate 1L culture and grow until OD_{550}= 0.5 (50million cells/mL)

3] Chill culture on ice 10 mins

4] Spin @ 5K RPM (Setting 6 on RT6000) for 5 mins.

5] Discard supernatant and gently resuspend cells in half volume of sterile, ice cold 50mM $CaCl_2$.

6] Place on ice for 15 min and spin as in 4]

7] Discard supernatant and Resuspend in 1/15 original vol. ice cold 50mM $CaCl_2$.

8] Aliquot into 200 µL fractions and freeze in liquid nitrogen prior to storage.

TRANSFORMATION OF FROZEN COMPETENT BACTERIA.

1.Thaw competent cells on ice. Do not allow temperature to rise over 4 degrees.
2. To 50ul of competent cells . Add 1 µl of plasmid DNA or ligation mix (1ng - 2ng).
3. Incubate for 30 min on ice.
4. Heat shock the cells for 45 sec's at 42 degrees
5. Incubate 2 min on ice.
6. Add 450 µl of L.B
7. Incubate 60 min at 37 degrees.
8. Plate 30 µl and 2 x 100 µl on L-amp (or other appropriate antibiotic) plates.
9. Allow the LB containing the transformed cells to dry.
10. Place the plates upside down in a 37 degree incubator overnight

QUICK TRANSFORMATION

This will not work for freshly ligated DNA only for retransformation of plasmids and uses far more DNA than the conventional protocol outlined in B1.

1. Thaw competent cells on ice .
2. Add to 6 µl competent cells, 2 µl of plasmid DNA (100 ng-1 µg)
3. Place DNA/plasmid mix on ice for circa 2 mins.
4. Then incubate at 42 degrees for 2 mins.
5. Place breifly on ice.
6. Add 100 µl of L.B.
7. Plate on L-amp (or other appropriate antibiotic) plates.
8. Allow the LB containing the transformed cells to dry.
9. Place the plates upside down in a 37 degree incubator overnight

RESTRICTION DIGEST

To cut the DNA with the desired enzyme, set up the reaction in the following order:

Small Scale		Large Scale
dd water	15 µl	60 µl
10 x Buffer	2 µl	20 µl
DNA	1 µl	100 µl
Enzyme	2 µl	20 µl

The 10 x buffer will vary depending on the enzyme used and can be determined by consulting with the enzyme chart. The buffer (10x) will always compose a tenth of the reaction. The enzyme should never compose more than a tenth of the reaction (although less can be used), this is because the buffer in which the enzyme is stored contains glycerol, and large amounts of glycerol willl inhibit the reaction. The activity of the enzyme is given on the tube and refers to one unit as the amount of enzyme required to cut a particular DNA target in one hour. If you look at this and the amount you are using, you will see that we routinely use far more than is required.

Small-scale Isolation of Plasmid DNA from Bacteria (Mini-preps)

1. Inoculate 5 mL LB containing the appropriate antibiotic with a single bacterial colony (SCI). Incubate O/N on shaker/tube rotator at 37 °C.

2. Harvest cells either in culture tube or by transferring to an Eppendorf tube – centrifuge at 14,000 rpm for 1 min at RT.

3. Wash cells by resuspending in 1 mL of H_2O and harvest by centrifugation at 14,000 rpm for 1 min at RT. Remove water, leaving the bacterial pellet as dry as possible.

4. Resuspend cell pellet by vortexing in 100 µL of an ice cold solution of GET – incubate 5 min at RT.

GET Buffer

50 mM glucose
10 mM EDTA (pH 8.0)
25 mM Tris HCl (pH 8.0)

5. Add 200 µL of a **freshly** prepared solution of 0.2 N NaOH/ 1% SDS. Lyse cells by inverting the tube rapidly 2 or 3 times (do not vortex). Store on ice for 5 min.

NaOH/SDS Solution

0.9 mL 0.22 N NaOH
0.1 mL 10% SDS

6. Neutralize solution by adding 150 µL of an ice cold solution of 3 M potassium acetate (pH 4.8).

Neutralize Solution

60 mL	5 M potassium acetate
11.5 mL	glacial acetic acid
28.5 mL	water

Mix contents by flicking the tube vigorously. Store on ice 10 min.

7. Centrifuge sample for 5 min at 14,000 rpm at RT.

8. Transfer supernatant (400-450 µL) to a fresh tube. Add 4 µL of RNaseA (10mg/mL stock) incubate 30 minutes at 37C, and then add an equal volume of phenol/chloroform:isoamyl alcohol/TE (25:24:1; 10 mM Tris/1 mM EDTA-buffered phenol) and extract by vortexing for 30 sec. Centrifuge for 2 min at 14,000 rpm at RT.

9. Transfer supernatant to a fresh tube and precipitate DNA with 2X volumes of 95% EtOH. Mix by inverting several times and place at -20 °C for 30 min.

10. Collect precipitated DNA by centrifugation for 5 min at 14,000 rpm at RT.

11. Decant supernatant and wash pellet 2X with 500 µL 70% EtOH. Air dry pellet briefly or place in vacuum desiccator.

12. Resuspend DNA in 50-100 µL TE (pH 8.0) or water. Quantify spectrophotometrically.

Analysis of DNA on Agarose Gel

To analyse whether the DNA has been successfully cut, the reaction is run out on a agarose gel. Agarose is purified from seaweed and forms a molecular sieve through which the DNA can migrate when subject to an electric current.

Protocol:

To make a 1% agarose gel :

1. Weigh 1 g of agarose and place in a 250 ml flask.
2. Add 100 ml of 1 x TBE to the flask.
3. Melt the agarose by micro-waving on high power for 2 mins.
4. Check that the agarose is completely melted, if not microwave for a further 30 secs.
5. Allow melted agarose to cool for a minute and then add 5 µl of 10 mg/ml ethidium bromide. **Caution: Ethidium bromide is highly mutagenic, so be extremely careful not to get any on your skin. If you do pour bleach on it, to neutralise the ethidium and then wash throughly with lots of water.**
6. Seal the dams of the gel tray, by pipetting agarose along them and allowing to set.
7. Pour the gel, the gel should come between half way and two-thirds of the way up the teeth of the comb. These teeth will later form the wells. Remove any bubbles.
8. Allow the gel to set, this can be speeded up by placing the gel in the fridge.

To run the gel:

9. Remove the comb and dams carefully. And cover the gel in 1 x TBE.
10. Add 4 µl of DNA loading buffer, to the 20 µl digestion reaction (The buffer should consistute 1/6th of the total volume).
11. A DNA ladder, and a sample of uncut plasmid should also be prepared as follows

dd water	17 µl
plasmid or ladder	1 µl
Loading buffer	4 µl

12. Carefully load the samples into the wells and run the gel at 90 volts until the orange dye is close to the bottom of the gel. The electrodes should be attached such that the black (positive) electrode is at the end of the gel where the wells are located, and the red (negative) electrode is at the botom of the gel. This is because DNA is negatively charged and will run to the positive electrode.
13. Photograph the gel.

SDS-PAGE GEL ELECTOPHORESIS

Theory:

SDS-page gels consist of two layers, an upper stacking gel and a lower seperating gel. The stacking gel is of a lower pH than the seperating gel. As protein samples run through the stacking gel and hit the seperating gel, the samples become concentrated into a very small volume increasing the ability of the resolution of the gel.

First pour the seperating (bottom) gel

(For large gels, an approz. 2 cm space should be left between the bottom of the comb and seperating gel. For mini gels, a 1 cm space sould be left.)

	Mini Gel (10ml)	Large gel (50 ml)
Seperating buffer (4X)	2.5ml	12.5ml
ddH_2O	to 10 ml	to 50 ml
Acrylamide (30%, 29:1):		
15%	5.0 ml	25.0 ml
12%	4.0 ml	20.0 ml
10%	3.33 ml	16.67 ml
8%	2.67 ml	13.33 ml
7%	2.33 ml	11.67 ml

Degas samples at least 5'.

Add 10% APS and TEMED immediately before pouring

10% APS	50 ul	250 ul
TEMED	5 ul	25 ul

After pouring the seperating gel, overlay liquid gel with isobutanol. This will allow the polymerization reaction to occur more quickly as air inhibits the reaction. Polymerization will take about 30 min to an hour.

Pour the Stacking gel

(Before pouring, dump off iso-butanol and rinse top of gel with water.)

Stacking buffer (4X)	1.5 ml	6.0 ml
Acrylamide (30%, 29:1)	0.84 ml	3.36 ml
ddH2O	3.66 ml	14.64 ml

Degas samples at least 5'.

Add 10% APS and TEMED immediately before pouring

10% APS	18 ul	272 ul
TEMED	6 ul	24 ul

After pouring, place comb into gel and let polymerize (15 to 30 min.)

SARNOW TRANSLATIONAL EXTRACT BUFFERS

Sarnow 5X Buffer A — 1 L

150 mM Hepes (Free Acid)	35.74 g
500 mM Potassium Acetate	98.15 g
10 mM Magnesium Acetate	2.15 g

Mannitol Buffer A — 500 mL

1 X Buffer A	100 mL (5 X Buffer A)
8.5% Mannitol	42.5 g
2 mM DTT	2 mL (500 mM stock)

Mannitol Buffer A / PMSF — 100 mL

Mannitol Buffer A	100 mL
0.5 mM PMSF	8.71 mg

Buffer A / PMSF — 100 mL

1X Buffer A	20 mL (5 X Buffer A)
2 mM DTT	400 λ (500 mM stock)
0.5 mM PMSF	8.71 mg

6X Translation Buffer — 10 mL

132 mM Hepes	1.32 mL	(1 M Stock, pH7.4)
720 mM Potassium Acetate	1.44 mL	(5 M Stock)
12 mM Magnesium Acetate	120 λ	(1 M Stock)
4.5 mM ATP	450 λ	(100 mM Stock)
0.6 mM GTP	60 λ	(100 mM Stock)
150 mM Creatine Phosphate	490 mg	
10.2 mM DTT	204 λ	(0.5 M Stock)
DEPC water	6.40 mL	

10 mg/mL Creatine Kinase — 10 mL

100 mg Creatine phosphokinase
5 mL Glycerol
200 λ Hepes (1 M Stock, pH 7.4)

4.8 mL DEPC water

Preparation of Yeast S30 Lysate for in vitro Translation

The whole procedure - especially the Sephadex chromatography – should be carried out in the cold room!

1) Inoculate 100 ml YAPD with 5 ml stationary phase culture (yeast MBS strain)
2) Grow during the day at 30 C
3) Inoculate 3 x 2 L YAPD with 0.5 ml of this culture
4) Grow to an OD_{600} of ~ 1.0
5) Harvest cells in 1 L bottles and spin down 10 min @ 5000 rpm
6) Pour off supernatant carefully but quickly
7) Add 100 mL 1 x buffer A to one bottle and resuspend cells, transfer to a second bottle and resuspend cells. Repeat with the other four bottles
8) Transfer to three ~200 mL centrifuge bottles, adjust volume to 175 mL each
9) Spin down cells @ 5000 rpm, 5 min.
10) Resuspend pellets in 25 mL buffer A each, transfer to pre-weighed 50 mL Falcon tubes, add buffer A to ~50 mL each.
11) Spin down @ 5000 rpm, 5 min.
12) Weigh cell pellets.
13) Resuspend cell pellets with 1/10 volume ("w/v") buffer A, yielding a viscous, thick cell paste
14) Add PMSF to 0.5 mM, mix well.
15) Drip the cell paste into liquid nitrogen (use a small flat dewar or a plastic beaker in liquid nitrogen.)
16) The yeast pellets can be collected into 50 mL Falcon tubes and kept at -80 C
17) Crush the yeast pellets with a mortar and pestle in liquid nitrogen: Add some nitrogen to the mortar to pre-cool. Add ~10 g yeast beads and crush/grind for about 20 minutes (about 5 cycles and adding/evaporating nitrogen). Make sure to obtain a very fine ground powder.

18) Set up a 50 mL Falcon tube on ice with a funnel. Carefully pour the yeast powder into the tube and let it thaw on ice. This takes about 4 to 5 hours. Use this time to prepare the Sephadex G25 column (see below).
19) Spin down the cell debris (4000*g, 5 min)
20) Remove supernatant to 1.5 mL polyallomer ultracentrifuge tubes.
21) Spin 6 min @ 38,000*g in the Beckman TLA100 rotor
22) Remove supernatant to fresh ultracentrifuge tubes and spin again under the same conditions.
23) Apply the supernatant to the top of the Sephadex column.
24) Collect fractions by hand into 1.5 mL Eppendorf tubes (~0.5 mL per fraction).
25) Measure A_{260} of each fraction (use 5 mL in 1 mL H_2O)
26) Pool all fractions with $A_{260} > 0.45$
27) Aliquot the yeast lysate (150 µL each), freeze in liquid N_2 and store at -80 C

Sephadex G25 Column:

- Add 8.5 g Sephadex G25* to 100 mL buffer A and allow the resin to hydrate 4 hours to overnight.
- Pack a column (~13 x ~2.5 cm) with the resin.
- Equilibrate with 50 mL buffer A + 0.5 mM PMSF.
- Make sure that the column doesn't dry out.

* use "Sephadex G25 Superfine, DNA Grade" from Sigma

Treatment with Micrococcal Nuclease:

1) To 250 µL of lysate, add 3 µL of 40 mM $CaCl_2$, 3 µL of Nuclease S7 (15 kU/mL). Check different nuclease concentrations/incubation times for every lot of lysate.
2) Incubate at room temperature for 10 min.
3) Add 5 µL of EGTA. The lysate can be stored at -80 C.

AGAROSE NORTHERN BLOT

Preparation of 1.4% agarose gel

Combine: 20 mL 10X MOPS Buffer
148 mL dH_2O
2.8 g agarose.
Dissolve agarose by boiling; cool to approximately 60 °C then add 32 mL formaldehyde (37% stock)

Sample preparation

Resuspend RNA sample in 5 μL of RNA Loading Dye
(RNA Loading Dye: 5 mL Formamide
1.8 mL Formaldehyde (37% stock)
1 mL 10X MOPS Buffer
800 μL EtBr (10mg/mL)
1.4 mL dH_2O (to 10 mL final volume)
Divide and freeze in 100 μL aliquots)
Heat RNA samples at 65 °C for 10 minutes.
Run gel in 1X MOPS Buffer 6-8 hours at 80-100 volts (or O/N at 35V)

Parker Transfer Protocol

Pare down gel to size and wash briefly (1-2 min) in 2X SSC.
Place gel upside down on damp transfer pads (soaked in 20X SSC), place membrane (presoaked in 2X SSC), 3 layers of Whatmann paper (presoaked), and a 1-2 inch stack of paper towel – all cut to similar size of gel. Add approximately 500 g of weight to transfer stack. Leave O/N.

DNase I Treatment of RNA

Dilute RNA to 1 μg/μL.

Combine into a 200 μL reaction volume

50 μL RNA (@1 μg/μL)
20 μL 10X DNase I Digestion Buffer (see below)
20 μL DNase I (RNase-free; Invitrogen)
110 μL DEPC-dH_2O

Incubate reaction at 37 °C for 45-60 min.

Extract sample once with 200 μL P/C/I (P/C/TE in Parker Lab), followed by two additional extractions with 200 μL each of $CHCl_3$.

Precipitate RNA with the addition of 65 μL 8 M NH_4OAc and 700 μL 95% EtOH and incubation at -20 °C O/N.

Collect RNA by centrifugation; wash once with 500 μL 70% EtOH and air dry.

Resuspend RNA in DEPC-dH_2O and quantify spectrophotometrically. Evaluate RNA integrity by formaldehyde agarose electrophoresis.

10X DNase I Digestion Buffer

400 mM Tris-HCl pH 7.5
25 mM $MgCl_2$
10 mM $CaCl_2$
(made with DEPC-dH_2O and autoclaved; store in aliquots at -20 °C)

IN SITU HYBRIDIZATION OF mRNA AND IMMUNOFLUORESCENCE

CELL PREPARATION

1. Grow a 25 mL culture of yeast cells overnight in YEPD to early log phase (OD_{600} = 0.35-0.50). Remove 5 mL to a test tube and fix cells by adding 700 μL 37% formaldehyde (5.2% final conc.); incubate 90-120 min on rotator at 24 °C.

2. Collect cells by centrifugation at 2,000 X g for 2 min. Wash cells 2X with 5 mL of 0.1 M potassium phosphate buffer pH 6.5 and 1X with 5 mL solution P.

3. Resuspend cell pellet in 1 mL solution P and add DTT to 25 mM (final conc.). Incubate at room temp for 10 min.

4. Add 30 μL of 10 mg/mL zymolase (in solution P) and incubate 15-30 min on rotator at room temperature (rotate gently). While cells are being treated with zymolase, coat slide wells with 0.3% (or 0.1%) polylysine (incubate 5 min at room temperature; rinse 1X with water and air dry).

5. Collect cells by gentle centrifugation (6,000 rpm) and resuspend in 1 mL of solution P. Apply 40 μL of the cell suspension to each well and incubate 15 min at room temperature.

6. Gently aspirate off unadhered cells using an aspirator fitted with a Pasteur and P-200 tip (just touch the outside of well).

7. Permeabilize cells with 30 μL 0.5% N-P40 (in solution P) for 5 min at room temperature. While permeabilizing, make 0.1 M triethanolamine pH 8.0.

8. Aspirate off the N-P40 solution and rinse wells 1X with 40 μL of solution P. Equilibrate cells in 40 μL freshly prepared 0.1 M triethanolamine pH 8.0 for 2 min at room temperature.

9. Block polar groups with 40 μL 0.25% acetic anhydride/0.1 M triethanolamine for 10 min at room temperature.

HYBRIDIZATION (beginning with this step, never let the wells dry out)

10. Boil salmon sperm DNA for 5 min then place on ice for 5 min. Add ssDNA to prehybridization buffer to a final conc. of 500 μg/mL. Add 40 μL prehyb solution to each well and incubate 1 hour at 37 °C in humidified chamber.

11. Just before adding probe, boil ssDNA for 5 min then place on ice for 5 min. Add ssDNA to prehyb buffer to a final conc. of 500 μg/mL and add digoxigenin-labelled oligo$(T)_{50}$ probe also to prehyb (may try dilutions of probe; 1 μL in 300 μL; 1/10, 1/100, etc.), vortex well.

12. Aspirate off prehyb and add 40 μL of the hybridization solution containing probe to each well. Hybridize overnight in humidified chamber at 37 °C.

13. Remove hybridization solution and wash wells as follows:

1X with 2X SSC for 1 min at room temp
1X with 2X SSC for 1 hour at room temp
1X with 1X SSC for 1 hour at room temp
1X with 0.5X SSC for 30 min at 37 °C
1X with 0.5X SSC for 30 min at room temp

14. Block cells with 40 μL antibody blocking buffer for 1 hour at room temperature. Add anti-digoxigenin Fab-FITC (or rhodamine) at 1:200 in antibody blocking buffer. Incubate overnight at room temperature. (If doing a double label with another antibody, dilute second antibody appropriately and add here).
15. If doing a double label, wash wells 3X for 5 min each with 40 μL antibody blocking buffer before adding secondary antibody at desired dilution. Incubate 1 hour at room temperature.

16. Remove antibody solution and wash wells as follows:

1X with Ab wash solution 1 for 1 min at room temp
1X with Ab wash solution 1 for 10 min at room temp
1X with Ab wash solution 1 for 30 min at room temp
1X with Ab wash solution 2 for 10 min at room temp
1X with Ab wash solution 2 for 30 min at room temp

Staining and Mounting Slides

17. Stain nuclei with DAPI solution for 5 min at room temperature. Wash wells 2X for 5 min each with 40 μL of Ab wash solution 2. Air-dry slide and mount in mounting medium. Seal with clear nail polish.

Required Materials

- 37% formaldehyde
- 0.1 M potassium phosphate buffer pH 6.5
- Solution P: 0.1 M potassium phosphate buffer pH 6.5, 1.2 M sorbitol
- 1 M DTT
- 10 mg/mL zymolase in solution P
- 0.1% or 0.3% polylysine (Sigma, >150 kDa)
- 0.5% N-P40 in solution P (prepare fresh)
- 0.1 M triethanolamine (TEA) pH 8.0 (prepare fresh; pH using 1 M NaOH)
- 0.25% acetic anhydride in 0.1 M TEA pH 8.0
- Prehyb solution: 50% deionized formamide, 4X SSC, 1X Denhardt's, 125 μg/mL tRNA; 10% dextran sulfate
- Sheared salmon sperm DNA
- Digoxigenin-labelled probe: probe is critical, oligo should be uniform length (5' and 3' hydroxyl, RP-1 purified). The probe is labeled using the Boehringer-Mannheim Genius-6[a] kit.
- 2X SSC, 1X SSC, and 0.5X SSC

- antibody blocking buffer: 0.1 M Tris pH 9.0, 0.15 M NaCl, 5% heat inactivated FCS, 0.3% Triton X-100
- anti-digoxigenin Fab-FITC (or rhodamine; Roche)
- Ab wash solution 1: 0.1 M Tris pH 9.0, 0.15 M NaCl
- Ab wash solution 2: 0.1 M Tris pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$
- DAPI solution: 1 μg/mL in Ab wash solution 2
- Mounting medium: dissolve 100 mg p-phenylenediame in 10 mL PBS, adjust to pH 8.0 with 0.5 M sodium carbonate pH 9.0. Bring volume to 100 mL with glycerol. Store in light-protected bottle at -20 °C.
- clear nail polish

KINASING OLIGONUCLEOTIDES FOR USE AS PROBES IN NORTHERN BLOTTING

Set up the following reaction mixture:

300 ng oligonucleotide
2 μL 10 polynucleotide kinase buffer
3 μL [^{32}P] γ-ATP
1.5 μL polynucleotide kinase (PNK)
H_2O to 20 μL

Incubate at 37 °C for 90 min. Increase reaction volume to 200 μL and add 1 μL of blue dextran dye and 1 μL of red dye. Remove unincorporated nucleotide from sample using a P6 sepharose spin column. Begin with 1 mL syringe; plug end with cotton and tamp down with plunger. Fill with P6 sepharose (or G25 SephaDex). Place syringe in 14 mL Falcon tube and spin for 2 min at 2,000 rpm (in table top centrifuge). Discard flow-thru and repeat with additional sepharose until column material is almost to top of syringe. Place column in new 14 mL Falcon tube and load the 200 μL reaction volume to the top of the column. Spin column for 2 min at 2,000 rpm. Collect flow-thru (should be blue, while red dye should remain on column). Determine radioactivity by counting 1 μL in a cintilation counter. Store probes at -20 °Cwhen not required.

SYNTHESIS OF RANDOM-PRIMED DNA PROBES FOR NORTHERN HYBRIDIZATION

Set up the following reaction mixture:

50 ng – 1 μg DNA fragment in 3 μL volume
10 μL of ddH_2O

Heat to 100 °C for 3min, and while cooling, add:

5 μL OLB
5 μL [^{32}P] α-dATP (approx 50 μCi)
1 μL BSA (10 mg/mL)

Allow mixture to cool to room temperature (to facilitate primer/DNA annealing), then add:

1 μL Klenow DNA polymerase

Incubate at room temperature for 90 min. Increase reaction volume to 200 μL and add 1 μL of blue dextran dye and 1 μL of red dye. Remove unincorporated nucleotide from sample using a G50 sepharose spin column. Begin with 1 mL syringe; plug end with cotton and tamp down with plunger. Fill with G50 sepharose. Place syringe in 14 mL Falcon tube and spin for 2 min at 2,000 rpm (in table top centrifuge). Discard flow-thru and repeat with additional sepharose until column material is almost to top of syringe. Place column in new 14 mL Falcon tube and load the 200 μL reaction volume to the top of the column. Spin column for 2 min at 2,000 rpm. Collect flow-thru (should be blue, while red dye should remain on column). Determine radioactivity by counting 1 μL in a cintilation counter. Store probes at -20 °C when not required.

ISOLATION OF PLASMID DNA – RAPID LYSATE PROCEDURE

1. Inoculate 5 mL LB containing the appropriate antibiotic with a single bacterial colony (SCI). Incubate O/N on shaker/tube rotator at 37 °C.

2. Harvest cells either in culture tube or by transferring to an Eppendorf tube – centrifuge at 14,000 rpm for 1 min at RT.

3. Wash cells by resuspending in 1 mL of 40 mM KH_2PO_4 and harvest by centrifugation at 14,000 rpm for 1 min at RT. Remove phosphate buffer, leaving the bacterial pellet as dry as possible.

4. Resuspend cell pellet by vortexing in 100 µL of an ice cold solution of GET – incubate 5 min at RT.

GET BUFFER
50 mM glucose
10 mM EDTA
25 mM Tris HCl (pH 8.0)

5. Add 200 µL of a freshly prepared solution of 0.2 N NaOH/ 1% SDS. Lyse cells by inverting the tube rapidly 2 or 3 times (do not vortex). Store on ice for 5 min.

NAOH/SDS SOLUTION
0.9 mL 0.22 N NaOH
0.1 mL 10% SDS

6. Neutralize solution by adding 150 µL of an ice cold solution of 3 M sodium acetate (pH 4.8). Mixcontents by flicking the tube vigorously. Store on ice 10 min.

7. Centrifuge sample for 5 min at 14,000 rpm at RT.

8. Transfer supernatant (400-450 µL) to a fresh tube. Add an equal volume of phenol/chloroform:isoamyl alcohol/TE (25:24:1; 10 mM Tris/1 mM EDTA-buffered phenol) and extract by vortexing for 30 sec. Centrifuge for 2 min at 14,000 rpm at RT.

9. Transfer supernatant to a fresh tube and precipitate DNA with 2X volumes of 95% EtOH. Mix by inverting several times and place at -20 °C for 30 min.

10. Collect precipitated DNA by centrifugation for 15 min at 14,000 rpm at RT.

11. Decant supernatant and add 500 µL 70% EtOH, Centrifuge 10 mins at 14,000 rpms at RT. Air dry pellet briefly or place in vacuum desiccator.

12. Resuspend DNA in 50-100 µL dH_2O.

13. Add 2 µL of RNaseA (stock concentration of 10 mg/mL)

14. Incubate at 37°C for 1 hour

15. Add 200 µL of dH_2O

16. Add an equal volume of phenol/chloroform and extract by vortexing for 30 sec. Centrifuge for 2 min at 14,000 rpm at RT.

17. Transfer supernatant to new tube. Add 1/10th volume 3 M NaOAc and 2X 95% ETOH. Mix by inverting several times and place at -20 °C for 30 min.

18. Collect precipitated DNA by centrifugation for 15 min at 14,000 rpm at RT.

19. Decant supernatant and add 500 µL 70% EtOH, Centrifuge 10 mins at 14,000 rpms at RT. Air dry pellet briefly or place in vacuum desiccator.

20. Resuspend DNA in 50-100 µL dH_2O.

Quantify spectrophotometrically.

RNase H Digestion of RNA

1. ETOH ppt 20 µg of RNA and 300 ng cleavage oligo [can ppt as much as 40 µg of RNA and still maintain the 300 ng of oligo].

2. Resuspend pellet in 10 µL of 1X Hybridization Mix (see below).

3. Heat sample at 65 °C for 10 minutes. Cool slowly to 30 °C. Pulse spin down.

4. Add 9.5 µL of 2X RNase H Buffer and 0.5 µL RNase H. Mix well.

5. Incubate sample at 30 °C for 60 minutes.

6. Add 180 µl Stop Mix.

7. Precipitate RNA by adding 500 µL of EtOH. Freeze at -80 °C for 60 min. (You must ppt the RNaseH reaction. This is required to get rid of the Mg++ present in the 2X buffer. If you heat the samples in step 9 with Mg++ the RNA will be destroyed.)

8. Spin down sample for 10 min at RT. Wash RNA pellet with 300 µL of 70% EtOH. Dry either at RT or under vacuum in Speedvac (once again, don't overdry).

9. Resuspend sample in 10 µL of Loading Dye. Heat sample at 100 °C for 5 min prior to loading on 6-8%denaturing acrylamide gel.

Prepare solutions using DEPC-treated water and store in aliquots at -20 °C

<u>10X Hybridization Mix</u>
0.25 M Tris-HCl, pH 7.5
10 mM EDTA
0.5 M NaCl

<u>Stop Mix</u>
0.04 mg/mL tRNA
20 mM EDTA
300 mM NaOAc

<u>2X RNase H Buffer</u>
40 mM Tris-HCl, pH 7.5
20 mM $MgCl_2$
100 mM NaCl
2 mM DTT
60 µg/mL BSA
*Add 0.5 µL RNase H to
9.5 µL buffer for each reaction*

<u>Formamide Loading Dye</u>
100% deion formamide
5 mM EDTA
0.25% bromophenol blue
0.25% xylene cyanol

Cleavage oligo for PGK1 = oJC591

YEAST GLASS BEAD RNA ISOLATION

1. Set up the following tubes:
   Tube 1: 500 µL phenol/chloroform/LET
   Tube 2: 500 µL chloroform
   Tube 3: 40 µL 3 M NaOAc (made with DEPC dH_2O)

2. Resuspend frozen yeast cell pellet (in 2 mL Eppy) in 150 µL LET.

3. Add 150 µL phenol/LET.

4. Add equal volume (approx. 300-400 µL) of glass beads and vortex at RT in MultiMixer for 5 min at top speed.

5. Add 250 µL DEPC H_2O and 250 µL phenol/chloroform/LET.

6. Vortex in MultiMixer for an additional 5 min followed by centrifugation at RT for 5 min at 14,000 rpm.

7. Remove aqueous phase (approx 450 µL - top layer) and add to Tube #1. Vortex 60 sec and spin 3 min at 14,000 rpm.

8. Remove aqueous phase and add to Tube #2. Vortex 60 sec and spin 3 min at 14,000 rpm.

9. Remove aqueous phase and add to Tube #3; mix well. Add 1 mL 100% EtOH, mix, and place at -80 °C O/N.

10. Collect RNA by centrifugation at RT for 10 min at 14,000 rpm. Wash pellet with 500 µL 70% EtOH and recentrifuge for 5 min. Drain supernatant and dry pellet (either air dry or dry in SpeedyVac [no heat]).

11. Resupend pellet in 50-150 µL DEPC dH_2O and quantify spectrophotometrically (4 µL in 1000 µL dilution).

LET
25 mM Tris, pH 8.0
100 mM LiCl
20 mM EDTA
(all reagents should be made in DEPC-treated dH_2O)

Recipe for 6% polyacrylamide gel

15 mL 19:1 polyacrylamide
10mL 10X TBE
46 g urea
H_2O to 100 mL

_____

Degas; add 200 μL APS (20%) and 100 μL TEMED

Recipe for pre-hybe for Northern Blots probed with Radiolabelled Oligo

Prehyb/hyb

20 mL 100X Denhardt's
60 mL 20X SSC
120 mL dH_2O
2 mL 10% SDS

_____
200 mL total volume

Wash blots with 6X SSC/0.1% SDS twice at RT and once at 50 °C

Recipe for pre-hype for Northern Blots probed with Random primed probes

40 mL 20X SSC
80 mL formamide
6.4 mL 10X SDS
16 mL 100X Denhardt's
16 mL salmon sperm DNA (10 mg/mL)
1.6 mL dH_2O

_____

mRNA Decay Analysis – Galactose 'Shut-off'

(Note: must have a reporter gene downstream of the galactose-inducible promoter [i.e. LEU2PM])

1. Start a 200 mL minimal/synthetic media + 2% galactose/1% sucrose culture in a 500 mL Erlenmeyer flask (with appropriate amino acids added) to grow overnight at 30 °C (starter can be an overnight primer culture or from a plate). Note: may have to grow cells at 24 °C or other temperature.

2. Grow culture to approximately OD_{600}=0.40 - 0.45.

3. Harvest the cells by centrifugation of the 200 mL culture in four 50 mL conical tubes at 4000 rpm for 2 min. Remove supernatant and resuspend cells in 15 mL minimal/synthetic media (NO SUGAR) with appropriate amino acids added. Combine resuspended cells into one 50 mL conical tube and collect the cells by centrifugation for 2 min at 4000 rpm.

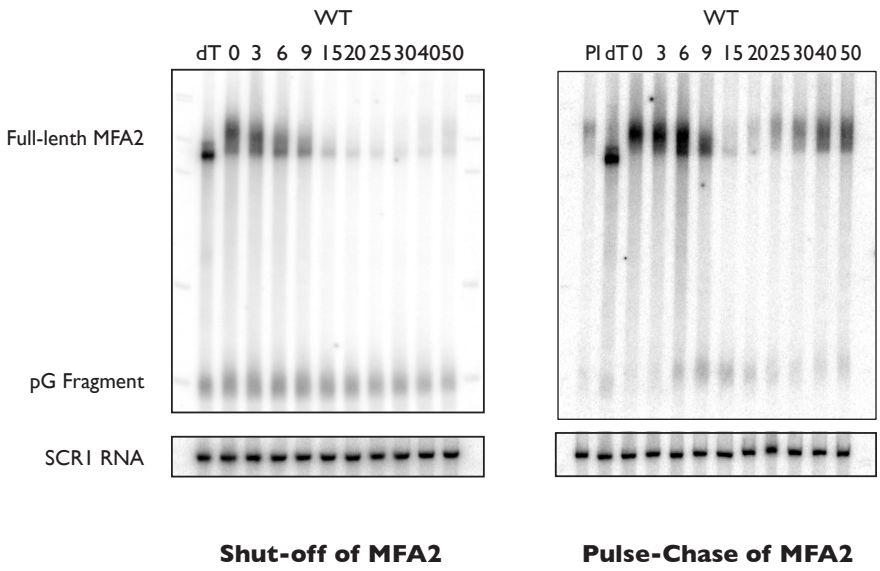
4. Drain supernatant from pelleted cells, and resuspend pellet in exactly 20 mL minimal media (NO SUGAR) with appropriate amino acids added.
5.
6. Remove a 2 mL aliquot of resuspended cells (t = 0), transfer to a 2 mL Eppendorf tube, spin for 10 sec in a bench-top centrifuge, remove supernatant by aspiration, and flash freeze cell pellet in a dewer of liquid.nitrogen.

7. Transfer the remaining 18 mL of resuspended cell culture to a 50 mL flask containing 2 mL of 40% dextrose (dextrose at a final concentration of 4% - 'shut-off' of galactose promoter) and commence shaking flask in a 30 °C water bath.

8. Remove 2 mL aliquots from the culture at the following time points:
   0, 3, 6, 9, 12, 15, 20, 30, 40, 50 min or
   0, 3, 6, 9, 15, 20, 25, 30, 40, 50 min
   Treat samples as above (i.e. pellet cells and flash freeze).

9. Isolate RNA from cell pellets and analyze decay by Northern blotting.

This shows the difference between information you can gain from a GAL shut off experiment vs. a pulse-chase.



PRIMER EXTENSION ANALYSIS

LABELLING OF OLIGONUCLEOTIDE PRIMER

Combine into a 25 µL reaction volume

10 µL oligonucleotide primer (1 pmol/µl)	total = 10 pmol
2.5 µL 10X PNK buffer (Roche)	
2 µL γ ^{32}P-ATP	3 µL in thesis
1.5 µL Polynucleotide Kinase (Roche; 10 U/µL)	total = 15 U
9 µL DEPC-dH_2O	

Incubate reaction at 37 °C for 10 min followed by incubation at 65 °C for 10 min.

Bring reaction up to 200 µL with the addition of 175 µL DEPC-dH_2O and extract once with 200 µL P/C/I (P/C/TE in Parker Lab).

Remove aqueous phase and to it add 15 µL 8M NH_4OAc and 1.5 µL glycogen; precipitate oligonucleotide primer with the addition of 650 µL of 95% EtOH and incubation at -20 °C O/N.

Collect precipitate by centrifugation at 14,000 rpm for 10 min; wash pellet once with 500 µL 70% EtOH and air dry.

Resuspend sample in DEPC-dH_2O to 0.5 pmol/µL (i.e. 20 µL) and store at -20 °C.

PRIMER EXTENSION ANALYSIS

Combine into a 10 μL reaction volume

1.1 μL total cellular RNA (5 μg total)
4 μL ^{32}P-labelled oligonucleotide (@ 0.5 pmol/μL; 2 pmol total)
2 μL 5X PE buffer – Mg^{2+}
1 μL 0.1 M DTT
1.9 μL DEPC-dH_2O

Incubate at 60 °C for 5 min; chill briefly on ice.

Add 1 μL M-MLV RTase (200 U/μL)
2 μL 5X First strand buffer
5 μL 10 mM dNTP mix
1 μL 0.1 M DTT
1 μL DEPC-dH_2O

Incubate reaction at 37 °C for 45 min.

Stop reaction with the addition of 40 μL FA dyes. Analyze 20 μL on a 6% denaturing polyacrylamide gel.

Roche 10X PNK buffer

500 mM Tris-HCl pH 8.2
100 mM $MgCl_2$
0.1 mM EDTA
50 mM DTT
1 mM spermidine

Thesis PNK reaction buffer (Gibco-BRL)

700 mM Tris-HCl pH 7.6
100 mM $MgCl_2$
1 M KCl
10 mM β-mercaptoethanol

5X PE BUFFER – MG^{2+} (same as First strand buffer but without the Mg)

250 mM Tris-HCl pH 8.3
375 mM KCl

NORTHERN BLOT HYBRIDIZATION USING A RIBOPROBE

TRANSCRIPTION OF AN ANTISENSE RNA

2 µL	10X transcription buffer
2 µL	100 mM DTT
0.5 µL	40 U/µL RNasin (RNase inhibitor)
4 µL	5X rNTP mix (2.5 mM each of ATP, CTP, GTP; and 0.075 mm UTP)
2 µL	linearized plasmid DNA (0.5 µg/µL)
2.5 µL	α ^{32}P UTP (800 Ci/mmole)
6 µL	DEPC-treated ddH_2O
1 µL	T_7 RNA polymerase
20 µL	

Incubate reaction mixture at 37 °C for 60 min.
Purify RNA transcript away from rNTPs etc. using a G50 spin column.

PREHYBRIDIZATION/HYBRIDIZATION SOLUTION & CONDITIONS

For 100 mL

50 mL	deionized formamide
25 mL	20X SSC
2 mL	50X Denhardts solution
12.5 mL	200 mM Na-phosphate buffer pH 7.0
5 mL	salmon sperm DNA (10 mg/mL)
2 mL	0.5 M EDTA
2 mL	10% SDS
1.5 mL	DEPC-treated ddH_2O

Prehybridize at 42 °C for 1 hour.
Hybridize overnight at 42 °°C.

WASHING OF BLOT

2X SSC/0.1% SDS at room temperature (5 min)
2X SSC/0.1% SDS at room temperature (5 min)
0.1X SSC/0.1% SDS at 65 °C (60 min)

Polysome Protocol

Pouring gradients using the Biocomp gradient maker (the preferred method for making gradients).

The Biocomp gradient maker is the most reliable and consistent means to generate polysome gradients. We generally pour 15-45% weight/weight gradients. This means that we start with two solutions. The first is 15g of sucrose in 85 mL of sucrose gradient buffer. The second is 45g of sucrose in 55 mL of sucrose gradient buffer.

Use Beckman 14X89mm Polyallomer Tubes (cat#331372). Using the provided Biocomp standard, mark a line on each tube.

Fill the polyallomar tube to line with 15% sucrose (approximately 5.5 mL).

Using a long syringe layer the 45% sucrose beneath the 15% sucrose until interface between two reaches drawn line.

Add the long caps to top and insert into gradient maker. See specific instructions for this instrument for further directions. Typically, this procedure takes 15 mins from set-up to gradient. Place gradient in 4C and use immediately (within a few hours).

Pouring Step-Gradients (an alternative method if Biocomp gradient maker is not available)

Step gradients can be made well ahead of the time of performing the experiment. They will keep at -80 for months and should be pulled out of the freezer and thawed only the night before performing the sedimentation.

Make the following stock solutions

50%	41.25%	32.5%	23.75%	15%	Stocks
41.67 mL	34.38 mL	27.08 mL	19.8 mL	12.5 mL	60% Sucrose
5.0 mL	5.0 mL	5.0 mL	5.0 mL	5.0 mL	10X Sucrose Buffer
100 µL	100 µL	100 µL	100 µL	100 µL	0.5 M DTT
3.23 mL	10.52 mL	17.82 mL	25.1 mL	32.4 mL	Water

Set up twenty Beckman 14X89mm Polyallomer Tubes (cat#331372).

1] Add 2.4 mL of 50% sucrose sol.

2] Freeze completely at -80C (Approximately 30 mins)

3] Add 2.4 mL of 41.25% sucrose sol.

4] Freeze completely at -80C (Approximately 30 mins)

5] Add 2.4 mL of 32.5% sucrose sol.

6] Freeze completely at -80C (Approximately 30 mins)

7] Add 2.4 mL of 23.75% sucrose sol.

8] Freeze completely at -80C (Approximately 30 mins)

9] Add 2.4 mL of 15% sucrose sol.

10] Freeze completely at -80C (Approximately 30 mins)

Cover with parafilm to prevent condensation from accumulating on top.

Growth Conditions for Polysome Analysis

For Polysomes under normal conditions

1] Grow a 25 mL overnight in either YPD or MIN media at appropriate temperature

2] Innoculate 200 mL of YPD or MIN

3] Harvest at OD = 0.4 (See Harvesting protocol below)

4] Add 212µL of cycloheximide (53µg/mL stock in isopropanol)

5] WORK QUICKLY and KEEP EVERYTHING COLD

6] Pour culture into ice cold 50 mL conicals.

7] Spin for 5 mins at 4 C, pour off supernatant

8] Resuspend in residual media and transfer to cold 2 mL eppendorff tube

12] Spin quickly and store the pellet at -80 C until ready to perform sedimentation.

Polysome Lysis/Sedimentation Analysis Protocol

If using steps gradients, the night before you look at polysomes you need to take out the appropriate number for gradients you need from the -80. Place these gradient to thaw at 4 C in someplace where they will not be bumped or disturbed in any way. Thawing of the step gradient sets up the linear gradient distribution

of sucrose from the bottom of tube to top. Alternativly use the Biocomp gradient maker the day of procedure.

Also go turn on the ultracentrifuge and place it and the SW-Ti41 rotor at 4C

1] Make up 10 mL of 1X lysis buffer (See recipes)

2] Weigh an empty 2 mL eppendorff tube (Zero to this weight)

3] Weigh each cell pellet

4] Resuspend pellet in 1.5 mL of 1X Lysis Buffer per gram of cells (usually around 400 µL)

5] Add 1/2 volume of glass beads

6] Vortex at 4 C for 3 mins

7] Place on ice 2 mins

8] Vortex at 4 C for 3 mins

9] Place on ice 2 mins

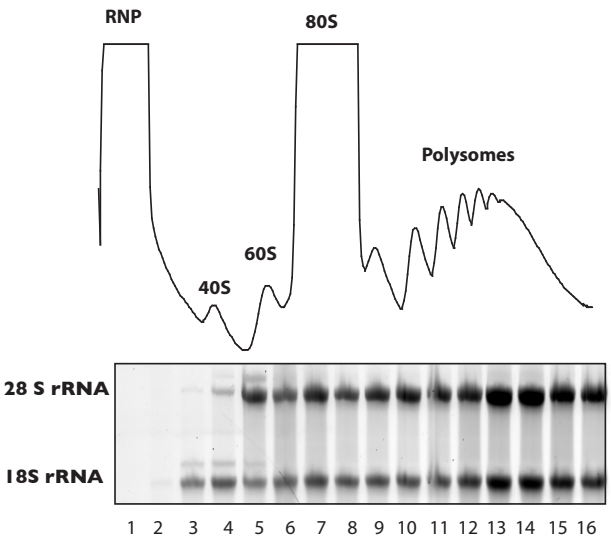
10] Vortex at 4 C for 3 mins

11] Puncture a hole in the bottom of the 2 mL tube with a red hot 18 gauge needle

12] Place tube in 15 mL conical

13] Spin at 4000 RPM for 2 mins at 4 C

14] Transfer supernatant to ICE COLD Beckman polycarbonate tube (11X34 mm; order number 343778). Keep on ICE. Balance tubes with lysis buffer



15] Spin in table top ultracentrifuge using TLA 120.2 rotor at 29,000K for 10 mins at 4C.

16] Measure the OD of 5 µL of each sample in 1000 µL of Lysis buffer

17] Calculate the OD/µL of sample using the following equation. You'll be adding a total of 20 OD units per gradient. This OD amount is variable however depending on analysis. For

example, when looking at proteins it is better to use 7.5-10 OD units. RNA analysis requires 20 OD units.

$$20 \text{ OD units} / (OD_{260}/5) = \mu L \text{ to add to gradient}$$

20 = OD units you wish to add

OD_{260} is what you determine empirically

5= The dilution factor you used to measure the OD, in this case 5µL /1000µL

17b] Add 1/9 volume of 10% Triton-X-100 to the 20 OD cell lysate, the final con. of Triton will be 1%.

18] Add the appropriate amount of lysate to each gradient (this is typically around 100 µL, if not something is wrong, check your math).

19] Balance each tube using lysis buffer Make sure someone show you how to do this step. It is imperative that your tubes are perfectly balanced to each other. Make no mistakes as it can be dangerous and costly to you and the ultracentrifuge.

20] Place gradients in SW-41 Ti rotor and spin at 41,000 RPMs for 2:26 hours at 4C under pressure. The Acceleration profile should be #0 and the Deceleration profile should be #0. IF YOU HAVE NEVER USED AN ULTRACENTRIFUGE MAKE SURE SOMEONE TRAINS YOU FIRST.

20] DO NOT LEAVE ULTRACENTIFUGE UNTIL YOU POSITIVE THAT IT IS WORKING PROPERLY AND IT HAS OBTAINED THE MAXIMUM SPEED (about 5-10 mins).

21] Three hours late, fractionate. This is something you have to see rather than read about, so have some one teach you at this point.

POLYSOME BUFFER RECIPIES

1X Lysis Buffer

cycloheximide (53 µg/mL stock	106 µL
Lysis Buffer (10X)	10 mL
Heparin (50mg/mL)	1000 µL
DTT (0.5M)	200 µL
DEPC H_2O	88.67 mL

100 mL total

10X Sucrose Gradient Buffer

Final Concentrations	For 250 mL
0.5 M Tris-Acetate pH 7.0	62.5 mL (2M TrisOAC stock)
0.5M NH_4Cl	6.68 g
0.12M $MgCl_2$	6.12 g
Use DEPC H_2O to bring up volume	

60% Sucrose

60g Sucrose in 100 mL of DEPC H_2O

10X Lysis Buffer

Final Concentration	For 250 mL
0.1M Tris pH 7.4	25 mL (1M Tris pH 7.4)
1M NaCl	50 mL (5M NaCl)
0.3M $MgCl_2$	75 mL (1M $MgCl_2$)
Use DEPC H_2O to bring up volume	

This is the stereotypical read-out that one gets following a polysome analysis. Above is a OD_{255nm} trace that is taken during fractionation. The pattern you see is representative of the amount of RNA within each fraction. As tRNA and ribosomal RNAs comprise most of the total cell RNA concentration, what you are really seeing is the distribution of tRNAs and ribosomal RNA (hence ribosomes) across the gradient. Below is a EtBR stained gel showing the location of the 25S RNA (60S subunit), and the 18S rRNA (40S) subunit. Note that the 18S rRNA is first seen under the 40S peak, and the 25S rRNA isn't seen until you get the 60S peak, it these rRNA that you are actually measuring in the trace.

The RNP fraction is mainly tRNAs, non-translating mRNA, and other non-translating RNA species. The 80S peak is mainly representative of free monosomes (40S + 60S) which are not associated with mRNA. Recall the ribosome has a propensity to associate in an inactive monosome form. The peaks to the right are polysomes. The first peak following the 80S peak equals mRNA + two ribosomes, the next peak is mRNA + 3 ribosomes, etc.....

QUICKSITE MUTAGENESIS

Mutagenesis Protocol
5 µL Cloned PFU Buffer (10X)
1 µL Vector (pJC154)
3 µL Oligo 1 (41.67ng/µL)
3 µL Oligo 2 (41.67ng/µL)
1 µL dNTP (6.25 mM)
1 µL PFU Turbo Polymerase
36 µL dH_2O

Cycle parameters

95°C 30s
Cycle 18 Times 95°C 30s
55°C 1.0 min
68°C 17.0 mins
4°C forever

Dpn1 Treatment
Add 1 µL of Dpn1 to PCR reactions
Incubate at 37 C for 1 hour

Transformation

Add 2 µL of Dpn1 treated PCR reaction to 50 µL of competent cells
Transform as usual.

Reference: The ribosomal DNA (rDNA) of Saccharomyces cerevisiae

The ribosomal DNA (rDNA) of Saccharomyces cerevisiae is encoded by the RDN1 locus, an approximately 1-2 Mb region consisting of 100-200 tandem copies of a 9.1 kb repeat, on the right arm of chromosome XII (reviewed in 1). Each repeat contains the genes for 5S, 5.8S, 25S, and 18S rRNAs (RDN5, RDN58, RDN25, and RDN18), as well as three types of spacer regions: internal transcribed spacers (ITS1, ITS2), external transcribed spacers (5' ETS, 3' ETS) and nontranscribed spacers (NTS1, NTS2). As in other eukaryotes, genes encoding 18S, 5.8S, and 25S rRNAs are transcribed by RNA polymerase I as a single precursor, the 35S pre-rRNA, that also includes the ITS1 and ITS2 sequences. Transcription starts in the 5'ETS and terminates in the 3' ETS. The majority of transcripts terminate at a terminator 93 base pairs downstream of the 3' end of 25S rRNA, while a minority terminate at a site 211-250 nucleotides downstream (2, 3). The 5S rRNA is transcribed separately, and on the opposite strand, by RNA polymerase III.

Processing of the 35S pre-rRNA occurs in the nucleolus, and initiates with co-transcriptional cleavage in the 3' ETS. The transcript is then extensively modified and rapidly processed (reviewed in 1). Each ribosomal RNA is present in a single copy in a yeast ribosome: 18S rRNA is a component of the 40S ribosomal subunit, and the 25S, 5.8S, and 5S rRNAs are components of the 60S subunit (4).

Note that the systematic sequencing of the yeast genome included only two of the 100-200 rDNA repeats (5). Within SGD, each of the two annotated repeats is represented by several locus entries. The RDN1 locus represents the entire 1-2Mb repeat region. RDN37-1 and RDN37-2 represent the primary 35S transcripts of the two repeats. RDN25-1 and RDN25-2, RDN18-1 and RDN18-2, and RDN58-1 and RDN58-2 represent the 25S, 18S, and 5.8S rRNAs encoded by these transcripts, respectively.

RDN5-1 and RDN5-2 are within the RDN1 locus. RDN5-3 through RDN5-6 are located at sites distal to RDN1, in a 3.6 kb repeated region. Only RDN5-1 represents the complete 5S rDNA sequence. RDN5-2 through RDN5-6 are variant genes (6).

Reference: RNA polymerase usage

pol I 18S, 5.8S, and 25S

pol II mRNA, snRNA, snoRNA

pol III tRNAs, snU6RNA, 5S RNA

Reference: Approximate Size of ribosomal RNAs

60S	25S	4718 nt
	5.8S	160 nt
	5S	120 nt
40S	18S	1874 nt

Calculation of mRNA half-lives

Following a transcriptional pulse-chase or shut-off experiment the decay rate and half-live of a mRNA can be calculated as follows assuming first order kinetics.

k = decay constant
$[RNA]_0$ = concentration of mRNA at time 0
$[RNA]_t$ = concentration of mRNA at time t

$$k = \frac{\ln [RNA]_0 - \ln [RNA]_t}{t}$$

$$t_{1/2} = \frac{0.693}{k}$$

Preparation of Competent E. coli Cells

1. Streak LB plate (no ampicillin) with XL1-Blue cells (stored as glycerol stock at -80 °C).Grow overnight at 37 °C.

2. Pick single colony and inoculate 10 mL LB culture. Grow overnight at 37 °C with aeration.

3. In A.M. use 2 mL of overnight culture to inoculate 500 mL culture of LB, grow at 37 °C with aeration.

4. When cells density is OD_{600} =0.5 (not higher), place culture flask on wet ice for 10 min. Pour culture into prechilled centrifuge bottles; harvest cells by centrifugation in 4 °C Sorval centrifuge at 5000 rpm for 5 min.

5. Remove supernatant (drain pellet) and resuspend cells gently in 10 mL ice cold, sterile 0.1 M $CaCl_2$ (perform resuspension step while centrifuge bottles are sitting in wet ice, do not vortex cells). Once resuspended, bring volume up to 100 mL.

6. Harvest cells again by centrifugation at 4 °C at 5000 rpm for 5 min.

7. Gently resuspend cells in 10 mL ice cold 0.1 M $CaCl_2$. Once cells are resuspended, bring volume up to 100 mL. Incubate cells on wet ice for 60 min.

8. Harvest cells at 5000 rpm for 5 min. Resuspend cells in 25 mL of sterile 85 mM $CaCl_2$/15% glycerol.

9. Aliquot 250 µL into labeled 1.5 mL Eppendorf tubes (tubes should be either on dry ice or dry ice/EtOH bath while aliquoting). Store cells at -70 °C. Transformations typically require 50-75 µL per DNA sample.

Preparation and Use of G418

Dissolve G418 in dH_20 to a stock concentration of 20 mg/mL.

Store at – 20 °C.

Use at a final concentration of 300 µg/mL (i.e. 7.5 mL per 500 mL of solid media).

TANDEM AFFINITY PURIFICATION

PROCEDURE

1. Grow 3-6 liters of cells to 2×10^7 to 3×10^7 per ml (OD = 1.0-1.3).

2. Pellet cell using centrifuge bottles and wash once with ice cold water.

3. Wash once with ice cold NP-40 buffer while transferring to 50ml centrifuge tubes.

4. Pellet cells and resuspend the cells in 10ml NP-40 buffer.

5. Prechill a mortar and pestle with dry ice. Add liquid nitrogen and allow to completely cool. When the liquid nitrogen evaporates, add cells dropwise to the pestle to freeze, occasionally add a little liquid nitrogen to keep the cell cool. Grind with the pestle, very hard, for 15 minutes. Do not allow cell to thaw.

6. Transfer lysate to an ice cold beaker and thaw at room temperature. When the edges are thawed, add 20ml of NP-40 buffer containing protease inhibitors.

7. Transfer crude lysate to 40ml Nalgene tubes and spin at 16,500 rpm for 30 minutes in a SA-600 rotor or equivalent.

8. Add 1 ml of Sepharose 6B (Sigma) beads (1:1 slurry with NP-40 buffer). Incubate on a rotating platform for 30 minutes at 4 °C. Pellet the beads. Transfer the supernatant into a new 50-ml Falcon tube.

9. Add 400 ul of IgG Sepharose 6 Fast Flow (Amersham) (Previously prepared in a 1:1 slurry with NP-40 buffer) and incubate on a rotating platform for at least 2 hours at 4 °C.

10. Pour the lysate/IgG Sepharose suspension onto a BioRad Poly-Prep chromatography column with a reservoir. Pack by gravity.

11. Wash beads with 30 ml of NP-40 buffer.

12. Wash beads with 10 ml of TEV C-buffer.

13. Close the bottom with a stopper and add 1 ml of TEV C-buffer and 5ug of TEV. Plug the top of the column and incubate on a rotating platform between 2 hours and overnight at 4 °C.

14. Drain eluate into a new column sealed at the bottom.

15. Wash out old column with 1.0 ml of TEV C-buffer.

16. Add 3 ml of CBB buffer to the TEV supernatant plus 3 ul of 1M $CaCl_2$ per ml of TEV eluate. Add 300 ul of calmodulin Sepharose (Amersham) in CBB buffer (1:1 slurry) and incubate on the rotating platform for 1 hour at 4 °C.

17. Wash: a. Twice in 1 ml of CBB (0.1% NP-40); b. Once in 1 ml of CBB (0.02% NP-40).

18. Plug the bottom of the column and add 1 ml of CEB.

19. Elute first 1-ml fraction into a microfuge tube.

20. Plug the bottom of the column and add 1 ml of CEB.

21. Elute second 1-ml fraction into a microfuge tube.

22. Combine the fractions and split into 500-, 750-, and 750-ul portions. Place in non-siliconized microfuge tubes.

23. Adjust aliquots to 25% TCA with 100% TCA and place on ice for 30 minutes with periodic vortexing.

24. Spin at maximum speed in a microfuge for 30 minutes at 4 °C.

25. Wash once with ice cold (-20 °C) acetone containing 0.05N HCl and spin for 5 minutes at maximum speed at 4 °C

26. Wash once with ice cold (-20 °C) acetone and spin for 5 minutes at maximum speed at 4 °C.

27. Remove supernatant and dry in a speed vacuum for approximately 10 minutes.

Use the 500-ul fraction for silver staining on a 10% gel. Use 750-ul fraction for mass spectrometry.

NP-40 Buffer

10 mM sodium phosphate buffer (pH 7.2)
150 mM sodium chloride
1% NP-40
50 mM sodium vanadate
1 mM DTT
Protease inhibitors

TEV C-buffer

25 mM Tris (pH 8.0)
150 mM sodium chloride
0.1% NP-40
0.5 mM EDTA
1 mM DTT

Calmodulin Binding Buffer (CBB)

25 mM Tris (pH 8.0)
150 mM sodium chloride
1 mM magnesium acetate
1 mM imidazole
2 mM calcium chloride
10 mM β-mercaptoethanol

Calmodulin Elution Buffer (CEB)

25 mM Tris (pH 8.0)
150 mM sodium chloride
1 mM magnesium acetate
1 mM imidazole
0.02% NP-40
20 mM EGTA
10 mM β-mercaptoethanol

WESTERN BLOTTING OF PROTEINS FROM YEAST WHOLE CELL EXTRACTS

1. Set up a 5 mL overnight culture of yeast strain(s) to be analyzed. Grow at appropriate temperature.

2. Use overnight culture to inoculate a 50 mL culture – grow to OD600=0.5 (OD units can be used to determine abundance of yeast culture to be harvested – alternatively, harvest all samples when they reach 0.5).

3. Harvest cells by centrifugation at 4000 rpm for 5 min at 4 °C.

4. Resuspend cell pellet in 1 mL sterile, ice cold dH20 (or appropriate solvent; e.g. media) and transfer to a 1.5 mL Eppendorf tube.

5. Pellet cells at 14,000 rpm for 60 sec at RT; remove supernatant (can freeze cell pellets at -80 °C here).

6. Resuspend pellet in 200 µL of ice cold 5M urea and place samples at 95 °C for 2 min.

7. Add a small volume (<200 µL) of glass beads and vortex samples for 5 minutes.

8. Add 500 µL of ice cold Solution A (125 mM Tris-HCl pH 6.8, 2% SDS) and vortex 1 min.

9. Place samples at 95 °C for 2 min.

10. Pellet cell debris and glass beads by centrifugation at 14,000 rpm for 2 min.

11. Remove supernatant to a fresh tube (avoid glass beads) and add an equal volume of 2X SDS Sample buffer (250 mM Tris-HCl pH 6.8, 4% SDS, 200 mM DTT, 20% glycerol, 0.1% bromphenol blue).

12. Place samples at 95 °C for 5 minutes and put on ice. Load 20 µL of protein sample on SDS PAGE gel. Run gel in 1X SDS-PAGE running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) at constant current (~35 mA – volts will be >300).

13. Gels can be stained with 0.05% (w/v) Coomassie brilliant blue R-250 in 45% methanol and 10% acetic acid, and destained with a 5% ethanol/5% acetic acid solution.

Pouring SDS PAGE Mini Gels

SDS PAGE Gels

	7.5%		10%		12.5%	
	1 gel	2 gels	1 gel	2 gels	1 gel	2 gels
40% acrylamide (37.5:1)	0.94 mL	1.88 mL	1.25 mL	2.50 mL	1.55 mL	3.10 mL
1 M Tris-HCl pH 8.8	1.875 mL	3.75 mL	1.875 mL	3.75 mL	1.875 mL	3.75 mL
10% SDS	0.05 mL	0.10 mL	0.05 mL	0.10 mL	0.05 mL	0.10 mL
dH_2O	1.94 mL	3.88 mL	1.625 mL	3.25 mL	1.325 mL	2.65 mL
glycerol	0.25 mL	0.50 mL	0.25 mL	0.50 mL	0.25 mL	0.50 mL
10% APS	13 µL	25 µL	13 µL	25 µL	13 µL	25 µL
TEMED	8 µL	15 µL	8 µL	15 µL	8 µL	15 µL

Stacking gel (4.5%)

	1 gel	2 gels
40% acrylamide (37.5:1)	0.313 mL	0.626 mL
1 M Tris-HCl pH 6.8	0.182 mL	0.364 mL
10% SDS	0.028 mL	0.056 mL
dH_2O	2.277 mL	4.554 mL
10% APS	10 µL	20 µL
TEMED	5 µL	10 µL

WESTERN BLOTTING AND IMMUNODECTION

Transfer proteins from PAGE gel to Immobilon-P PVDF membrane. Prewet membrane in methanol at RT (2 min) and then store in 1X Western transfer buffer until ready to use – do not let the membrane dry out. Assemble transfer sandwich using sponges (two) and filter paper [(six), filter paper should be somewhat larger than gel and membrane] pre-wetted in 1X Western transfer buffer. Transfer at a constant current of 250 mA (approximately 70V) for 2 hours (or overnight at 20V) at 4 °C while stirring in Western transfer buffer (1 L of 1X Western transfer buffer = 100 ml 10X Western transfer buffer stock [-methanol], 200 ml methanol, 700 ml dH2O).

10X Western transfer buffer stock [-methanol]

250 mM Tris base	-	30.3 g Tris base
1.92 M glycine	-	144.1 g glycine
Store solution at 4 °C.		dH2O to 1L (pH should be ~8.3; do not adjust).

IMMUNODECTION

Disassemble transfer sandwich and place membrane (protein side up) in 1X TBS/0.1% TWEEN with 5% milk. Block membrane for 1 hour at RT with shaking (or overnight at 4 °C).

2. Wash membrane 3 times for 15 minutes each at RT with 1X TBS/0.1% TWEEN.

Treat membrane with primary antibody in 1X TBS/0.1% TWEEN with 5% milk at RT for 60 min.

Wash membrane 3 times for 15 minutes each at RT with 1X TBS/0.1% TWEEN.

Treat membrane with secondary antibody in 1X TBS/0.1% TWEEN with 5% milk at RT for 60 min.

Wash membrane 3 times for 15 minutes each at RT with 1X TBS/0.1% TWEEN.

Detect signal using and ECL detection kit as per manufacturer's instructions.

10X TBS (pH 7.6)

250 mM Tris	-	30 g Tris base
27 mM KCl	-	2 g KCl
1.4 M NaCl	-	80 g NaCl
Store at 4 °C.		dH20 to 1L; pH to 7.6.

1X TBS/0.1% TWEEN

100 mL 10X TBS
900 mL dH2O
1 mL TWEEN 20
Store at 4 °C (to prevent bacterial growth).

1X TBS/0.1% TWEEN + 5% milk

10 mL 10X TBS
90 mL dH2O
0.1 mL TWEEN 20
5 g milk powder
Make fresh and mix well (alternatively, add 5 g milk powder to 100 mL 1X TBS/0.1% TWEEN solution).

YEAST IMMUNOPRECIPITATION

1] Grow a 50 mL culture to an O.D.= 0.5. Pellet cells and either store at -80 or proceed with lysis.

2] Add IP buffer to cell pellet (amount should equal twice volume of pellet). Transfer to a 2mL microcentrifuge tube. Add 1 volume of glass beads

3] Vortex 3 mins at 4°C, incubate on ice for 2 mins, vortex 3 mins at 4°C.

4] Separate lysate from bead via hot needle puncture method, i.e. puncture hole in bottom of 2mL tube with hot needle, place atop 15 mL conical, and spin for 2 mins at 4000 RPM at 4°C.

5] Transfer supernatant to new 1.5mL tube and spin at 4000 RPM for 2 mins.

6] Transfer supernatant to new tube.

Preparation of beads

7] Add matrix suspension of choice (i.e. anti-Flag, anti-MYC, etc...) to new 1.5mL tube. Add enough to get 50µL of packed volume.

8] Wash beads 3X with lysis buffer. Wash by gently resuspending in 500 µL of buffer, and spin down under low speed (4000 RPM) and removing supernatant.

9] Add lysate from step 6 to washed beads.

10] Incubate at 4°C for 3 hour to overnight with gentle rocking.

11] Spindown beads and save supernatant.

12] Wash beads 3X with wash buffer.

13] Elute bound proteins either with antigen, or by boiling in SDS-Page buffer.

14] Run SDS-PAGE and Western.

Lysis Buffer

50 mM Tris pH7.5
50 mM NaCl
2 mM MgCl2
2 mM β-mercaptoethanol**
1X complete protease inhibitor cocktail EDTA free**
0.1% NP40
**Add BME and Protease inhibitors fresh before use.

Wash Buffer

150 mM Tris pH7.5
50 mM NaCl
2 mM MgCl2
0.1% NP40

GST PURIFICATION

Growth, induction, and lysis

1] Grow 5 mL of E. coli bearing expression vector overnight

2] Innoculate 400 mL of LB media with 4 mL and incubate at 30 C until O.D. reaches 1.0

3] Add 100 mM IPTG to a final concentration of 1.0 mM and incubate an addition 4 hours.

4] Centrifuge at 4C for 10 mins

5] Place pellet on ice

6] Resuspend in 20 mL of ice-cold 1X PBS

7] Sonicate in short burst. Avoid frothing and check efficiency by microscopic examination.

8] Add 1mL of 20% Triton X-100 and mix gently for 30 mins at 4C

9] Centrifuge 10 mins at 4C and transfer supernatant to fresh tube.

Purification

1] Gently resuspend Glutathione Sepharose 4B

2] Remove a sufficient slurry to equal a bed volume of 200 µL (400 µL slurry)

3] Sediment the media by centrifugation at 500g for 5 mins, decant supernatant

4] Wash beads by adding 3 mL of cold 1XPBS

5] Sediment by centrifugation at 500 g for 5 mins and decant

6] Add 3 mL of cold 1X PBS

7] Add entire slurry to lysate and incubate at room temperature 30 mins with gentle tumbling.

8] Transfer to a column and allow to drain at room temp

9] Wash three times with 2 mL of 1X PBS

10] Close column, and add 200 µL of elution buffer. Incubate at room temp for 10 mins

Elution Buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0

11] Allow to flow into tubes

12] Repeat elution twice more and pool the eluates.

pCp Labelling

Pellet RNA

1] Resuspend RNA in 22 µL of DEPC H_2O

2] Add 3 µL of 10X RNA ligase reaction buffer

3] Add 2 µL of 5' ^{32}P pCp (cytidine 3'5' bisphosphate)

4] Add 3 µL of RNA ligase

Incubate at 37 C for 1 hour

Add 200 µL of DEPC H_2O

Pass through two G-50 columns

ETOH ppt

Run on gel

TAP - AFFINITY PURIFICATION ALA WENQIAN

1. Grow 6 liters of cells to OD 600 1.2 – 1.3.

2. Pellet cell using centrifuge (4,000 rpm 5min), discard the supernatant. Wash the cell pellet with media, and transfer the resuspended cell into 50 ml tubes, pellet the cell by centrifugation at 4,000 rpm for 5 min. Freeze the cell pellet on dry ice for at least 20 min, then store at -80C until use.

3. To the yeast pellet add one volume of ice cold Buffer A. Melt the pellet on ice.

   Buffer A: 10 mM K-HEPES pH7.9; 10 mM KCl; 1.5 mM MgCl2; 0.5 mM DTT

   1 tablet protease inhibitors per 50 ml

4. Wash the French-press with water. Then, run 20 ml Buffer A through the press.

5. Break the cells by passing them 3 times through the French-press (1,000 to 1,200 psi).

6. Transfer the cell lysate to ice cold centrifugation tubes, centrifugation at 16,500 rpm for 20min at 4C. Collect the supernatant.

7. Adjust the salt concentration to 10 mM Tris-HCl; 150mM NaCl; 0.1% NP-40, and 0.5mg/ml Heparin using 1M Tris-HCl pH 8.0; 5 M NaCl; 10% NP-40; 50 mg/ml Heparin.

8. Add 1 ml of Sepharose 6B (Sigma) beads (1:1 slurry with IPP150 Buffer). Incubate on a rotating platform for 30 minutes at 4 °C. Pellet the beads. Transfer the supernatant into a new 50-ml Falcon tube.

   IPP150 Buffer: 10mM TrisHCl pH8.0; 150mM NaCl; 0.1% NP-40

9. Add 400 ul (beads volumn) of IgG Sepharose 6 Fast Flow (Amersham) (Prepared in a 1:1 slurry with IPP150 buffer) and incubate on a rotating platform for 3 hours at 4 °C.

10. Pour the lysate/IgG Sepharose suspension onto a BioRad Poly-Prep chromatography column with a reservoir. Pack by gravity.

11. Wash beads with 30 ml of IPP150 Buffer.

12. Wash beads with 10 ml of TEV C-buffer (-EDTA)

    TEV C-Buffer (-EDTA): 10mM TrisHCl pH 8.0; 150mM NaCl; 0.1% NP-40; 1mM DTT

13. Close the bottom with a stopper and add 1 ml of TEV C-buffer (-EDTA) and 120 units of TEV. Plug the top of the column and incubate on a rotating platform overnight at 4°C.

14. Drain eluate into a new column sealed at the bottom.

15. Wash out old column with 1.0 ml of TEV C-buffer.

16. Add 3 ml of IPP150 CBB Buffer to the TEV supernatant plus 3 ul of 1M CaCl2 per ml of TEV eluate. Add 400 ul (beads volumn) of calmodulin Sepharose (Amersham) in IPP150 CBB buffer (1:1 slurry) and incubate on the rotating platform for 1 hour at 4 °C.

IPP150 CBB Buffer: 10mM TrisHCl pH8.0; 150mM NaCl; 1mM Mg(Ac)2; 1mM imidazole; 2mM CaCl2; 0.1% NP-40; 10mM b-mercaptoethanol

17. Wash the beads three times with 1 ml of IPP150 CBB

18. Eluate the proteins bound on the beads using 0.5ml IPP150 CEB Buffer six times, collect the eluant

IPP150 CEB Buffer: 10mM TrisHCl pH8.0; 150mM NaCl; 1mM Mg(Ac)2; 1mM imidazole; 20mM EGTA; 0.1% NP-40; 10mM b-mercaptoethanol

19. Adjust aliquots to 20% TCA with 100% TCA and place on ice for 30 minutes with periodic vortexing.

20. Spin at maximum speed in a microfuge for 30 minutes at 4 °C.

21. Wash once with ice cold (-20 °C) acetone containing 0.05N HCl and spin for 5 minutes at maximum speed at 4 °C.

22. Remove the supernatant. Resuspend the protein.

Stripping proteins from Western blot membranes by Low pH

(For the Immobilon Transfer Membranes, Millipore)

Required Equipment and Solutions

Stripping solution: 25mM glycine-HCl, pH 2, 1%(w/v) SDS

Phophate buffered saline (PBS): 10mM sodium phosphate, pH 7.2, 0.9%(w/v) NaCl

Shallow tray, large enough to hold the membrane

Procedure

1. Place the blot in stripping solution and agitate for 30 minutes.
2. Place the blot in buffer and agitate for 10 minutes. Repeat with fresh buffer.
3. Proceed to the blocking step for the next round of immunodetection.

GRADIENT FRACTIONATOR PARAMETERS

UA-6 (UV detector)

Chart Speed = 60 cm/hr

Peak separator = off

Sensitivity = 1.0

Noise Filter = 0.5

Pump

Speed = 1.5 mL/min

Set on "remote start/stop"

Fraction Collector (Foxy Jr.)

20 tubes

Set for "Time"

30 sec.

Chart Recorder (Pharamacia)

Speed = 0.5 mm/sec

Cal = on

Range = 2 V

PCR Amplification of DNA from a Single Bacterial Colony

Resuspend either an entire single bacterial colony or cells harvested from 500 L of an O/N liquid culture in 50 L lysis buffer.

Lysis Buffer

1% Triton-X 100 (v/v)

20 mM Tris-HCl pH 8.5

2 mM EDTA.

Heat sample for 95 °C for 10 min.

Centrifuge samples for 30 sec at 14,000 rpm (at RT) to remove cell debris.

Use a 2 L aliquot of the supernatant for a 25 L PCR reaction.

Useful Electrophoresis Information

Properties of Nucleic Acids

Common Conversions of Nucleic Acids

I. Weight conversions

1 μg = 10^{-6} g
1 ng = 10^{-9} g
1 pg = 10^{-12} g
1 fg = 10^{-15} g

II. Spectrophotometric conversions

1 A_{260} unit of double-stranded DNA = 50 μg/ml
1 A_{260} unit of single-stranded DNA = 33 μg/ml
1 A_{260} unit of single-stranded RNA = 40 μg/ml

III. Molar convertions

1 μg of 1,000 bp DNA = 1.52 pmole (3.03 pmoles of ends)
1 μg of pBR322 DNA = 0.36 pmole
1 pmole of 1,000 bp DNA = 0.66 μg
1 pmole of pBR322 DNA = 2.8 μg

IV. Protein convertions

1 kb of DNA = 333 amino acids of coding capacity = 3.7 x 10^4 MW
10,000 MW protein = 270 bp DNA
30,000 MW protein = 810 bp DNA
50,000 MW protein = 1.35 kp DNA
100,000 MW protein = 2.7 kp DNA

Range of Separation in Gels Containing Different Amounts of Agarose

Amount of agarose in gel (% [w/v])	Efficient range of separation of linear DNA molecules (kb)
0.3	5–60
0.6	1–20
0.7	0.8–10
0.9	0.5–7
1.2	0.4–6
1.5	0.2–3
2.0	0.1–2

Relative Sample Migration Rates*

Cell type	Voltage	Bromophenol Blue migration rate
Sub-Cell Model 96	200 V	5.15 cm/hr
Sub-Cell Model 192	200 V	6.20 cm/hr
Sub-Cell GT cell, 15 x 15 cm gel	75 V	3.0 cm/hr
Wide Mini-Sub cell GT, 15 x 10 cm gel	75 V	4.5 cm/hr
Mini-Sub Cell GT cell, 7 x 10 cm gel	75 V	4.5 cm/hr

* These sample migration rates were determined based on a 0.5 cm thick 1.0% agarose gel using Bio-Rad's Molecular Biology Certified Agarose in 1x TAE electrophoresis buffer (diluted from Bio-Rad's Premixed 50x TAE Buffer). Migration rates will vary depending on the voltage, current and type of agarose or buffer used.

DNA Size Migration with Sample Loading Dyes

Agarose Concentration (%)	Xylene Cyanol	Bromophenol Blue
0.5–1.5	4–5 Kb	400–500 bp
2.0–3.0**	750 bp	100 bp
4.0–5.0**	125 bp	25 bp

** Sieving agarose such as AmpliSize agarose.

Gel-Loading Buffers

Buffer type	6x Buffer	Storage temperature
I	0.25% bromophenol blue 0.25% xylene cyanol FF 40% (w/v) sucrose in water	4 °C
II	0.25% bromophenol blue 0.25% xylene cyanol FF 15% Ficoll (Type 400) in water	Room temp.
III	0.25% bromophenol blue 0.25% xylene cyanol FF 30% glycerol in water	4 °C
IV	0.25% bromophenol blue 40% (w/v) sucrose in water	4 °C
V	*Alkaline loading buffer* 300 mN NaOH 18% Ficoll (Type 400) in water 0.15% bromocresol green 0.25% xylene cyanol FF	4 °C

Commonly Used Electrophoresis Buffers

Buffer	Working solution	Concentrated stock solution (per liter)
Tris-acetate (TAE)	1x: 0.04 M Tris-acetate 0.001 M EDTA	50x: 242 g Tris base 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA (pH 8.0)
Tris-phosphate (TPE)	1x: 0.09 M Tris-phosphate 0.002 M EDTA	10x: 108 g Tris base 15.5 ml 85% phosphoric acid (1.679 g/ml) 40 ml 0.5 M EDTA (pH 8.0)
Tris-borate (TBE)	0.5x: 0.045 M Tris-borate 0.001 M EDTA	5x: 54 g Tris base 27.5 ml boric acid 20 ml 0.5 M EDTA (pH 8.0)
Alkaline (TBE)	1x: 50 mN NaOH 1 mM EDTA	1x: 5 ml 10 N NaOH 2 ml 0.5 M EDTA (pH 8.0)

Effective Range of Separation of DNAs in Polyacrylamide Gels

Acrylamide (% [w/v])[a]	Effective range of separation (bp)	Xylene cyanol FF[b]	Bromophenol blue[b]
3.5	1,000–2,000	460	100
5.0	80–500	260	65
8.0	60–400	160	45
12.0	40–200	70	20
15.0	25–150	60	15
20.0	6–100	45	12

[a] *N,N'*-methylenebisacrylamide is included at 1/30th the concentration of acrylamide.
[b] The numbers given are the approximate sizes (in nucleotide pairs) of fragments of double-stranded DNA with which the dye comigrates.

In vivo 35S labeling / TCA precipitation

Measuring Translation Kinetics

1] Grow 100 mL cells in synthetic media (+dex complete)

2] Harvest 7.5 mL at OD = 0.5

3] Resuspend in 20 mL of either

synthetic media (+dex-met/cyt) or (-dex -met/cyt)

4] Add 120uL cold methonine (10ug/mL)

5] Add 3 uL 35S met (15uCi)

6] Harvest 1mL aliquots at 0, 2,4,6,8,10 mins

7] Add 1 mL of 20% trichloroacetic acid

8] Heat at 95C for 20 mins in glass tubes

9] collect on filter

10] Wash filter with 10mL 10% TCA

11] Wash filter with 10mL 95% EtOH

12] Quantitate using scintillation counter

Yeast Ribosome Transit Time Measurement

1. Inoculate yeast strain into 500ml –Met SD media, grow the culture to OD 0.5-0.6.

2. Pellet the cell by centrifugation at 4000 rpm for 3min.

3. Re-suspend the cell pellet in 50ml –Met SD media.

4. Pulse-label the cell with 35S Met, record as time 0.

5. At 1min, 1.5min, 2min, 2.5min, 3min, 4min, 5min, take 7ml of the culture into 7ml ice cold 2 X Buffer A.

Buffer A: 200ug/ml Cycloheximide; 50mM sodium azide, 50mM NaCl, 10mM Tris-HCl PH7.4, 5mM $MgCl_2$, 5mM NH_4Cl, 0.5mg/ml heparin, 1mM DTT, protease inhibitor

6. Pellet the cell by centrifugation at 4000rpm for 2min at 4C.

7. Wash the cell once with 5ml ice cold Buffer A, then pellet the cell by centrifugation at 4000 rpm for 2min at 4C

Note: An alternative approach to step 1-7 is to grow a small volume of cell (100ml) to log phase, pulse-label the cell, take 10ml from each time point, add the cell pellet into the same amount carrier cells.

8. Re-suspend the cell pellet in 500ul ice-cold Buffer A, and transfer to a 2ml tube

9. Add ½ volume of glass beads

10. Vortex at 4C for 3min, place on ice for 2 min; Vortex at 4C for 3min, place on ice for 2 min; Vortex at 4C for 3min

11. Puncture a hole in the bottom of the 2 ml tube, and place the tube in 15m conical tube

12. Spin at 4000 rpm for 2 min at 4C

13. Transfer the supernatant to ice cold 1.5 ml tube, spin at 13,000rpm for 20 min at 4C to remove the cell debris

14. Transfer the supernatant to ice cold 1.5 ml tube

15. OD the supernant. For the sample collected at each time points, use the same amount OD for the following analysis.

16. For the sample collected at each time points, one part is subject to Total Protein Analysis, the other part is subject to Complete Protein Analysis.

For the Total Protein Analysis:

1. Add TCA to final con 10%
2. Heat at 95C for 20mins
3. Collect on glass fiter
4. Wash filter with 10ml 10% TCA
5. Wash filter with 10ml 95% EthOH
6. Quantitate using scintillation counter

For the Complete Protein Analysis:

1. Transfer the sample to ultracentrifugation tubes
2. Pellet the ribosome by centrifugation at 45,000rpm/4.5h/4°C/50.2 Ti in a Beckman ultracentrifuge.
3. Collect the post-ribosomal supernatant fraction.
4. Add TCA to final con 10%
5. Heat at 95C for 20mins
6. Collect on glass filter
7. Wash filter with 10ml 10% TCA
8. Wash filter with 10ml 95% EthOH
9. Quantitate using scintillation counter

Oligo dT Cellulose Selection

Materials

First gather all the necessary materials for the procedure. You will need oligo dT beads, column buffer as 1X and 2X (1X 0.5M NaCl, 0.2M Tris pH 7.5, 10mM EDTA, 0.1% SDS), chromatography columns, 2mL microcentrifuge tubes, 65deg DEPC water, clamps and column stand to accommodate all samples.

It is important to use the same amount of RNA for each sample. Calculate the volume of RNA needed for equal numbers of micrograms of RNA. If the volumes are reasonably low add DEPC water until all samples are at the same volume. If the concentration of RNA is low and large volumes are required do an ethanol precipitation and resuspend in a lower volume.

Prepare the Column

First swell the beads by soaking 0.07g of oligo dT cellulose in 1X column buffer for about 12 minutes.

Fill the chromatography columns with about 200ul of the swollen beads then equilibrate the column with about 17ml of 1X column buffer.

Column Selection

You want the RNA in a total volume of 200ul in 1X column buffer (100ul of 2X column buffer and up to 100ul of RNA in water)

Boil the RNA in buffer for 90sec then quick cool on ice. Dilute with 200ul of 1X column buffer to a total volume of 400ul.

Pass the RNA over the column 5 times boiling and quick cooling each time. Collect the Flow Through in a 2ml tube labeled poly(A) minus. The Flow Through contains the poly (A) minus RNA.

Wash the column 5 times with 400ul of 1X column buffer, collecting the washes in a 2mL tube labeled wash. The wash will also contain poly (A) minus RNA.

Elute the poly (A) plus RNA by washing with 400ul of warm DEPC water pre-heated at 65deg. Collect the flow through in a 2ml or 1.5ml tube labeled poly (A) plus.

Ethanol precipitate the poly (A) minus, wash, and poly (A) plus eluants. Wash with 70% Ethanol and resuspend in DEPC water. The poly (A) minus and wash can now be combined. The ending volumes of poly (A) plus should be the sample as the poly (A) minus + wash. Add equal volume of loading dye. (The total volume should not be more than 30ul so resuspend with 15ul for the poly (A) plus, 7.5ul for both the minus and wash with 15ul of loading dye)

Generating pCp

33 µL gamma ATP (25 µM Final)

4 µL 10 X Kinase Buffer

2 µL OptiKinase (USB)

1 µL 833 µM 3'-CMP

Incubate 1 hour at 37 C and then heat inactivate at 65 C for 10 mins.

'QUICK CHANGE' SITE-DIRECTED MUTAGENESIS PROTOCOL

(WORKS EVERY TIME – AND USES LESS ENZYME!!!)

plasmid DNA (5 ng/µL)	1 µL
mutagenic oligo A (62.5 ng/µL)	1 µL
mutagenic oligo B (62.5 ng/µL)	1 µL
10x Pfu turbo buffer	2.5 µL
dNTP mix (10 mM)	0.5 µL
Pfu turbo DNA polymerase	0.5 µL
dH_2O	18.5 µL
	(final volume 25.0 µL)

* make reactions up in pools/master mixes if possible.

* small amount of plasmid template ensures very little or no parental plasmid background and means screening fewer clones. Template should be 'clean' – a 'Zyppy' prep works very efficiently.

* PCR as recommended in instruction manual by Stratagene – remember that the extension time is approximately 1 minute per 1 kbp of DNA; and, the greater the change/mutation to be introduced, the greater number of cycles required.

- typically analyze 7.5 µL on agarose gel to visualize amplification (if you see DNA, it must be amplified product since amount of template added is so low). If you do not see any product, a second mutagenesis reaction using 50 ng of plasmid DNA should be tried.

- digest remaining 17.5 µL of SDM PCR reaction with 0.5 µL DpnI (or 1 µL of *Dpn*I diluted 2-fold in 1× NEB 4 enzyme reaction buffer) for 90 min at 37 °C.

- transform 50 µL of XL1-Blue competent cells with 2.0 µL of SDM PCR reaction, plating entire amount of cells. Expect 10-100 transformants per plate. Using more than 2.0 µL of PCR reaction for transformation often leads to a lesser number of transformants.

- screening more than one or two colonies is typically unnecessary – using this procedure, clones are correct 100% of the time!

ANCHOR AWAY

Visualization of anchoring and P bodies

Anchor Away is a technique to selectively and rapidly anchor a protein of interest to a desired subcellular localization. The technique is described in Haruki et al Molecular Cell 31, 925–932, 2008. When anchor is tagged with FKBP12 and the protein of interest is tagged with an FRB (FKBP12-rapamycin-binding) domain in the presence of rapamycin a tight ternary complex is formed.

We have the following strains:

yJC579 PMA1 (plasma membrane) tagged with the anchor FKBP12
yJC580 RPL13A (ribosome) tagged with the anchor FKBP12
yJC586 DCP2 tagged with FRB in the PMA1 anchor tagged strain
yJC587 DCP2 tagged with FRB-GFP in the PMA1 anchor tagged strain
yJC588 DCP2 tagged with FRB in the RPL13A anchor tagged strain
yJC589 DCP2 tagged with FRB-GFP in the RPL13A anchor tagged strain (doesn't fluoresce as well as the PMA1 anchored strain)

(Note the cells autofluoresce because of the red pigment seen in cells with an ade mutation. The vacuole glows with our microscope)

Grow a 50ml culture to mid log phase OD600 of 0.4 in SD complete
(Prior to harvesting make up 1ml of media to resuspend in for visualization that will match the treatment conditions - SD media containing 1ug/ml of Rapamycin, SD media containing DMSO 1ul into 1ml, no sugar media containing 1ug/ml of Rapamycin, and no sugar media containing 1ul into 1ml DMSO)
Harvest cells by centrifugation at 4,000 rpm for 2 min and resuspend in 5ml of SD complete
Aliquot 1ml each tube and treat with specific conditions.

Tube 1 + Rapamycin
DAPI stain (final concentration of 4ug/ml) 4ul of 1ug/ul DAPI treat for 10 minutes @ room temperature with agitation
Treat with Rapamycin (final concentration 1ug/ml) 1ul of 1ug/ul RAP for 15 minutes @ room temperature with agitation
Spin the cells down and resuspend in 100ul of media containing 1ug/ml final concentration RAP
Spot 5ul onto a slide and visualize under the microscope

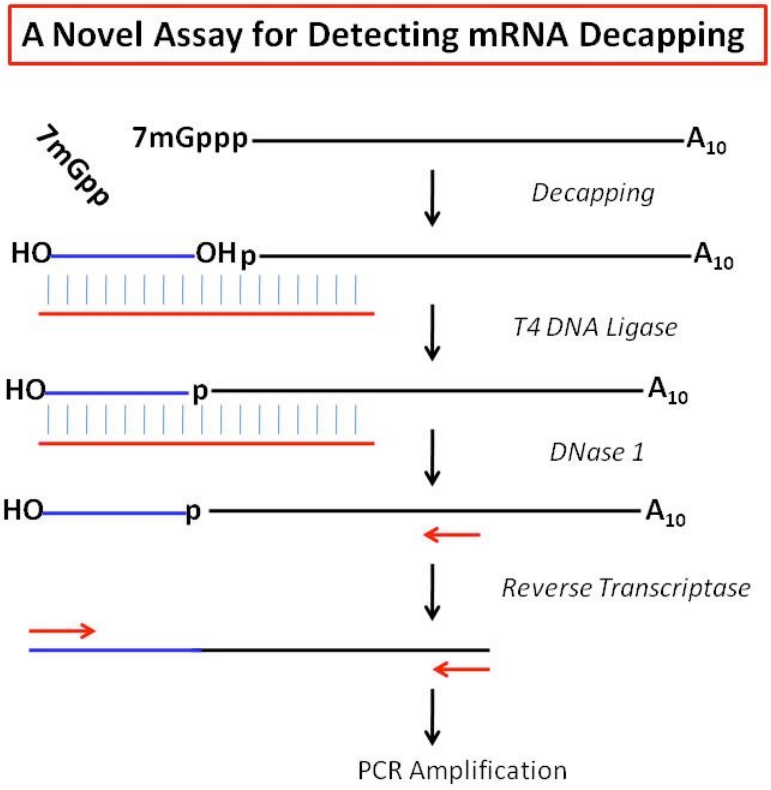
Tube 2 - Rapamycin
DAPI stain (final concentration of 4ug/ml) 4ul of 1ug/ul DAPI treat for 10 minutes @ room temperature with agitation
Treat with 1ul of DMSO for 15 minutes @ room temperature with agitation
Spin the cells down and resuspend in 100ul of media containing DMSO
Spot 5ul onto a slide and visualize under the microscope

Tube 3 + Rapamycin + Stress
DAPI stain (final concentration of 4ug/ml) 4ul of 1ug/ul DAPI treat for 10 minutes @ room temperature with agitation
Treat with Rapamycin (final concentration 1ug/ml) 1ul of 1ug/ul RAP for 15 minutes @ room temperature with agitation
Spin cells down and resuspend in 1ml of no sugar media containing 1ug/ml RAP and incubate for 10 minutes @ room temperature with agitation
Spin the cells down and resuspend in 100ul of no sugar media containing 1ug/ml final concentration RAP
Spot 5ul onto a slide and visualize under the microscope

Tube 4 - Rapamycin + Stress
DAPI stain (final concentration of 4ug/ml) 4ul of 1ug/ul DAPI treat for 10 minutes @ room temperature with agitation
Treat with 1ul of DMSO for 15 minutes @ room temperature with agitation
Spin cells down and resuspend in 1ml of no sugar media containing DMSO and incubate for 10 minutes @ room temperature with agitation
Spin the cells down and resuspend in 100ul of no sugar media containing DMSO
Spot 5ul onto a slide and visualize under the microscope

SPLINT LIGATION RT-PCR FOR DETECTING DECAPPED MRNA

Overview:



Splint-ligation:

RNA (15ug total RNA or RNA from gradient fractions):	X ul
20 pm/ul DNA splint:	1ul
30 pm/ul RNA adaptor:	1ul
500mM KCl:	1ul
1mM EDTA:	1ul
dH2O to:	10 ul

65°C 7min, 56°C 5min, 42°C 5min, 37°C 5min, 30°C 5min, then add following buffer:

10 X T4 DNA ligase Buffer:	1.5ul
RNasin (Promega):	1.0ul
T4 DNA ligase (NEB):	1.0ul
dH2O	1.5ul
Total:	15ul

Incubate at RT overnight (16hrs)

Continued on next page

Note: *RNA adaptor*: oJC706

DNA splint: When design DNA splint, make sure its length is above 70nt, so that the ligation is efficient (>95%)and quantitative.

Note: The concentration of DNA splint and RNA adaptor, and the ratio of RNA adaptor to DNA splint require optimization for each individual gene. For PGK1 and RPL41A, 1 ul 1pm/ul DNA splint, 1ul 3pm/ul RNA adaptor (oJC706) gives best results (highest signal to noise background)

DNase 1 treatment:

Add 2ul 50mM CaCl2, 2ul DEPC dH2O, 1ul DNase 1 (Roche) to the reaction tube, incubate at RT for 60min.
P/C/L extraction
Add 400ul LET to the reaction tube, then add 400ul P/C/L, vortex, spin, and collect the supernatant. Add 40ul 7.5M NH4OAC and 1ul glycoblue to the tube, and 1ml 95% Ethanol, precipitate at -80^{0}C.

Primer Extension:

Pellet the RNA, and re-suspend the RNA pellet in the 12ul following buffer:

5X First strand buffer:	2.4ul
Gene Specific primer (0.7pm/ul):	2.0ul
dH2O:	7.6ul
Total:	12ul

65^{0}C 7min, 56^{0}C 7min, 42^{0}C 7min

Then add 8ul of the following buffer:

5X First strand buffer:	1.6ul
100mM DTT:	2.0ul
10mM dNTP:	2.5ul
Superscript 2 (invitrogen):	0.5ul
dH2O:	1.4ul
Total:	8.0ul

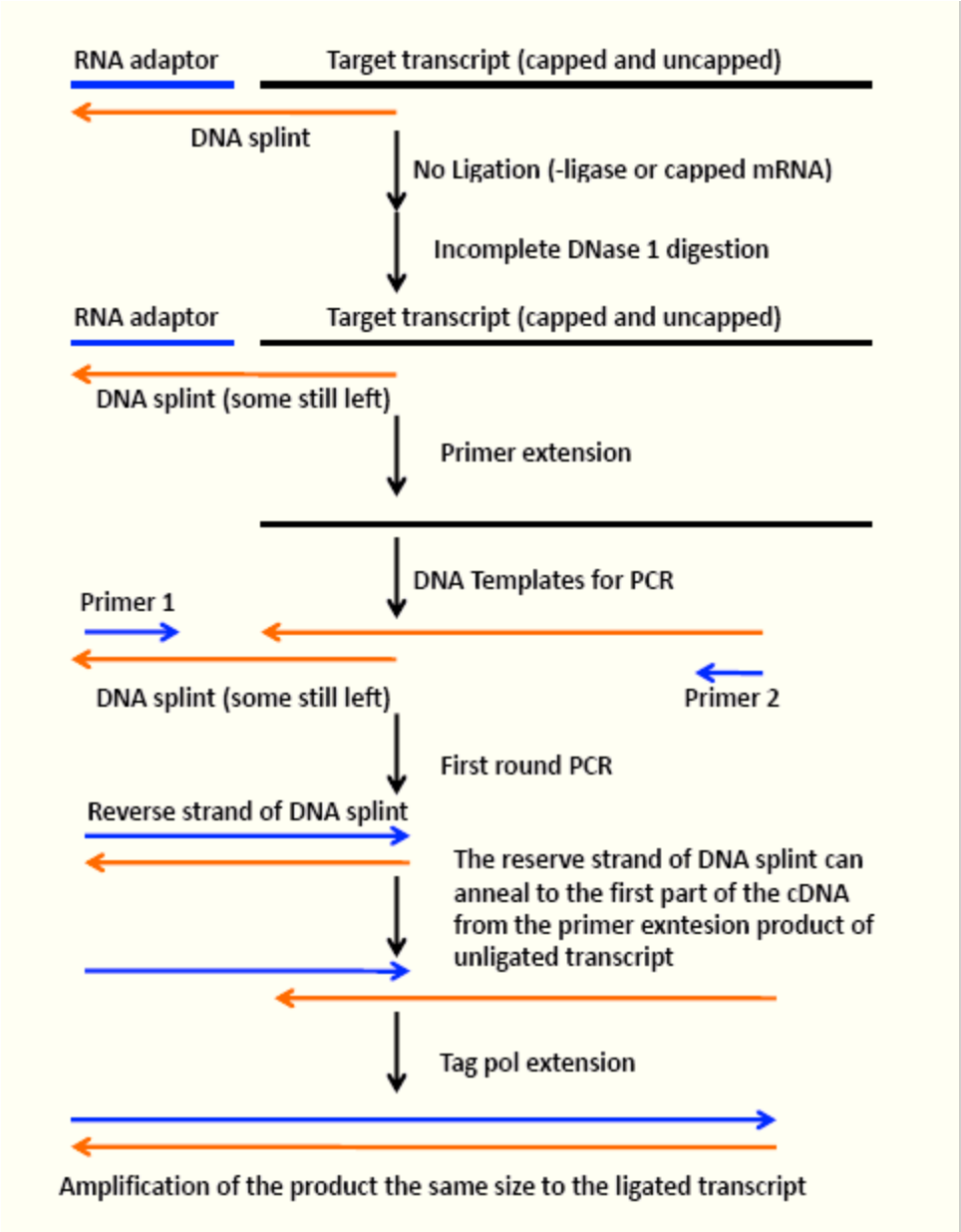
Incubate at 42^{0}C for 1 hour, then 65^{0}C 15min

PCR to detect the cDNA:

2ul of the primer extension product is used for PCR. oJC707 (binds to the RNA adaptor) and the gene specific primer used for primer extension are used for PCR amplification. The cycle number is usually 25-27 for endogenous mRNAs, depending on expressing level; for reporter, such as PGK1, 20-22 cycle is enough in WT cells.

Gel analysis:
The PCR product (usually below 200bp) is subject to 8% native PAGE gel analysis and EB staining.

Important control:
It is important to incorporate a minus T4 ligase ligation control to monitor complete removal of DNA splint, because if the DNA splint is not removed completely, a product of the same length can be generated as shown below. Usually DNase 1 treatment results in great reduction of signal intensity in minus ligation samples compared to the ligated samples.



False positive caused by incomplete DNase 1 digestion.

AFFINITY PURIFICATION OF FLAG TAGGED RIBOSOME FOR DETECTING mRNA DECAY FACTORS

Grow ribosome-tagged yeast cells and control cells in appropriate media to early log phase (OD = 0.4). Usually 125ml culture is enough.

Add 157ul 50mg/ml cyclohexmide to the culture, mix, pellet the cell by centrifugation at 4000rpm for 2 min. Store the cell pellet at -80C until use.

Prepare the anti-FLAG M2-agarose beads: For each IP, 50ul beads (beads volume) will be used; wash the beads 5 times in 500 ul ice cold 1× binding buffer. Store the washed beads on ice until use.

1× binding buffer: 50 mM Tris-HCl pH 7.5, 12 mM Mg(OAc)2, 0.5 mM DTT, 100 ug/ml cycloheximide, protease inhibitor)

Re-suspend the cell pellet in 400ul lysis buffer, add equal volume glass beads, vortex 3 times, each time 3 min, with 2 min interval between each vortex, in cold room.

Lysis Buffer: 20 mM HEPES pH 7.4, 12 mM Mg(OAc)2, 100 mM KOAc, 0.5 mM DTT, 100 ug/ml cycloheximide, protease inhibitor)

Puncture a hole in the bottom of the tube, and then place the tube in 15 ml conical tubes

Spin at 4000 rpm for 2min at 4C

Transfer the supernatant to ice cold 1.5 ml eppendorff, then spin at 10,000rpm for 10min at 4C to give the final lysate.

OD the cell lysate at 260nm. 10 OD cell lysate is used for each IP.

10 OD cell lysate is mixed with 2×binding buffer, then added to the washed agarose beads.

Binding is allowed for 2 hours at 4C on a rotating platform, with low rotating speed. Then the beads are pelleted by brief centrifugation.

Wash the beads five times with 300ul ice-cold IXA-100 buffer

IXA-100 buffer: 50 mM Tris-HCl pH7.5, 100 mM KCl, 12 mM Mg(OAc), 1mM DTT, 100 ug/ml cycloheximide,

Note, the salt concentration is adjustable, for example, IXA-200 means 200 mM KCl.

Elution is achieved by incubating the beads in 50ul of IXA-100 buffer containing the FLAG-peptide at 100 ug/ml for 20 min at 4C on a rotating platform with low rotating speed.

10ul 6X SDS-PAGE loading dye was added to the elutant.

SDS-PGAE and western are performed to detect XRN1-HA or DCP2-HA

CIRCULARIZATION RT-PCR

Circularization RT-PCR (cRT-PCR) can be used to detect monophosphorylated RNA (i.e. – decapped mRNA). The 5' monophosphate can be ligated to the 3' hydroxyl of the same RNA molecule, and unique products across the 5'-3' junction are detectable by RT-PCR. Include –ligase, -RT controls.

Ligation

DEPC dH2O
10 µL 10X T4 RNA Ligase buffer
10 µL 100% DMSO
0.5 µL (5 units) T4 RNA ligase (Promega)
Input RNA in DEPC dH2O (determine amount empirically for each RNA)
Final Volume – 100 µL
Incubate 37°C for 30 min
Clean up by phenol/chloroform extraction, ethanol precipitation
Resuspend in DEPC dH2O

RT

Mix 1:
DEPC dH2O
2 µL 5X first strand buffer
1 µL 100 mM DTT
1 µL (2 pmol) gene specific primer
ligated RNA (determine amount empirically for each RNA)
Final Volume = 10 µL
Incubate 65°C for 10 min -> ice 1-2 min

Make mix 2:
2 µL 5X first strand buffer
1 µL 100mM DTT
1 µL 10 mM each dNTP
DEPC dH2O
0.5 µL SuperScript II RT (Invitrogen)
10 µL mix 1 + 10 µL mix 2
incubate 37°C for 45 min, 65°C for 15 min
can store at -20°C

PCR

Use Taq (NEB), set up standard 20 µL PCR reactions using gene specific primers, cDNA from above. Primers should be a forward primer in the 3' end of the RNA, and a reverse primer in the 5' end of the RNA such that the primers would be facing away from each other in the original linear RNA (and genomic DNA). Annealing temperature is based on primers; number of cycles for linear detection determined empirically for each RNA.

Separate products on agarose gels, visualize bands by ethidium bromide staining.

POLY(A) TAILING ASSAY

The poly(A) tailing assay is used to analyze the poly(A) tail distribution for a given mRNA. Yeast poly(A) polymerase (yPAP) is used to add poly(G):poly(I) tails to RNA, and a primer with a unique adaptor at the 5' end, a stretch of C's, and 2-3 T's (to anchor the primer at the poly(A) tail) is used for reverse transcription. PCR using a primer complementary to the adaptor of the RT primer and a forward gene specific primer allows detection of products whose only differences in size will be the poly(A) tail length. The gene specific primer should be designed to be as close to the 3' end of the mRNA as possible, so different tail lengths can be resolved. PCR with the forward gene specific primer and a gene specific reverse primer complementary to the cleavage and poly(A) site should be used as an A0 control. Also include -yPAP, -RT controls.

DNase I treatment of RNA

DEPC dH2O
10 µL 10X DNase I buffer
1 µL DNase I (Roche)
RNA
Final volume = 100 µL
Incubate at 24°C for 15 min
Add 10 µL 25 mM EDTA, incubate 65°C for 10 min
Clean up by phenol/chloroform extraction, ethanol precipitation
Resuspend in DEPC dH2O

Tailing reaction (latest protocol from USB)

DEPC dH2O
4 µL 5X tail buffer mix
RNA
2 µL tail enzyme mix
Final volume = 20 µL
Incubate at 37°C for 60 min in a thermal cycler
Add 2 µL 10X tail stop solution
Can store at -20°C

RT

5 µL tailed RNA
4 µL 5X RT buffer (has NTPs, primer)
2 µL 10X RT enzyme mix (USB)
9 µL dH2O
Incubate at 44°C for 60 min in a thermal cycler, then 92°C for 10 min
Dilute RT reaction with 20 µL dH2O

PCR

dH2O
Diluted RT reaction (up to 5 µL, determine empirically)
1 µL 10 µM forward gene specific primer
1 µL 10 µM reverse primer complementary to tail adaptor

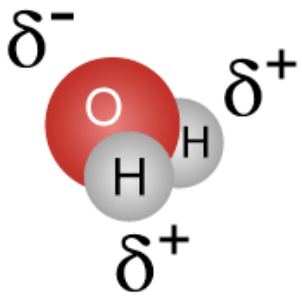
5 µL 5X PCR buffer (with Mg and NTPs)
1 µL HotStart Taq (1.25 U)
Final volume = 25 µL
Cycling: 94°C 2 min -> 30-35X of 94°C 10 sec, 60°C 30 sec, 72°C 30 sec -> 72°C 5 min -> 4°C hold (cycling may need to be altered according to RNA analyzed)
Separate products on 3% agarose gels, visualize bands by ethidium bromide staining of gel after run using 0.1 µg/mL ethidium for 30 min. Destain in water as needed.

THE BASIC PRINCIPLES OF HOW ETHANOL PRECIPITATION OF DNA AND RNA WORKS

Ethanol precipitation is a commonly used technique for concentrating and de-salting nucleic acid (DNA or RNA) preparations in aqueous solution. The basic procedure is that salt and ethanol are added to the aqueous solution, which forces the nucleic acid to precipitate out of solution. The precipitated nucleic acid can then be separated from the rest of the solution by centrifugation. The pellet is washed in cold 70% ethanol then after a further centrifugation step the ethanol is removed, and the nucleic acid pellet is allowed to dry before being resuspended in clean aqueous buffer. So how does this work?

A bit about solubility...

First we need to know why nucleic acids are soluble in water. Water is a polar molecule - it has a partial negative charge near the oxygen atom due the unshared pairs of electrons, and partial positive charges near the hydrogen atoms (see the diagram on the right). Because of these charges, polar molecules, like DNA or RNA, can interact electrostatically with the water molecules, allowing them to easily dissolve in water. Polar molecules can therefore be described as hydrophilic and non- polar molecules, which can't easily interact with water molecules, are hydrophobic. Nucleic acids are hydrophilic due to the negatively charged phosphate (PO3-) groups along the sugar phosphate backbone.



The role of the salt...

The role of the salt in the protocol is to neutralize the charges on the sugar phosphate backbone. A commonly used salt is sodium acetate. In solution, sodium acetate breaks up into Na+ and [CH3COO]-. The positively charged sodium ions neutralize the negative charge on the PO3- groups on the nucleic acids, making the molecule far less hydrophilic, and therefore much less soluble in water.

The role of the ethanol...

The electrostatic attraction between the Na+ ions in solution and the PO3- ions are dictated by Coulomb's Law, which is affected by the dielectric constant of the solution. Water has a high dielectric constant, which makes it fairly difficult for the Na+ and PO3- to come together. Ethanol on the other hand has a much lower dielectric constant, making it much easier for Na+ to interact with the PO3-, shield it's charge and make the nucleic acid less hydrophilic, causing it to drop out of solution.

The role of temperature...

Incubation of the nucleic acid/salt/ethanol mixture at low temperatures (e.g. -20 or -80C) is commonly cited in protocols as necessary in protocols. However, according to Maniatis et al (Molecular Cloning, A Laboratory Manual), this is not required, as nucleic

acids at concentrations as low as 20ng/mL will precipitate at 0-4C so incubation for 15-30 minutes on ice is sufficient.

The wash step with 70% ethanol…

This step is to wash any residual salt away from the pelleted DNA.

A few tips on nucleic acid precipitation…

Choice of salt

Use Sodium acetate (0.3M final conc, pH 5.2) for routine DNA/RNA precipitations

Use Sodium chloride (0.2M final conc) for DNA samples containing SDS since NaCl keeps SDS soluble in 70% ethanol so it won't precipitate with the DNA.

Use Ammonium acetate (2M final conc) for the removal of dNTPs, but do not use for preparation of DNA for T4 polynucleotide kinase reactions as ammonium ions inhibit the enzyme.

To increase the yield in precipitations of low concentration or small nucleic acid pieces (less than 100 nucleotides)

Add MgCl2 to a final concentration of 0.01M
Increase the time of incubation ice before centrifugation to 1 hour.

Preparation of Dialysis Tubing

Cut the tubing into pieces of convenient length (10-20cm).
Boil for 10 minutes in a solution of 2% sodium bicarbonate and 1mM EDTA.
Rinse the tubing thoroughly (at least three times) with distilled water.
Place tubing in 500mL bottle and fill with distilled water so tubing is submerged.
Autoclave tubing according to the liquid cycle parameters.
Store at 4°C until use.

ECL Reagent: Homebrew

Advantages: almost no cost; stable/reproducible, stored in frozen aliquots. This recipe make enough ECL for 9500 blots!! Total cost is about $100.

STOCK Luminol:

250 mM 3-aminopthalhydrazide (Sigma A8511-5G)
To make add 266 mg and dissolve in 6 mL of DMSO
Store at - 20°C in 60 µL aliquots

STOCK Coumaric Acid:

90 mM coumaric acid (Sigma C9008)
To make dissove 38 mg in 2.5 mL of DMSO
Store at - 20°C in 25 µL aliquots

To probe one blot:

Solution A (stable 1 month at 4°C)

5 mL cold 100 mM Tris pH 8.8
22 µL 90 mM Coumaric Acid
50 µL 250 mM Luminol

Solution B (stable 1 month at 4°C)

5 mL cold 100 mM Tris pH 8.8
3 µL H_2O_2

Mix Solution A with Solution B and apply to blot 1 min
Signal will last for 10 mins

YEAST GENOMIC DNA PREPARATION

Grow 10-25 mL of a yeast culture to at least OD600 ~1.0 (overnight or two day culture).
Collect cells by centrifugation at 4,000 rpm for 2 min. and resuspend cell pellet in 1 mL of dH2O; transfer suspension to a 2 mL Eppendorf tube and pellet cells at 14,000 rpm for 30 sec. Remove supernatant and store pelleted cells at -80°C.
Resuspend cell pellet in 200 µL DNA extraction buffer.

DNA extraction buffer (5 mL)
100 µL Triton X-100
500 µL 10% SDS
100 µL 5M NaCl
50 µL 1M Tris-HCl pH 8.0
10 µL 0.5M EDTA pH 8.0
4.24 mL dH2O

Add 200 µL P/C/L and an equal volume (to approx. 1 mL marker on Eppendorf) of glass beads.
Vortex 15 min. at RT.
Add 300 µL DNA extraction buffer (minus the SDS) and 300 µL P/C/L.
Vortex 5 min. followed by centrifugation for 5 min. at 14,000 rpm.
Transfer the approx. 450 µL of aqueous phase to a fresh 1.5 mL Eppendorf tube; precipitate with 1 mL 95% EtOH at -80°C for 30 min.
Pellet the DNA by centrifugation at RT for 10 min., wash pellet once with 500 µL 70% EtOH, and air dry pellet.
Resuspend DNA pellet in 400 µL TE (pH 8.0), and 20 µL RNase A (10 mg/mL), and incubate at 37°C for 30 min.
Extract DNA once with an equal volume of P/C/L and transfer aqueous phase to a fresh Eppendorf tube.
Add 125 µL 2M NH4OAc* and precipitate DNA with 1.0 mL 95% EtOH by incubating for 30 min. at -80°C.
Collect DNA by centrifugation for 10 min. at 14,000 rpm, wash pellet once with 500 µL 70% EtOH, and air dry pellet.
Resuspend DNA pellet in 50-100 µL dH2O.

*Precipitation of DNA with 2M NH4OAc is used to help decrease the co-precipitation of dNTP's and oligomers.

RNA Extraction from Polysome Gradient Fractions

The protocol of RNA extraction from polysome gradient fractions is modified from protocols described in literature (Cigan et al., 1991; Nielsen et al., 2004).
:

1) Add 1250 µl cold ethanol to each faction, mix well, precipitated overnight at -80°C. Note: the volume of each gradient fraction is about 625 µl.
2) Pellet the RNA-protein by centrifugation at 14,000rpm for 30 min in a cold room.
3) Wash the RNA-protein pellet with 500 µl of 75% ethanol.
4) Add 450 µl LET (LET: 25mM Tris-HCl pH 8.0, 100 mM LiCl, 20 mM EDTA) and 50 µl 10% SDS, vortex 5 min to get the pellet re-suspended.
5) Extract the RNA twice with Phenol/CHCl3/LET (each time, vortex for 5min, then centrifuge for 5 min).
6) Transfer the supernatant to an empty Eppendoff tube.
7) Add 40 µl 7.5M NH4OAC, 1 µl of glycoblue (Ambion), and 1ml of cold 95% ethanol, precipitate at -800C overnight.
8) Pellet the RNA by centrifugation at max speed of a bench top centrifuge for 20 min, wash the pellet with 500 µl 75% ethanol.
9) Re-suspend the RNA pellet in appropriate amount of H2O for downstream analysis, such as Northern blot and primer extension analysis.

Note: for the gradient fractions from formaldehyde cross-linked cells, the RNA extraction procedure is the same as above, except changing step 5 to: Add equal volume (500ul) of Phenol/CHCl3/LET, vortex 15s, incubate at 70°C for 15 min (vortex every 1 min for 10s), to reverse cross-linking, then centrifuge for 5 min. Transfer the supernatant to a new Eppendoff tube, Add equal volume (500 µl) of Phenol/CHCl3/LET, vortex 15s, incubate at 70°C for 15 min (vortex every 1 min for 10s), to reverse cross-linking, then centrifuge for 5 min.

FORMALDEHYDE CROSS-LINKING OF YEAST CELLS

1) Grow the yeast cells in appropriate media at 240C to early log phase (OD600 = 0.4).

113

2) Add 50 mg/ml cycloheximide to final concentration of 100 μg/ml, and at the same time add 37% formaldehyde to final concentration of 0.25%.
3) Incubate the culture at 240C with shaking at 200 rpm for 5 min.
4) Then add 2.5 M glycine to final contraction of 125 mM, incubate the culture at 240C with shaking at 200 rpm for 10 min.
5) Harvest the culture by spinning at 4,000 rpm for 2 min. Discard the supernatant.
6) Re-suspend the pellet in the residual medium, and transfer it to a 2 ml Eppendoff tube.
7) Spin at 10,000 rpm for 30 s. Discard the supernatant.
8) Put the cell pellet on dry ice for 5 min, then transfer to -800C. Store until use.

MAPPING SINGLE END READS IN GALAXY (HTTP://MAIN.G2.BX.PSU.EDU/)

1. Get data using the "Get Data" Tool at the left side bar. Alternatively use data that is already in a saved history if that data has already been uploaded.

2. Make sure the data is in fastqsanger format. If the data is not in fastqsanger run the reads through the fastq groomer under the heading "NGS: QC and manipulation" at the left.

3. Check the quality of the reads by going to "NGS: QC and manipulation" at the left and click on "compute quality statistics" and choose the reads that you will be analyzing (the fastqsanger file). Click Execute.

4. While the quality statistics are being generated you can set up the next step in the workflow. Click on "Draw quality score boxplot" under "NGS: QC and manipulation" at the left. Choose the output of the previous step and click execute.

5. Click on the eye icon when the job is finished. Look at the quality of the reads the higher the numbers on the y axis indicate better quality. Want the quality to be at least above 20 and best if it is above 28. Note where the highest quality is so that the reads can be trimmed accordingly by bowtie.

6. Map the reads by clicking on "Map with Bowtie for Illumina" under "NGS: Mapping" at the left. Select the appropriate reference genome, singel-end, the FASTQ file is the the fastqsanger file. If you need to trim the reads you will change Bowtie settings to use to full parameter list and change the trim bases off the reads to reflect the number of bases you will be trimming off the end of the read. Click execute.

7. The output of Bowtie is a file in SAM format. You need to filter the SAM file so go to "NGS: SAM Tools" at the left and click on "Filter SAM." The data to filter is the output of Bowtie. Click on "Add a new flag." To filter out reads that did not map click "The read is unmapped" and set the states for this flag to No. Click execute.

8. Convert the SAM output of the filtered reads to BAM by clicking on "SAM-to-BAM" under "NGS: SAM Tools." Use the default parameters and convert the filtered data. Click execute.

9. When this is done click on the SAM-to-BAM at the right and then click on the link to display at UCSC main to look at the data on the UCSC genome browser.

10. Digital ribosomal read removal
"Filter and Sort" then clicked "Select." I selected the SAM file of the merged data, under "that" selected "NOT Matching," under "the pattern:" I copied and pasted the regular expression "chrXII (45[1-9][0-9][0-9][0-9]|46[0-8][0-9][0-9][0-9])" Note: The TAB between the "chrXII and (45" must be a TAB. Best to just cut and past from this text.

11. Separate by strand
Next we separated the resulting dataset based on strand. To do this we went to to "Filter and Sort" the clicked "Filter" then chose the ribo filtered file to filter by strand and we put in "c2==0" or "c2==16" under "with following condition." The c2==0 gives the plus strand or Watson strand and this expression means to keep all of the lines with that value. The c2==16 returns everything then on the minus strand or the Crick strand.

CUFFLINKS WORKFLOW

Begin with a Bowtie-mapped SAM file that has been filtered to remove ribosomes and separated by strand.

To run a SAM file through Cufflinks, the data in the file must be sorted by chromosomal location. This can be done with an imported workflow (recommended), or manually:
Galaxy has a public workflow that allows you to input a SAM file, and will return a SAM file that has been sorted as needed for Cufflinks. To import this workflow, go to https://main.g2.bx.psu.edu/u/jeremy/w/sort-sam-file-for-cufflinks and click "Import workflow" at the top of the page. To access this workflow, open the history with the file to be sorted. Click "Workflow" at the top of the page, click "Imported: Sort SAM file for Cufflinks" and select "Run." Use your SAM file as the input dataset. The output file will be a sorted SAM file that is suitable for Cufflinks.
Alternative, you can perform each step of the above workflow individually instead of importing the workflow. These steps are:
Select (under Filter and Sort) on your input SAM file, that "NOT matching" the pattern "^@" This will select your data only, and remove the headers for the sorting step
Select (under Filter and Sort) on the same input SAM file, that "matching" the pattern "^@" This will maintain the headers for your data in their own separate file
Sort (under Filter and Sort) the output file from step i on column "c3" with flavor "Alphabetical sort" everything in "Ascending order". Add new column selection and sort on column "c4" with flavor "Numerical sort" everything in "Ascending order" This will sort your data based on chromosome name (column 3) and then position (column 4).
Concatenate (under Operate on Genomic Intervals), Concatenate your output from step ii with your output from step iii. This will merge your data headings back into the file containing your data.

Run Cufflinks on the sorted SAM file. Select Cufflinks under NGS: RNA Analysis. Use the SAM file sorted in step 2 above as your input file.
To add a Reference Annotation, you must import a reference GTF file from the UCSC Browser. On the UCSC browser home page, select "Tables" at the top of the page. Locate the correct genome and version [i.e. genome S. cerevisiae, assembly June 2008 (SGD/sacCer2)]. Selected "SGD Genes" as track, and be sure "genome" is selected under region. Select "GTF - gene transfer format" as the output format, and select Send

output to "Galaxy." Click "get output," and then "Send query to Galaxy" on the following page.
You do not have to use a Reference annotation - if you do not, your cufflinks will not be assigned to specific genes, but this also prevents loss of unannotated transcripts such as antisense non-coding RNAs.

To find changes between two files, run Cuffdiff under NGS: RNA Analysis. Select a cufflink GTF file (from step 3 above) to define your Transcripts. Choose your sorted SAM files (from step 2 above) for each of the SAM or BAM file of aligned RNA-Seq reads that you are comparing.
For consistency, select your WT or control sample as your cufflink file. Also input your WT or control sample as the first SAM file, and use your experimental sample as the second SAM file.
Cuffdiff will return 11 different output files. The one you should focus on is the 4th one, "Gene differential expression testing," which should contain FMPK values for your first SAM file as column 8 (value_1), FMPK values for your second SAM file as column 9 (value_2), fold changes between the samples in column 10 [log2(fold change)], p values, and statistical significance among other data.

RNA IMMUNOPRECIPITATION FROM YEAST CELLS

Yeast strains for analyzing the association of HA-tagged Upf1 with endogenous RNAs were inoculated into 5 mL YPD and grown overnight at 30 °C. Strains expressing plasmid-based reporters were inoculated into 5 mL synthetic dextrose lacking uracil (SD–URA) liquid media. The following morning, overnight cultures were diluted to an OD_{600} of 0.075-0.125 into 100 mL of YPD (for endogenous targets) or synthetic minimal–URA media containing 2% galactose, 1% sucrose (for plasmid-based reporters).

1) Grow cells to an OD_{600} of 0.6 and harvest at RT by centrifugation for 2 min at 4,000 rpm.

2) Decant media, resuspend cell pellets into 1 mL dH_20 by vortexing, and transfer to a 2 mL Eppendorf tube. Pellet cell suspension for 30 sec at 13,300 rpm. Remove dH_20 by aspiration and flash freeze pellets on dry ice (cell pellets can be stored at -80 °C until needed).

3) Resuspend cell pellet in 400 µL of cold lysis buffer (200 mM KCl, 5 mM $MgCl_2$, 150 mM HEPES pH 7.0, 0.5% NP-40, and 1 mM DTT).

4) Add ~200-300 µL of acid washed glass beads; parafilm seal lids and conduct cell lysis as follows: vortex for 3½ min at 4 °C followed by an incubation on wet ice for 2 min. Repeat 4x.

5) Use a hot, 21-gauge needle to puncture the bottom of the 2 mL Eppendorf tube: immediately place tube in a 15 mL conical on wet ice. Separate cell lysate from the glass beads by spinning Eppendorf/conical at 4 °C for 2 min at 3,000 rpm.

6) Transfer lysate to a new 1.5 mL Eppendorf tube and spin for an additional 2 min at 8,000 rpm at 4 °C. Transfer cleared lysate to a new 1.5 mL Eppendorf tube.

7) Quantify protein lysate by diluting 5 µL into 1 mL dH_20 and determining OD_{260} using a UV spectrophometer.

8) Pre-bind antibody by diluting 15 OD units of cell lysate into cold lysis buffer (200 mM KCl, 5 mM $MgCl_2$, 150 mM HEPES pH 7.0, 0.6% NP-40, and 1 mM DTT also containing freshly added 0.66 mM vanadyl ribonucleoside complex, 1 µL Sigma Protease Inhibitor Cocktail [fungal extract specific], 15 units Roche DNAse I recombinant [RNAse-free], and 4 µL Covance Anti-HA Monoclonal Antibody) to a total final volume of 600 µL in a 1.5 mL Eppendorf tube.

9) Incubate for 1 hour at 4 °C with gentle agitation (i.e. rotator or Nutator). Transfer lysate to a new 1.5 mL Eppendorf tube containing 75 µL of Protein G Dynabeads prewashed with 400 µL lysis buffer. Incubate lysate + beads for 1 hour at 4 °C with gentle agitation.

10) Following incubation, separate unbound lysate from beads and bound material using an Invitrogen DYNAL magnetic bead separator. Wash beads and bound material on a rotator at RT as follows:

   a. (2x) 600 µL lysis buffer plus 0.1% SDS, 1 M urea for 5 min.

   b. (2x) 600 µL lysis buffer for 5 min.

   c. (1x) 600 µL lysis buffer for 20 min.

****increasing wash times may increase signal to background ratio*

11) Following the final wash, resuspend samples in 600 µL of lysis buffer and transfer to a new 1.5 mL Eppendorf tube (this eliminates any contamination that may have stuck to the original tube).

12) Place tube on magnet, remove lysis buffer, and resuspend beads and bound material in 400 µL TE pH 7.0.

FOR PROTEIN ANALYSIS:

13) Resuspend beads by pipetting to homogeneity, then quickly remove 5 µL of resuspended solution into a new Eppendorf tube containing 10 µL elution buffer (20 mM Tris-HCl pH 7.5, 1.5% SDS, 50 mM EDTA). Heat at 95 °C for 5 min.

14) Directly after heating, place tube on magnet, remove supernatant and transfer elution into a new Eppendorf tube. Add 3 µL of 6x SDS loading dye.

15) Heat at 95 °C for 5 min and run samples on 6% SDS PAGE gel and transfer to PVDF membrane for Western blotting.

FOR RNA ANALYSIS

16) Add 400 µL of P/C/T (Phenol/Chloroform/TE) to the resuspended beads and bound material (from step 12) and vortex sample for 5 min at RT. Subsequently, centrifuge samples at 13,300 rpm for 5 min. Place tube on magnet and remove top aqueous layer (~400 µL) to a new 1.5 mL Eppendorf tube containing 400 µL of chloroform.

17) Vortex for 5 min at RT and subsequently centrifuge at 13,300 rpm for 5 min. Transfer the top aqueous layer to a new 1.5 mL Eppendorf tube containing 40 µL 3M NaOAc pH 5.2 and 1 µL glycoblue (to aid RNA precipitation). Mix, then add 2.5x volumes of 95% EtOH (~1.0 mL) and precipitate sample overnight by incubating at -20 °C.

18) Collect RNA by centrifugation (13,300 rpm) for 10 min at RT. Remove supernatant by pipetting and wash RNA pellet once with 500 µL 75% EtOH (invert tube 2-3 times to mix). Centrifuge (13,300 rpm) for 10 min at RT and gently decant supernatant. Quickly pulse centrifuge tubes and remove remaining residual EtOH with a pipette. Air dry RNA pellet ~5 min.

19) Resuspend RNA pellet in 10.5 µL DEPC-treated dH_2O and store at -80 °C.

REVERSE TRANSCRIPTION REACTION OF RIP RNA

20) Heat RNA at 65 °C for 5 min prior to RT reaction; immediately place on ice.

21) Combine in 0.2 mL PCR tube:

1.5 pmol gene-specific RT primer

5 µL RNA (from RIP, step 19 above) **or** 1µg DNAse-treated total input RNA

1 µL 10 mM dNTP

DEPC dH_20 to 13 µL

22) Heat reaction (convenient to use PCR machine here) at 70 °C for 5 min; slow cool reaction as follows: 30 sec @ 65 °C, 30 sec @ 60 °C, 30 sec @ 55 °C, 30 sec @ 50 °C, and 5 min @ 4 °C.

23) Spin down tubes and add:

4 µL 5X First Strand Buffer

1 µL 0.1 M DTT

1.4 µL DEPC dH_20 (2 µL in –reverse transcriptase reaction)

0.6 µL Superscript III RTase

24) Incubate at 55 °C for 60 min. Subsequently, heat reaction at 70 °C for 15 min. Store at -20 °C until used in PCR reaction (we use 2 µL per PCR reaction).

YEAST CHROMATIN IP

1. Grow culture to OD~0.8
2. Transfer 50ml of culture to conical and add 1.4ml 37% Formaldehyde
3. Incubate nutator 15 min RT
4. Quench cross-linking by adding 3.4ml 2M Glycine
5. Incubate RT 5 minutes
6. Centrifuge cells (5 minutes 3000rpm), discard supt
7. Wash cells with 10ml ice-cold TBS
8. Resuspend in 1ml ice cold TBS, move to epi
9. Centrifuge, discard supt, put on ice (or freeze in N2)

Everything after this point is cold with protease inhibitors

10. Resuspend pellet in 400µl lysis buffer, add 400µl glass beads
11. Vortex 10 minutes, ice 15 minutes
12. Drain extract (hole in bottom of epi) into new epi, rinse beads with 400µl lysis buffer
13. Sonicate – Branson sonicator, micro tip, constant output, 15%-20% power, 6X 10 second pulse with 2 minutes on ice between each pulse
14. Spin max, 5 min, 4C°, move supt to new epi
15. Spin max, 15 min, 4C°, move supt to new epi
16. Add 30µl protein A sepharose slurry and incubate 1 hour 4C° nutator
17. Spin 7.5K, 2 min, 4C°, move supt to new epi
18. Add primary antibody and incubate 4 hours – O/N 4C° on nutator
    Be sure to take 50µl of sample for input sample
19. Add 30µl protein A sepharose slurry and incubate 1hr, 4C° nutator
20. Spin 7.5K, 2 min, 4C
21. Keep 500µl supt for sizing if needed, discard rest of supt
22. Add 1ml lysis buffer, incubate 5 min, nutator, 4C, then spin 7.5K, 2 min, 4C, discard supt
23. Repeat with 1ml lysis buffer-500
24. Repeat with 1ml LiCl/detergent
25. Repeat with 1ml TBS

Don't need cold or protease inhibitors anymore

26. Add 100µl 1%SDS/1XTE to beads, mix, incubate 10min 65C°
27. Spin max, 15sec, and transfer eluate to new epi
28. Add 150µl 0.67%SDS/1XTE to beads, mix
29. Spin max, 15sec, and transfer eluate to previous eluate
30. Incubate input and IP samples for 6+ hrs 65C°
    *For input samples – add 200µl 0.67%SDS/1XTE**
31. Add 250µl ProK solution to samples and incubate 2hrs, 37C°
32. Add 55µl 4M LiCl to samples
33. Add 500µl P/C/IAA to samples
34. Vortex 1 min
35. Spin max 10 min RT
36. Transfer aqueous to new epi and add 1ml EtOH

37. Spin max, 15 min, RT
38. Discard supt, dry pellet
39. Resuspend samples in 100µl TE
40. Store at -20C°
41. Analysis of your choice…

1X TBS
- 150mM NaCl
- 20mM Tris-HCl, pH7.6

Lysis Buffer
- 0.1% Deoxycholic acid
- 1mM EDTA
- 50mM HEPES/KOH, pH7.5
- 140mM NaCl
- 1% Triton X-100

Lysis Buffer-500
- 0.1% Deoxycholic acid
- 1mM EDTA
- 50mM HEPES/KOH, pH7.5
- 500mM NaCl
- 1% Triton X-100

LiCl/detergent wash
- 0.5% Deoxycholic acid
- 1mM EDTA
- 250mM LiCl
- 0.5% NP-50
- 10mM Tris-HCl, pH8

Proteinase K solution
- 1ml 20ml/ml glycogen
- 5ml 20mg/ml Proteinase K
- 244.5ml 1X TE, pH7.6

mRNA-specific Ribonucleoprotein Purification

Before Experiment:

1. Grow 200 mL culture of cells to OD_{600} = 0.4. Cross-link with formaldehyde to 0.25% for 15' at 30°C (1.35 mL of 37% formaldehyde per 200 mL culture), followed by quenching with glycine to 0.125 *M* for 5' at 30°C (11 mL of 2.5*M* per 200 mL culture). Harvest cells at 4000 RPM for 1', and decant media. Resuspend pellet in residual media and transfer to 2 mL eppendorf tube. Pellet cells at 13,200 RPM and flash freeze on dry ice.

Day 1:

2. Perform polysome lysis procedure as described:
    a. Supplement 1x lysis buffer (**without heparin or CHX**) with DTT to 1 mM
    b. Resuspend each cell pellet in 400 µL 1X lysis buffer with brief vortex
    c. Add ½ volume of glass beads
    d. Vortex at 4 C for 3' (During vortexes, fast cool tabletop centrifuge)
    e. Ice 2'
    f. Vortex at 4 C for 3'
    g. Ice 2'
    h. Vortex at 4 C for 3'
    i. Puncture the bottom of the 2 mL tube with a red hot 18 gauge needle and place in 15 mL conical vial. Spin at 2000 RPM for 2' at 4 C.
    j. Transfer supernatant to a cold 1.5 mL eppendorf tube, and adjust volume for any lost buffer/sample by bringing up to 400 µL with additional lysis buffer.
    k. With remaining cell pellet, resuspend in lysis buffer and transfer to 2 mL tube. Pellet at 13,200 RPM. Use pellet to repeat lysis (steps b-j) and combine with first lysate.
    l. Quantify lysate OD_{260} and OD_{280} by diluting 5 µL in 995 µL dH_2O, using lysis buffer in dH_2O as a blank.
    m. Bring the volume of 800 µL lysate (2 lyses of a single 200 mL pellet) up to 3 mL with TE buffer (2.2 mL). Save a small amount of input, ideally 1/10 for RNA or 1/20 for protein.

n. Prepare 5 mL hybridization reactions for each sample in a 15 mL conical vial. (This can be scaled up to 10 mL in a 15 mL conical vial with no decrease in efficiency)

3 mL lysate in TE buffer	
500 mM LiCl	(500 µL 5 M stock)
0.5% SDS	(250 µL 10% stock)
50 mM EDTA	(500 µL 0.5 M stock)
10 mM Tris, pH 7.5	(50 µL 1 M stock)
14% formamide	(700 µL)
Fungal protease inhibitors	5 µl

3. Immobilize biotinylated oligo to streptavidin Dynabeads

   a. Resuspend Dynabeads completely by pipetting, and transfer enough beads to 1.5 mL eppendorf tube(s) for all samples. Use 250 µL beads per 200 mL cell pellet.

   b. Place beads on magnet and remove supernatant.

   c. Wash beads with an equal volume of 1x B&W buffer.

   d. Remove wash, and repeat twice.

   e. Incubate beads with biotinylated oligo for 15' at room temperature, in a total volume 4x the original bead volume:

      a. 2x volume of 2x B&W buffer

      b. 4 nmol biotinylated oligo per 100 µL beads (10 µL of 400 pmol/µL stock per 100 µL beads)

      c. DEPC dH_2O to 4x volume

   f. Wash beads 2x in 1x B&W buffer, in ½ volume as incubation. At each step, place beads on magnet and remove and discard supernatant.

   g. Resuspend beads in 1x B&W buffer to original volume, and aliquot volume of beads needed per sample into individual tubes.

4. Anneal RNA to Beads

a. Use an aliquot of each sample in hybridization buffer to resuspend Dynabeads, and transfer to 15 mL vial. Parafilm lid.

b. Incubate overnight at room temperature, rotating.

Day 2:

5. Briefly spin conical vials to collect liquid.
6. Remove supernatant from beads. Will need to do this in several steps; keep adding hybridization solution to same 1.5 mL eppendorf to collect all beads in one tube. Save supernatant if going to be analyzed.
7. Wash beads:
   a. Wash beads 2x in Wash Buffer 1, using ~twice the original volume of beads
   b. Wash beads 3x in Wash Buffer 2, using ~twice the original volume of beads. After collecting supernatant, briefly spin down beads to collect any residual wash buffer to help ensure complete removal of SDS.
8. Elute RNA and Proteins
   a. Resuspend beads in 75 µL dH_2O per 100 µL beads (93.5 µL for 125 µL beads).
   b. Heat beads at 70°C for 2 minutes to break annealing interaction
   c. Place beads <u>immediately</u> on magnet and remove eluate. If any beads are carried over into eluate, place new tube on magnet and transfer eluate one additional time to remove beads.
9. RNA Analysis:
   a. Precipitate input samples by bringing volume up to 400 µL with 0.2 M NaCl (20 µL 4M NaCl) + 340 µL DEPC dH_2O + 1.5 µL glycoblue and precipitating overnight at -20°C with 1 mL 95% EtOH. Precipitate supernatant samples in 500 µL by adding 25 µL 4M NaCl + 1.5 µL glycoblue, and precipitate overnight at -20°C with 1 mL 95% EtOH. Precipitate elution samples in a total volume of 400 µL, with 0.2 M NaCl (20 µL of 4M stock), DEPC dH_2O to 400 µL, 1.5 µL glycoblue, and 1 mL 95% EtOH at -20°C overnight.

b. Pellet RNA at 13.2 K for 10 minutes. Wash in 500 µL 75% EtOH, vortex for 10 seconds, centrifuge at 13.2 K for 10 minutes, and remove EtOH.

c. Resuspend pellet in 450 µL LET + 50 µL 10% SDS by vortexing for 5 minutes.

d. Reverse crosslinking by heating at 65°C for 1 hour.

e. Extract from proteins 1 x P/C/L, 1 x chloroform.

f. Precipitate RNA overnight in 0.2 M NaCl (20 µL 4 M stock for 400 µL), 1 µL glycoblue, and 1 mL 95% EtOH.

g. Pellet, wash, and air dry RNA. Resuspend in LET for Northerns, or DEPC dH2O for qRT-PCR analyses.

10. Protein analysis, for gel

a. Concentrate proteins in SpeedVac on high heat until volume is ~20 µL (about 1.5-2 hours)

b. Precipitate proteins by adding 4x volumes of cold acetone. Incubate for 1 hour at -20C.

c. Spin at 14K RPM at 4°C for 10' to pellet proteins. Wash with 80% acetone.

d. Remove supernatant and air dry pellet.

e. Resuspend in 1x SDS sample buffer by vortexing 5' or until pellets are completely resuspended. Also run buffer along sides of tubes extensively.

f. Heat eluate at 70°C for 1 hour followed by 95°C for 5' to reverse crosslinks. Sample is now ready for SDS-PAGE gel.

11. Protein analysis, for MS*:

a. Heat eluate at 70°C for 1 hour + 95°C for 5 minutes to reverse crosslinks.

b. Concentrate eluate in SpeedVac on high heat until volume is ~20 µL (about 1.5-2hr)

c. Precipitate with 4x volumes of cold acetone. Incubate for 1 hour at -20C.

d. Spin at 14K RPM at 4°C for 10' to pellet proteins. Wash with 80% acetone.

e. Remove supernatant and air dry pellet.

f. Resuspend pellet 6 M urea, 100 mM Tris buffer pH 7.8.

*Alternatively, samples can be provided on-bead to MS facility for on-bead trypsin digestion and elution

1X Polysome Lysis Buffer (Store at -20 C)	50 mL
10 mM Tris pH 7.4	(5 mL 10X lysis buffer)
100 mM NaCl	(5 mL 10X lysis buffer)
30 mM $MgCl_2$	(5 mL 10X lysis buffer)
Roche complete EDTA-free protease inhibitor cocktail tablet	1 tablet per 50 mL
DEPC water to volume	45 mL dH_20
*1 mM DTT	100 µL 0.5M DTT

*Add fresh, day of, to small working aliquot of buffer

2x Binding and Washing Buffer (B&W Buffer)	50 mL
10 mM Tris-HCl (pH 7.5)	500 µL 1M Tris-HCl (pH 7.5)
1 mM EDTA	100 µL 0.5 M EDTA
2 M NaCl	25 mL 4 M NaCl
	24.4 mL DEPC dH_20

Wash Buffer 1 50 mL

10 mM Tris (pH 7.5)	500 µL 1 M Tris (pH 7.5)
1 mM EDTA	100 µL 0.5 M EDTA
250 mM LiCl	12.5 mL 1 M LiCl
0.1% SDS	500 µL 10% SDS
	36.4 mL DEPC dH_2O

Wash Buffer 2 50 mL

10 mM Tris (pH 7.5)	500 µL 1 M Tris (pH 7.5)
1 mM EDTA	100 µL 0.5 M EDTA
100 mM LiCl	5 mL 1 M LiCl
	44.4 mL DEPC dH_2O

RIBOSOMAL FOOTPRINTING

SUGGESTED TIMELINE

Preparation of ribosomal footprint library

In advance

1) Using a starter culture grown overnight at 30°C in SD Complete, inoculate 200mL SD Complete cultures to OD_{600} = 0.050-0.100. Grow at 30°C at 250 RPM to OD_{600} = 0.4.

2) Harvest cells:

a) Immediately before harvesting, treat culture with 400 µL of 50mg/mL cycloheximide, to a final concentration of 100 µg/mL.

b) Harvest cells as 200 mL cell pellets, flash freeze, and store at -80°C.

3) Cell lysis:

DAY 1

a) Lyse yeast by polysome lysis method.

i) Resuspend cell pellet in 400 µL polysome lysis buffer (without heparin; see polysome protocol for recipe).

ii) Add ½ volume of glass beads, and vortex 3'.

iii) Place on ice for 2', then vortex 3'. Repeat once more.

iv) Puncture a hole in the bottom of the 2mL tube with a red-hot 18 gauge needle. Place tube in 15 mL conical vial, and preclear lysate at 2000 RPM for 2 minutes at 4 °C.

v) Transfer supernatant to ice cold Beckman polycarbonate tube (11X34 mm) (*Or, 1.5 mL microfuge tube). Balance tubes with lysis buffer.

vi) Spin in table top ultracentrifuge using TLA 120.2 rotor at 29K RPM for 10' at 4°C. Recover 360 µL supernatant. (*While tabletop ultracentrifuge is broken, replace this with a 14K spin for 10' in tabletop centrifuge in cold room. We always performed the 14K spin here for consistency)

vii) Add 40 µL 10% Triton X-100, and incubate on ice for 5'.

d) Quantify lysate A_{260} using a 200-fold dilution. Dilute extract so the A_{260} = 0.250. Can be stored at -80°C. Expect 200 mL of cells to produce enough lysate for ~1-2 RNase I treatments (volume after dilution to 0.250 should be ~500 µL).

3a) Total RNA isolation:

a) Isolate whole-cell RNA as per standard protocol, from cells not treated with CHX.

b) DNase treat RNA.

b) Treat 5 µg aliquots of DNase-treated RNA with the Ribo-Zero kit to eliminate rRNAs, followed by size selection through the Zymo Clean and Concentrator column to remove small RNAs. Elute 2x 11 µL with RNase-free dH_2O – expect to recover 10 µL per elution.

4) Nuclease digestion

a) Treat 250 µL (12.5 A_{260} units total) of cell extract from 3d with 1.88 µL RNase I (titrate the precise amount*), and incubate for 1 hour at 24°C with gentle rotation on a nutator. Also prepare one untreated sample, kept on ice without enzyme for 1 hr. *Note, the precise amount of RNase I to be used may need to be titrated with each new enzyme lot or new conditions.

b) Stop digestion by adding 5 µL SUPERase-In to both samples and placing on ice. Load samples onto sucrose gradients as soon as possible.

4a) Total RNA fragmentation

a) Combine 20 µL total RNA from step (3a)b with 20 µL 2x alkaline fragmentation buffer.

b) Incubate at 95°C for 40 minutes (may need to titrate reaction length to optimize fragment size), and then immediately place on ice.

c) During incubation, prepare 560 µL stop/precipitation solution per reaction, and place on ice (60 µL 3M NaOAc pH 5.2, 2 µL glycoblue, 500 µL DEPC-treated dH_2O)

d) Add 40 µL fragmentation reaction to 560 µL stop/precipitation solution. Add 600 µL isopropanol and precipitate[2]. Resuspend RNA in 10 µL 10mM Tris-HCl pH 8.0. Process in parallel with footprint fragments, beginning at step 7a.

5) Purify monosomes by sucrose gradient centrifugation:

a) Add 250 µL RNase-treated extract to a 15-45% sucrose gradient.

b) Separate polysomes by density by centrifugation in Sw41Ti rotor at 41,000 RPM at 4°C for 2:26 hr.

c) Fractionate, and collect fractions containing monosomes (expect fractions ~5-7 for RNase-treated sample).

d) Precipitate RNA from sucrose overnight at -80°C with 1250 µl 95% EtOH.

e) Centrifuge RNA for 30' at 13.2K to pellet RNA. Wash with 500 µL 75% EtOH, centrifuge for 5', and remove ethanol.

DAY 2

f) Resuspend RNA in 450 µL LET + 50 µL 10% SDS by vortexing for 5'.

g) Extract RNA 1 x P/L, 1 x P/C/L, and 1 x chloroform.

h) Precipitate RNA (~250 µL remaining after extractions) with 1.5 µL GlycoBlue, 20 µL 4 M NaCl (to ~300mM), and 300 µL isopropanol[2]. Resuspend RNA in 10 µL 10mM Tris-HCl, pH 8.0.

i) Combine all fractions for each sample, and quantify using nano-spec.

6) Preliminary depletion of ribosomal RNA

a) Treat 2 5 µg aliquots of footprint fragment RNA with the RiboZero kit to eliminate the majority of rRNA. Follow RiboZero kit protocol.

b) Clean RNA through a Zymo Clean and Concentrator-5 column, being sure to recover small RNAs. Use 100 µL rRNA-depleted RNA, 200 µL Zymo binding buffer, and 450 µL absolute EtOH for first step.

c) Elute RNA in 2 x 6 µL nuclease-free water from Zymo column; approximately 5 µL is recovered per elution.

7) Size-selection of footprint fragments and additional rRNA-depletion

a) Run RNA on a 15% denaturing polyacrylamide gel (TBE-urea gel).
   i) Pre-run gel at 200V for 15 minutes in 1X TBE.
   ii) To footprint RNA, add an equal volume of 2x denaturing gel loading buffer to RNA samples.
   iii) Prepare a 10 bp DNA ladder (1 µl plus 4 µl 10 mM Tris pH 8.0 and 5 µL 2x denaturing sample buffer), and 1-2 lanes of mixed marker oligonucleotides (1 µL 10 uM lower marker oligo [oKB687], 1 µL 10 mM upper marker oligo [oKB686], 8 µL 10 mM Tris pH 8.0, and 10 µL 2x denaturing sample buffer)
   iv) Denature all samples at 80°C for 2 minutes
   v) Before loading the gel, clean each well by pipetting in running buffer several times. Load gel, skipping lanes around samples to be extracted from gel. Load 10 µL marker oligos on either side of RNA samples as number of lanes allows. Run gel for 65-75 minutes at 200 V.

b) Stain gel for at least 3' with SYBR Gold in 1x TBE, and visualize with UV. May take ~10-15' of staining for bands to be visible by UV box in dark room. Excise region indicated by marker oligo; also excise marker oligos to carry through as a control.

c) Extract RNA from gel (combining duplicate RiboZero treatments)[1].

d) Precipitate RNA by adding 1.5 µL glycoblue and 500 µL isopropanol[2]. Resuspend total fragmented RNA and size marker controls in 10 µl 10 mM Tris pH 8.0. Resuspend footprint RNA in 20 µL DEPC-treated dH_2O.

DAY 3

e) Repeat RiboZero treatment of single combined footprint RNA samples. This time, eliminate the 50°C incubation step. This will facilitate removal of smaller rRNA fragments still remaining in the 24-36nt size-selected material. Do not perform this second rRNA-depletion on the total RNA.

f) Repeat the Zymo Clean and Concentrator clean-up step as in 6b; elute in 2x11 µL (expect to recover ~10 µL per elution).

WARNING - THESE TWO STEPS MAY NOT BE NEEDED!!

8) Sequencing library preparation

a) Dephosphorylation of footprint RNA
   i) Dephosphorylate RNA: Add 10 µL 10mM Tris pH 8 and 13 µL water to 20 µL of footprint RNA. Add 33 µl water to 10 µL of total RNA and size markers. Denature RNA for 90s at 80°C. Equilibrate to 37°C. Add: 5 µL 10 X PNK reaction buffer, 1 µL SUPERase-In, and 1 µL T4 PNK. Incubate 1 hour at 37°C, and heat inactivate at 70°C for 10'.
   ii) Precipitate RNA by adding 39 µL water, 1 µL glycoblue, 10 µL 3 M sodium acetate, and 150 µL isopropanol[2]. Resuspend RNA pellet in 9 µL 10 mM Tris-HCl pH 8.0.

DAY 4

b) 3' linker ligation
   i) Combine 9 µL dephosphorylated RNA, 1 µL preadenylylated linker (0.5 µg/ul) and denature at 80°C for 90 seconds.

ii) Cool to room temperature.
iii) Set up ligation reaction with above RNA and linker, plus:

2 µl	10x Rnl2 buffer
6 µl	PEG 8000 (50% w/v)
1 µl	SUPERase-In
1 µl	T4 Rnl2(tr) RNA ligase

Incubate at room temperature for 2.5 hours, with slight agitation on a nutator (better mixing may help increase efficiency here).
iv) Add 338 µL water, 40 µL 3 M NaOAc, 1.5 µL GlycoBlue, and precipitate with 500 uL isopropanol[2]. Resuspend RNA in 5 µl 10mM Tris pH 8.0.
vi) Purify ligation reactions by polyacrylamide gel electrophoresis as in 7a-d, excising the larger ligation product and not unligated linker[2,1]. Also continue to carry through marker oligo ligation as a control.

DAY 5

vii) Resuspend ligation product in 10 µL 10 mM Tris pH 8.0.

c) Reverse transcription:
i) Combine ligated RNA with 2 µL 1.25 µM reverse transcription primer (titrate the precise amount), and denature for 2 minutes at 80°C. Place on ice. (*Note: RT primer should be gel purified after being received from IDT).
ii) Add

4 µl	5x First Strand Buffer
1 µl	10 mM dNTPs
1 µl	0.1 M DTT
1 µl	SUPERase-In
1 µl	SuperScript III.

iii) Incubate for 30' at 48°C.
iv) Add 2.2 µL 1N NaOH and incubate 20' at 98°C to hydrolyze RNA, leaving only first-strand cDNA.
v) Precipitate RT product with 156 µL water, 20 µL 3 M NaOAc, 2 µL GlycoBlue, and 300 µL isopropanol[2]. Resuspend in 5 µL 10 mM Tris pH 8.0.

d) Gel purify reverse transcriptase products from RT primer:
i) Run gel and excise as in step 7a. Also run 10 bp ladder as in 7a, and 2 µL 1.25 uM RT primer (plus 3 µL 10 mM Tris pH 8.0 and 5 µL 2x denaturing sample buffer) as size control.
ii) Excise product band (~130 nt) while avoiding unextended primer (100 nt band) and gel purify as in 7b-d[2,1], this time using DNA gel extraction buffer. Resuspend DNA in 15 µL 10 mM Tris-HCl pH 8.0.

DAY 6

e) Circularization:
i) In a PCR tube, combine:

15 µL	RT product
2 µL	10X CircLigase buffer
1 µL	1 mM ATP
1 µL	50 mM $MnCl_2$

1 µL CircLigase

ii) Incubate for 1hr at 60°C.

iii) Heat inactivate at 80°C for 10'.

iv) Precipitate cDNA out of reaction buffer by adding 10 µL 3M NaOAc, 70 µL water, 1.5 µL glycoblue, and 150 µL isopropanol[2]. Resuspend cDNA in 5 µL 10mM Tris pH 8.

f) PCR Amplification:

i) Prepare 100 µL PCR reaction per sample:

20 µL 5x Phusion HF buffer
2 µL 10 mM dNTPs
1 µL 50 uM forward library primer
1 µL 50 µM reverse library primer (different indexing primer for each sample)
70 µL water
5 µL circularized DNA
1 µL Phusion polymerase.

ii) Aliquot 16.7 µL into each of 5 tubes.

ii) PCR amplify with cycle: 98°C for 30 seconds initial denaturation
| 98°C for 10 seconds |
| 65°C for 10 seconds | x 14 cycles
| 72°C for 5 seconds |

iii) Remove reactions after 6, 8, 10, 12, or 14 cycles (First optimize the number of cycles for best amplification of each library).

g) Gel purify library

i) Run each PCR reaction on an 8% nondenaturing polyacrylamide gel, with 3.3 µL 6X nondenaturing loading dye.

ii) Also run 1 µL 10 bp ladder (plus 15.7 µL 10 mM Tris pH 8.0, 3.3 µL 6x nondenaturing loading dye).

iii) Run gel for 40' at 180 V.

iv) Stain gel for 3 minutes in SYBR Gold in 1x TBE and visualize by UV. Product band should be ~120 bp, and increase in intensity with more PCR cycles.

v) Excise product band from 1-2 lanes, where PCR is not saturated and few larger MW products are seen. Avoid smaller product derived from unextended RT primer.

vi) Extract DNA from gel[1] using DNA gel extraction buffer.

vii) Resuspend library in 15 µL 10 mM Tris-HCl pH 8.0. Quantify a 5-fold dilution by BioAnalyzer. Expect a peak ~170 bp.

METHODS

[1]Gel Extraction

1) Excise bands from acrylamide gel using a clean razor.
2) Transfer gel slices into a 0.5 mL tube with a 20-Ga hole punctured in the bottom and the lid cut off. Nest 0.5 mL tube in a 1.5 mL tube.
3) Spin tubes at high speed in a tabletop centrifuge for 2' to force gel slice through the hole. Transfer any remaining gel pieces from the 0.5 mL tube to the 1.5 mL tube with a pipette tip.
4) Add 400 µL RNA gel extraction buffer or DNA gel extraction buffer to the gel pieces. Freeze samples on dry ice for 30'.
5) Place tubes on a nutator at room temperature and rock overnight.
6) The following day, transfer the entire gel slurry to a microfuge spin filter (VWR 29442-752) using a 1 mL pipette with the tip cut off. Cut off the tip using a clean razor.
7) Spin the sample through the spin filter at high speed in a tabletop centrifuge for 2'. The gel fragments will remain in the top of the filter.
8) Transfer flow-through to a new 1.5 mL tube, and add 1.5 µL glycoblue. Precipitate with 500 µL isopropanol, as described in precipitation protocol.

[2]Isopropanol precipitation

1) To nucleic acid sample containing salt (e.g. 100 mM NaOAc) and a carrier (e.g. glycoblue), add at least an equal volume of isopropanol.
2) Precipitate on dry ice for at least 30' or overnight at -80°C.
3) Pellet nucleic acid by spinning at 14K for 30' at 4°C.
4) Draw off supernatant, and air dry pellet.
5) Resuspend nucleic acid as needed, usually in 10 mM Tris pH 8.

REAGENTS

2x Alkaline fragmentation buffer	To make 5mL:
2mM EDTA	20 µL 0.5M EDTA
100mM Na•CO_3, pH 9.2	600 µL 0.1M Na_2CO_3 (15 parts)
	4.4 mL 0.1M $NaHCO_3$ (110 parts)

*Will equilibrate with gaseous CO_2 over time to raise the pH; store in tightly capped, single-use aliquots at room temperature

2x Denaturing loading buffer
98% formamide
10 mM EDTA
300 µg/mL bromphenol blue
*Dissolve 15 mg bromphenol blue in 1 mL 0.5M EDTA. Add 200 µl to 9.8 mL formamide. Can substitute with other denaturing loading dyes, but not xylene cyanol. Store at RT.

RNA Gel Extraction Buffer	To make 10 mL:
300 mM NaOAc (pH 5.2)	1 mL 3M NaOAc
1 mM EDTA	20 µL 0.5M EDTA
0.25% (w/v) SDS	250 µL 10% SDS
	8.73 mL DEPC dH_2O

DNA Gel Extraction Buffer	To make 10 mL:
300 mM NaCl	750 µL 4M NaCl
10 mM Tris (pH 8.0)	100 µL 1M Tris (pH 8.0)
1 mM EDTA	20 µL 0.5M EDTA
	9.13 mL dH_2O

6X Non-denaturing Loading Dye	To make 10 mL:
10 mM Tris (pH 8.0)	100 µL 1M Tris (pH 8.0)
1 mM EDTA	20 µL 0.5M EDTA
15% (w/v) Ficoll 400	1.5 g Ficoll 400
0.25% bromphenol blue	0.025 g bromphenol blue
	dH_2O to 10 mL

Steady State RNA Analysis by Northern Blotting

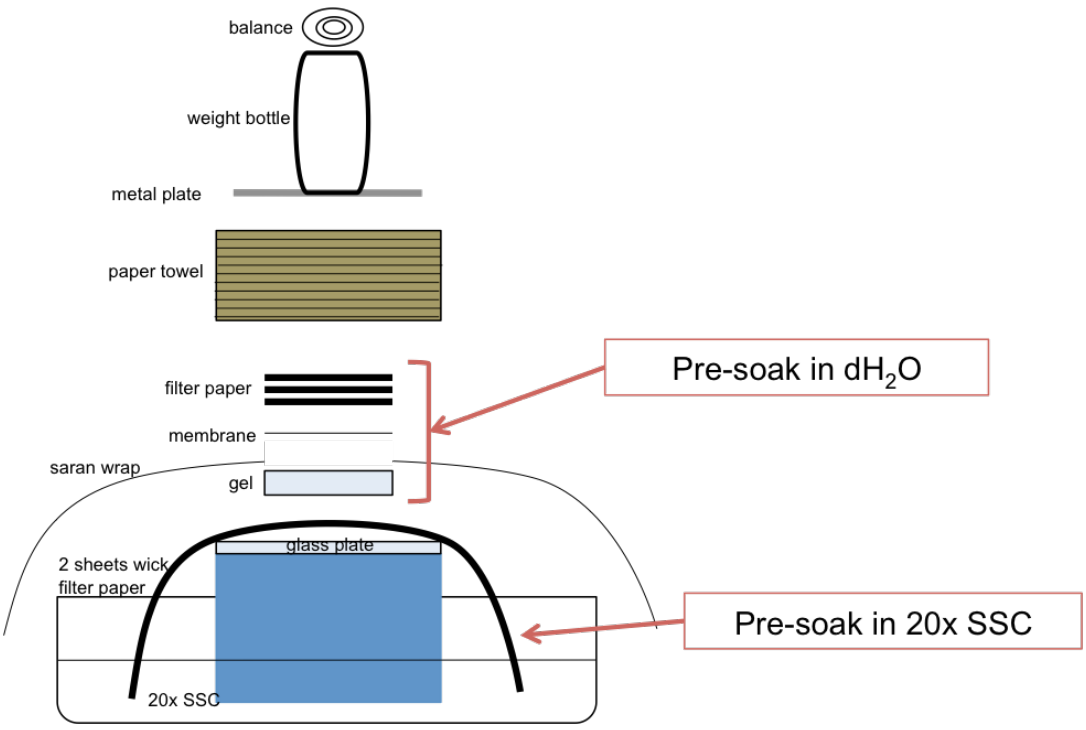
1. Grow cells for RNA isolation, keeping all media and cultures sterile.
    A. Inoculate 5 mL synthetic medium (SD Complete when no plasmid selection is required) the evening before, in a test tube. Incubate at 30°C, shaking, overnight.
    B. Use starter culture to inoculate 50 mL culture in 250 mL Erlenmeyer flask to OD_{600}=0.1.
    C. Grow at 30°C to OD_{600}=0.4.
    D. Collect cells in 50 mL conical vial at 4000 RPM for 5 minutes.
    E. Resuspend in 1 mL sterile dH_2O, transfer to 2 mL eppendorf tube, and collect cells by centrifugation at 13.2K for 2 minutes.
    F. Remove liquid and flash freeze cell pellet on dry ice. Store at -80°C until needed.

2. Isolate RNA from cell pellets as described in steady state RNA isolation protocol.

3. Quantify RNA by diluting 4 uL into 996 uL dH_2O, and measuring the absorbance at OD_{260} using the UV spectrophotometer.

4. Run agarose northern gel:
    A. Prepare gel based on recipe in research tower. Usually, a 125 mL standard gel is used, with either a 15- or 20-well comb. Level gel caster, and add formaldehyde in the fume hood once gel has cooled.
    B. Dilute RNA samples to a constant quantity (i.e. 30 ug ideally) in northern loading dye (NLD). Use at least an equal volume of NLD, for a total volume of up to 30 uL.
    C. Heat RNA samples at 65°C for 5 minutes.
    D. Transfer gel into running chamber so the RNA samples will run towards the positive (red) electrode, and fill chamber with 1x MOPS running buffer until gel is completely submerged. Remove comb gently.
    E. Load RNA samples into gel, and run at 100V for ~90 minutes. Bromphenol blue dye should be ~3/4 down the gel.

5. Set up northern transfer:
    A. Cut 2 filter paper wicks, the length of a full sheet of filter paper folded in half and the width of a glass plate. Soak these in 20x SSC.
    B. Take a UV image of the gel to check RNA quality.
    B. Trim all 4 edges off of gel to make it flat, but leave extra space if possible along sides. Measure gel dimensions, then soak gel in dH_2O.
    C. Cut 3 pieces of filter paper and 1 piece of HyBond northern blot membrane (labeled in top corner) to the same size as the gel, and soak these in dH_2O.
    D. Assemble transfer as follows (see figure):
        i. Glass pyrex dish, filled ~an inch deep with 20x SSC.
        ii. Blue tip box insert
        iii. Glass plate
        iv. 2 filter paper wicks, pre-soaked in 20x SSC, with both edges in 20x SSC in pyrex dish. Remove bubbles.
        v. Gel, pre-soaked in dH_2O, face down. Remove bubbles.
        vi. Saran wrap, with a hole cut out around the gel; border gel with tape to facilitate this.
        vii. Membrane, pre-soaked in dH_2O, face down on gel. Remove bubbles.
        viii. 3 pieces of filter paper, pre-soaked in dH_2O. Remove bubbles, and dry off any liquid droplets on saran wrap.
        ix. ~2 inches of brown paper towels, no wrinkles.
        x. Metal plate
        xi. Glass bottle weight
        xii. Balance
    E. Transfer overnight


balance
weight bottle
metal plate
paper towel
filter paper
membrane
saran wrap
gel
Pre-soak in dH2O
glass plate
2 sheets wick
filter paper
Pre-soak in 20x SSC
20x SSC

6. Probe northern blot:
    A. Disassemble transfer. Take image of gel to verify ribosomal RNA bands transferred out of gel successfully.
    B. UV crosslink membrane 2x on auto-crosslink setting in Stratalinker. Rotate 90° after the first crosslink.
    C. Wash membrane for 1hr at 65°C° in 0.1x SSC/0.1% SDS in hybridization oven.
    D. Pre-hybridize membrane for at least 1 hour at 42°C (or whatever temperature is being used for hybridization) in hybridization buffer.
    E. Add radiolabelled DNA oligo probe* and hybridize overnight at 42°C or empirically determined temperature.

7. Wash and develop northern blot:
    A. Warm 6x SSC/0.1% SDS buffer to dissolve SDS.
    B. Decant probe solution, and wash membrane 2x 15 minutes in 6x SSC/ 0.1% SDS at room temperature.
    C. Wash membrane for 15 minutes in 6x SSC/0.1% SDS at 50°C.
    D. Briefly dry membrane on filter paper. Wrap membrane in saran wrap, and tape into a **blanked** phosphorimager cassette. Expose as needed, usually overnight.
    E. Develop phosphorimager screen using Storm scanner upstairs in Biochemistry department. Quantify using ImageQuant software on computer in RNA Center.

*Radiolabel DNA oligo probe by setting up PNK reaction using 300 ng DNA oligo and radioactive γ-P^{32} ATP. Clean probe through G25 Sephadex column.

Preparation of RNA for cDNA Libraries

1. Isolate RNA as desired (steady-state, shut-off, polysome fractions, etc.).

2. Treat purified RNA with DNase I, through extraction and precipitation steps (see protocol). Resuspend RNA in DEPC-treated water.

3. Verify quality of RNA by running a small aliquot (as little as 1 µg) on an agarose northern gel.

4. Deplete ribosomal RNA from each RNA sample using the Epicentre Ribo-Zero rRNA Removal Kit (Ribo-Zero Magnetic Gold Kit [Yeast]), following the manufacturer's protocol.
    a. Standard is to treat 5 µg RNA per aliquot of beads.
    b. At the elution step, collect rRNA-depleted supernatant and proceed to the size-selection step described below; do not continue through the recommended steps for RNA purification via precipitation.

5. To remove small RNAs (less than ~200 nt, including tRNAs, 5S rRNA, and 5.8S rRNA), clean rRNA-depleted RNA sample through a Zymo RNA Clean and Concentrator-5 spin column (Zymo R1015).
    a. Add 25 µL DEPC-treated water to the ~95 µL RNA sample to bring the volume up to 120 µL.
    b. Add 80 µL anhydrous ethanol to the RNA sample
    c. Add 100 µL RNA Binding Buffer to the RNA sample. The final ethanol concentration is now 26.6% (optimized to maximize removal of RNAs <200 nt).
    d. Continue with the Zymo spin column manufacturer's protocol beginning at step 3.
    e. For the final elution step, elution in 2 x 10 µL DEPC-treated water is recommended.

6. Verify quality of RNA and depletion of rRNA by running a small aliquot (1/20 of recovered RNA can suffice, alongside an equivalent amount of pre-depletion RNA) on an agarose northern gel.
    a. No rRNA bands should be visible in the rRNA-depleted samples by ethidium stain.
    b. Radioactive probing for an abundant RNA (i.e. PGK1) should indicate little loss of non-rRNA species.

7. RNA is now depleted of rRNA and abundant small RNA species, and can be used to generate cDNA libraries for applications such as RNA-seq through the desired library preparation method.

CLICK-IT EU TAGGING – RNA DECAY ANALYSES

(Adapted from Click-IT chemistry and Ingolia 2012, Nat. Prot.)

1. Cell culture and RNA labelling pulse-chase

Seed cells in 60mm diameter dishes. When they reach 40% confluency, replace the media by EU (ethynyl-uridine)-containing media (EU final 0.2mM) (=pulse)

24h later: extract RNA from one plate = 0min time-point
Other plates: perform the chase: wash once with media without EU then replace by media containing ÒcoldÓ uridine at 5mM final

Extract RNA at the desired time-points.

1. RNA extraction with Trizol (protocol from RNA protocol book, Rio/Ares/ Hannon/Nilsen, 2011)

Add 1 to 2 mL Trizol per 60mm dish, resuspend cells by pipetting up and down (can be frozen at -80¡C at this stage).

Sit at Room Temperature (RT) for 5min.

Add 200µL chloroform per 1mL Trizol, vortex 3min and sit at RT for 3min.

Spin 16,000g (microcentrifuge) for 10min at RT.

Carefully transfer the aqueous phase to a new 1.5mL tube and add 1µL glycoblue and 500 µL isopropanol. Mix well, let sit 10min at RT then spin 10min at 16,000g at 4¡C.

Remove the supernatant then extract a second time: Resuspend the pellet in 150µL SDS extraction buffer then add 300 µL depc-treated H2O and 50µL NaOAc 3M pH5.2 (made in depc-H2O, final 0.3 M). Add 500µL P/C/L, vortex 5min and spin 5min at RT, 16,000g.

Extract the aqueous phase again with 400 µL chloroform (vortex 5min, spin 5min).

Precipitate with 40 µL NaOAc 3M, 1µL glycoblue and 1mL EtOH 100% at -20¡C (1h to overnight).

Spin 20min at 4¡C (16,000g). Wash with 70% EtOH/depc-H2O, dry then resuspend in 22µL of depc-H2O.

Measure with nanodrop.

1. DNAse treatment.

For each sample: perform DNAse treatment and RiboZero treatment in duplicates = use 2x 6-8µg of each RNA sample (samples will be combined after RiboZero treatment).

Add 2-5ng of each EU-tagged spike-in (here Luc and LysA fragments (0.5 and 1kb) transcribed in vitro with EU) in each sample.

6-8µg RNA + 2ng Spike-in + 0.5 µL SuperAse-In + 2µL DNAse I buffer + 0.5µL DNase I (5U) + depc-H2O to a total of 20µL.

Incubate at 37¡C for 30min

Bring up the volume to 200µL depc-H2O, add 200µL 2X SDS extraction buffer and resuspend. Add NaOAc final 0.3M + 500µL P/C/L and extract once (5min vortex, 5min spin).

Precipitate with 1µL glycoblue, NaOAc final 0.3M and 1mL EtOH 100%.

Spin, wash and resuspend in depc-H2O (up to 26µL).

1. RiboZero treatment = remove the ribosomal RNAs

Follow the protocol for each sample: *Illumina MRZG126/MRZG12324*

4µL reaction buffer + 10µL removal solution + RNA + water to 40µL total

Incubate at 68¡C for 10min

5min at RT then add to the 65µL of beads (prepared as: 225µL beads placed on magnetic stand: discard supernatant, wash twice with water and resuspend in 65µL of Magnetic bead resuspension solution)

Vortex, sit 5min at RT, then incubate at 50¡C for 5min

Directly put on magnetic bench. Purify the eluate on ZymoClean Columns following the columns kit protocol (RNA Clean and concentrator Kit, R1015 The epigenetics company).

Eluate twice with 11µL water (total 20-22µL).

1. RNA fragmentation

Combine the 20µL RNA with 20µL of (freshly mixed) 2X alkaline fragmentation buffer. Incubate at 95¡C for 25-30min.

Put on ice and stop immediately with 560µL stop/precipitation solution = 60µL NaOAc, 2µL glycoblue, 500µL depc-H2O. Precipitate with 800µL of isopropanol (on dry ice for 1h or at -80¡C overnight).

Spin 30min at 4¡C (16,000g). Wash once with 70%EtOH/depc-H2O. Dry and resuspend in 15.75µL depc-H2O.

1. Click-IT kit biotinylation

Follow the kit protocol: Click-IT nascent RNA kit C10365, Life technologies

Add 25µL component B

4µL CuSO4

2.5µL biotin azide

Mix and add to the 15.75µL RNA

Add 1.25 µL component E then 1.5µL component F exactly 3min after E

Mix gently and incubate at 24¡C in thermomixer 300-400 rpm for 30min

Add 1.5µL glycoblue + 50µL NH4OAc 7.5M + 900µL EtOH 100% and precipitate at -80¡C overnight.

1. RNA gel purification

Spin RNA 20min at 4¡C (16,000g)

Wash twice with 70% EtOH/depc-H2O

Resuspend in 10µL of Tris-HCl 10mM pH8

Add 10µL 2X denaturing loading dye

Prepare DNA ladder 10bp (Life Technologies, 10821-015): 1µL + 9µL Tris-HCl 10mM pH8 + 10µL dye (multiply if run in several lanes) and RNA size oligos markers (26nt, 34nt, 70nt): 1µL of each at 10µM + Tris + dye

Denature 2min at 80¡C

Run on NuPAGE denaturing polyacrylamide gel (TBE-urea 15% polyacrylamide, EC62152BOX) 200V (after pre-running the gel at 200V for 15min) for around 70min

Stain with SYBR Gold for 15min

Cut RNA between 20nt and 100nt with the help of the markers (also cut the markers to carry along as a control): transfer gel slices into a 0.5mL tube with a 20-Ga hole punctured in the bottom and the lid cut off. Nest this tube into a 1.5mL tube

Spin 2min (16,000g) to force the gel through the hole, transfer any remaining gel pieces

Add 500µL RNA gel extraction buffer

Freeze on dry ice for 30min

Rock overnight at room temperature

The next day, transfer the gel slurry (using a 1mL pipette with tip cut off) to a microfuge spin filter (VWR 29442-752) and spin for 2min (16,000g)

Transfer flow-through to a new 1.5mL tube, add 1.5µL glycoblue and precipitate with 700µL isopropanol (1h on dry ice or overnight at -80¡C).

1. Click-IT pulldown

Spin 30min at 4¡C (16,000g)

Dry pellet and resuspend in 10µL Tris-HCl 10mM pH8

Prepare beads: wash 50µL of C1 myOne streptavidin beads (Invitrogen, or T1 from the kit), twice, with 500µL Wash buffer 2 from the kit, then resuspend them in 50µL Wash buffer 2

RNA: add depc-H2O to 123µL total

Combine: 125µL RNA binding buffer (component G)

2µL SuperAse-In

The 123µL RNA (total 250µL)

Denature 5min at 68-70¡C

Add the beads and rock at RT for 30min

Wash: 5 times with 500µL of Wash buffer 1

Then 3 times with 500µL Wash buffer 2

Resuspend the beads in 20µL of Wash buffer 2

For the markers control sample: no pulldown; resuspend in 10µL Tris-HCl 10mM pH8 then go directly to step 9 dephosphorylation (add 33µL water and start from denaturation step).

1. Dephosphorylation (on beads)

Add 10µL of Tris-HCl 10mM pH8 and 10µL of depc-H2O to each beads/RNA sample

Denature at 80¡C for 90sec then equilibrate at 37¡C

Add 5µL 10X PNK reaction buffer, 1µL SuperAse-In, 1µL T4 PNK

Incubate 1h at 37¡C in thermomixer, interval or constant mixing (15Ó 1000rpm, 1min stand; or 900rpm)

Inactivate 10min at 70¡C

Place on magnetic stand, remove supernatant then wash the beads once with 500µL of Wash buffer 2

Resuspend the beads in 9µL Tris-HCl 10mM pH8

For the markers control sample: Instead of wash on magnetic stand, precipitate by adding 39µL water, 1µL glycoblue, 10µL 3M NaOAc and 300µL isopropanol. Resuspend in 9µL Tris-HCl 10mM pH8.

1. Linker ligation (on beads)

Combine the 9µL beads/RNA (and markers control sample) with 1µL preadenylylated linker (0.5µg/µL) and denature at 80¡C for 90sec

Cool to RT

Set up ligation reaction: beads/RNA

2µL 10X Rnl2 buffer

6µL PEG 8000 (in Rnl2 kit, NEB M0242S)

1µL SUPERase-In

1µL T4 Rnl2(Tr) RNA ligase

Incubate 30min at 24¡C in thermomixer 1000 rpm then overnight at 16¡C in thermomixer 1000rpm.

1. Reverse transcription (on beads) (Invitrogen kit: 18080-400, SuperScriptIII first strand reaction mix)

The next day, wash beads once with 500µL Wash Buffer 1 then once with 500µL Wash buffer 2

Resuspend in 10µL of Tris-HCl 10mM pH8 (estimated 12µL with beads)

For the markers control sample: Add 338µL water, 40µL 3M NaOAc, 1.5µL glycoblue and precipitate with isopropanol. Gel purify, excising the larger ligation product and not unligated linker. Resuspend in 12µL Tris-HCl 10mM pH8.

Add Reverse Transcription primer (purified from gel after reception from IDT) (2µL of 1.25µM, exact volume to determine after purification) and 2µL annealing buffer

Denature at 65¡C for 5-10min then place on ice

Add 20µL 2X first-strand reaction mix and 4µL of SSIII/RNAseOUT mix

Incubate at 48-50¡C in thermomixer 900rpm (or interval mixing) for 1h

Heat 8min at 90¡C in thermomixer 1200rpm to inactivate the reverse transcriptase and to elute the cDNA from the beads, collect the cDNA (around 35µL) by placing the beads on a magnetic stand.

For the markers control sample: add 2.2µL of 1N NaOH and incubate at 98¡C for 20min (hydrolyze RNA)

Precipitate by adding water to 300µL, 30µL NAOAc (0.3M final), 2µL glycoblue and 500µL isopropanol (-80¡C overnight or 1h on dry ice).

1. cDNA gel purification

Spin 30min at 4¡C (16,000g) then resuspend the cDNA in 10µL 10mM Tris-HCl pH8

Also prepare a sample of 10bp ladder and one of RT primer only

Add 10µL of 2X denaturing sample buffer to each sample and run on a 15% denaturing polyacrylamide NuPAGE gel (TBE-urea)

Excise the product band (as in 7) while avoiding the unextended primer (100nt band, use the sample with the primer only as a reference size) (the RT product here may not be visible, cut above the unextended primer size to the top)

Transfer gel slices into a 0.5mL tube with a 20-Ga hole punctured in the bottom and the lid cut off. Nest this tube into a 1.5mL tube. Spin 2min (16,000g) to force the gel through the hole, transfer any remaining gel pieces

Add 400µL of DNA gel extraction buffer

Freeze on dry ice for 30min then rock overnight at room temperature

The next day, transfer the gel slurry (using a 1mL pipette with tip cut off) to a microfuge spin filter (VWR 29442-752) and spin for 2min (16,000g)

Transfer flow-through to a new 1.5mL tube, add 1.5µL glycoblue and precipitate with 700µL isopropanol (1h on dry ice or overnight at -80¡C).

1. Circularization

Spin then resuspend the cDNA in 15µL of 10mM Tris-HCl pH8

Add: 2µL 10X CircLigase Buffer

1µL 1mM ATP

1µL 50mM MnCl2

1µL CircLigase

Incubate for 1h at 60¡C

Heat inactivate at 80¡C for 10min

Precipitate by adding 20µL 3M NaOAc, 140µL water, 2µL glycoblue and 400µL isopropanol

Resuspend in 10µL of 10mM Tris-HCl pH8. Add 40µL water.

1. PCR amplification

Prepare 100µL PCR reaction per sample:

20µL 5X Phusion HF buffer

2µL 10mM dNTPs

1µL 50 µM forward library primer

1µL 50µM reverse library primer (different indexing primer for each sample)

50µL water

25µL circularized DNA (keep ½ to re-do a PCR if not enough library material)

1µL Phusion polymerase

Aliquot 18µL into each of 5 tubes

PCR amplify: 98¡C for 1min initial denaturation

98¡C 30Ó

65¡C 30Ó

72¡C for 20Ó

Remove one tube per sample after 10, 12, 14, 16 and 18 cycles of amplification

1. Gel purify the library

Run each PCR reaction on an 8% nondenaturing polyacrylamide NuPAGE (TBE) with 4µL 6X nondenaturing loading dye (Also run 1µL 10bp ladder)

Run gel at 180V (around 50-60min) in 1X TBE

Stain 15min with SYBR gold in 1X TBE. Product band should be above a band from a smaller product derived from the unextended RT primer (around 145bp), and should increase in intensity with more PCR cycles. Excise product from lanes where PCR is not saturated (= avoid the large MW products)

Extract with DNA gel extraction buffer as in 12.

Resuspend library in 15µL 10mM Tris-HCl pH8.

Reagents/Materials/Methods:

SDS extraction buffer (Rio/Ares/Hannon/Nilsen book): 0.5% SDS, 1mM EDTA pH8, 20mM Tris-HCl pH 7.5

2X SDS extraction buffer (Rio/Ares/Hannon/Nilsen book): 1% SDS, 1mM EDTA pH8, 20mM Tris-HCl pH 7.5

P/C/L: Phenol/chloroform/LET (phenol buffered with LET = LiCl 100mM, EDTA 20mM, Tris pH8 25mM) then mixed with chloroform in proportions 1:1)

2X alkaline fragmentation buffer: 2mM EDTA, 100mM NaCO3 pH9.2 (=15 parts NA2CO3, 110 parts NaHCO3) (will equilibrate with gaseous CO2 over time to raise the pH: store in tightly capped, single-use aliquots at room temperature; or make fresh)

2X denaturing loading dye: 98% formamide, 10mM EDTA, 300µg/mL bromophenol blue (dissolve 15mg bromophenol blue in 1mL 0.5M EDTA; add 200µL to 9.8 mL formamide. Can substitute with other denaturing loading dyes, but not xylene cyanol; Store at RT)

RNA gel extraction buffer: 300mM NaOAc pH5.2, 1mM EDTA, 0.25% (w/v) SDS

DNA gel extraction buffer: 300mM NaCl, 10mM Tris pH8, 1mM EDTA

6X non-denaturing loading dye: 10mM Tris pH8, 1mM EDTA, 15% (w/v) ficoll 400, 0.25% bromophenol blue

RNA size markers:

oKB 687 26nt: 5′-AUGUACACGGAGUCGACCCAACGCGA-(Phos)-3′

oKB688 34nt: 5′-AUGUACACGGAGUCGAGCUCAACCCGCAACGCGA-(Phos)-3′

oJC3131 70nt: 5’-AUGUACACGGAGUCGAGCUCAACCCGCAACGCGAACCGAUCCUAGAGGUCUAGCAAGAUCGGCCAUGAAU-(Phos)-3′

Preadenylylated linker: Universal miRNA Cloning Linker 5′-rAppCTGTAGGCACCATCAAT–NH2-3′ (New England Biolabs, cat. no. S1315S)

RT primer: oKB 686: 5′-(Phos)-AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGC-(SpC18)-CACTCA-(SpC18)-TTCAGACGTGTGCTCTTCCGATCTATTGATGGTGCCTACAG-3′

Forward PCR primer: oKB689: 5′-AATGATACGGCGACCACCGAGATCTACAC-3′

Reverse PCR primers (different indexes): CAAGCAGAAGACGGCATACGAGAT-index-GTGACTGGAGTTCAGACGTGTGCTCTTCCG

Index in oKB 690: TGGTCA (read reverse-complement for sequencing analysis)

- oKB 691: CACTGT
- oKB 692: ATTGGC
- oKB 693: TCAAGT
- oKB 694: CTGATC
- oKB 695: TACAAG
- oJC 2796: CGTGAT
- oJC 2797: ACATCG

Gel extraction:

-Excise bands from polyacrylamide gel with clean razor

-Transfer gel slices into 0.5mL tube with 20-Ga hole punctured in the bottom and lid cut off; nest in a 1.5mL tube

-Spin at high speed in a tabletop centrifuge for 2min to force the gel slice through the hole. Transfer any remaining gel pieces from the 0.5mL to the 1.5mL tube with a pipette tip

-Add 400µL (500µL if big gel pieces) RNA/DNA gel extraction buffer; freeze on dry ice for 30min

-Place on a nutator at room temperature and rock overnight

-Following day: transfer the entire gel slurry to a microfuge spin filter (VWR 29442-752) using a 1mL pipette with the tip cut off (cut off the tip with a clean razor)

-Spin at high speed for 2min; the gel fragments will remain in the top of the filter

-Transfer flow-through to a new 1.5mL tube and add 1.5µL glycoblue; precipitate with isopropanol

Isopropanol precipitation:

-To nucleic acid sample containing salt (NaOAc) and a carrier (glycoblue), add a t least an equal volume of isopropanol

-Precipitate on dry ice for at least 30min or overnight at -80¡C

-Pellet by spinning at 14K for 30min at 4¡C

-Draw off supernatant, air dry pellet

-Resuspend, usually in 10mM Tris pH8.

In vitro transcription (Spike-in):

Plasmids (pBluescript SK+) containing LYSa (B.subtilis) or firefly Luciferase partial sequences: pJC879 and pJC880, respectively (constructed with gBlocks)

18µg digested with SacII for 4h at 37¡C then gel purified (GenElute Sigma), resuspension in 30µL water

Kit E2040S from NEB:

21µL digested plasmid + 3µL ATP 100mM

3µL GTP 100mM

3µL CTP 100mM

2µL UTP 100mM

1µL of 5-Ethynyl-UTP (Abcam) at 100mM

1uL water

3 uL transcription buffer

3 uL T7 mix

Incubate at 37¡C for 2h to 4h

DNAse treat for 15min at 37¡C

Add 200µL depc-H2O and extract with 300µL P/C/L (vortex 5min, spin 5min), then precipitate aqueous phase with NaOAc and EtOH

Resuspend in depc-H2O, measure concentration

Aliquot and keep at -80¡C

(Dilute to 1ng/μL before use).