

Dent Laboratory Protocols

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Yeast Protocols

Yeast Histone Preparation I

Method

Day 1

Inoculate 5 ml culture in YPD (or selective media) and grow overnight with shaking at 30°C.

Day 2

Inoculate 1-2 L of YPD (or selective media) with 0.5 ml overnight culture. Grow with shaking at 30°C.

Day 3

Dilute aliquot of cells 1/10 and count:

- For preparative experiments, cell densities can be high: $\sim 2 \times 10^8/\text{ml}$
- For analytical experiments, cell density should reflect log growth ($\sim 2 \times 10^7/\text{ml}$)

In Steps 5-12, add fresh protease inhibitors leupeptin and pepstatin to all buffers.

1. Spin cells at 3000 g for 5 min at 4°C.
2. Wash cell pellets in 200-400 ml sterile water and centrifuge again.
3. Resuspend cells in 50 ml DTT/Tris-HCl, pH 9.5. Incubate with shaking at 30°C, 15 minutes. Spin as above.
4. Resuspend pellet in 50 ml Sorbitol/Hepes buffer (Buffer #2). Re-spin.
5. Resuspend pellet in 50 ml Buffer #2 and add 1-2 ml zymolyase (10 mg/ml). Incubate at 30°C for 45-60 minutes or until well ghosted.
6. Add 100 ml ice-cold Buffer #3 (Sorbitol/Pipes/MgCl₂). Spin at 3.5K (JA10 rotor) for 5 minutes at 4°C.
7. Resuspend pellet in 50 ml ice-cold NIB buffer and hold on ice for 20 min. Spin at 4K (JA10 rotor) for 5 minutes at 4°C.
8. Repeat NIB wash 2X, and hold each for 20 minutes on ice.
9. Resuspend pellet in 50 ml A wash and hold on ice for 15 minutes. Spin as above for 5 minutes.
10. Repeat A wash and spin.

11. Resuspend pellet in 50 ml B wash and hold on ice for 5 minutes. Spin as above for 5 minutes.
12. Resuspend in 25 ml B wash and spin immediately.
13. Resuspend pellet in 10 ml cold 0.8M H₂SO₄ to extract histones. Hold on ice for 30 minutes, vortexing occasionally.
14. Spin at 10K for 10 min. Save supernatant, which contains the extracted histones. Measure volume with pipette.
15. Add 100% TCA to a final concentration of 20%. A large precipitate should form almost immediately. Hold on ice for 30 minutes.
16. Spin at 12K for 30 minutes. If supernatant still looks cloudy, pour into a fresh tube and spin again. Wash pellet in 10 ml acid acetone (acetone + 0.1% concentrated HCl) and spin for 5 min at 10K. Wash pellet in cold acetone and spin again at 10K 5 minutes. Pour off acetone and air-dry pellet.
17. Resuspend and combine pellets in a total of 1 ml of 10 mM Tris-HCl, pH 8.0. If pellet is hard to resuspend, add more Tris buffer, 0.5 ml at time. Try to keep histones as concentrated as possible.
18. Store histones at 20°C. Run 15 and 30 µl on a 22% SDS-PAGE gel.

Buffers

Store these buffers at RT except NIB and Buffer #3, which should be stored at 4°C for ease of use.

DTT / Tris

- 0.1 mM Tris-HCl, pH 9.4
- 10 mM DTT

Buffer 2

- 1.2 M Sorbitol
- 20 mM Hepes, pH 7.4

Buffer 3

- 1.2 M Sorbitol
- 20 mM Pipes, pH 6.8
- 1 mM MgCl₂

NIB

- 0.25 M Sucrose
- 60 mM KCl
- 15 mM NaCl
- 5 mM MgCl₂
- 1 mM CaCl₂
- 15 mM MES, pH 6.6
- 1 mM PMSF
- 0.8% Triton X-100

A Wash

- 10 mM Tris-HCl, pH 8.0
- 0.5 % NP-40
- 75 mM NaCl
- 30 mM Sodium Butyrate
- 1 mM PMSF

B Wash

- 10 mM Tris-HCl, pH 8.0
- 0.4 M NaCl
- 30 mM Sodium Butyrate
- 1 mM PMSF

Yeast Histone Preparation II

Method

1. Grow a 1- to 2-liter culture of diploid yeast to a density of approximately 2×10^8 cells/ml and pellet the yeast by centrifugation in a Beckman JA-10 rotor (or similar) for 5 minutes at 5000 rpm at 4°C.
2. Resuspend the cell pellet in sterile water (about 200 ml) and centrifuge as above. Discard the supernatant.
3. Resuspend the cell pellet in 50 ml of 0.1mM Tris, pH 9.4, 10mM DTT. Incubate for 15 minutes at 30°C with gentle shaking.
4. Centrifuge as above, wash the pelleted cells in 100 ml of Buffer 2 (no protease inhibitors), and re-centrifuge.
5. Resuspend the cell pellet in 50 ml of Buffer 2 with protease inhibitors. Add protease inhibitors at every step until the histones are extracted in step 10. Add 2 mls of 10 mg/ml zymolyase (dissolved in S Buffer) and incubate at 30°C with gentle shaking.

Periodically, examine a sample of cells microscopically to determine the optimal length of enzyme treatment. Place a 5- to 10- μ l sample of cells on a glass microscope slide and an equal volume of 1% SDS, near to the cells but not touching. Drop a small coverslip onto both droplets and view microscopically (You will need a 40X objective.).

You should see bright refractile yeast at the edge of the coverslip on the cell droplet side. Where the cell and SDS droplets have run together, you should see a mixture of bright refractile cells and 'ghosts'. 'Ghosts' are yeast cells that have lost their refractile cell wall in the presence of the SDS. They are often slightly larger than the intact yeast but should not be too much larger. They are difficult to see on a regular light microscope and it may take a little experience to see them. They are easy to see on a phase contrast scope.

Continue the incubation until about 95% of the yeast cells in SDS are ghosted. Typically, this takes 30-60 minutes.

6. Add of 100 ml of ice-cold Buffer 3, and centrifuge the cells in a JA10 rotor (5 minutes, 4°C, 3.5K).
7. Resuspend the cell pellets are in 50 ml of ice-cold NIB and hold on ice water for 20 minutes, and spin at 4K in a JA-10 rotor for 5 minutes. Repeat this wash 2X.

8. Wash the cells 3X in A wash, holding on ice water for 15 minutes for the first two washes.
9. Wash the cell pellet in Buffer B one time; hold on ice water for 5 minutes. After centrifugation, resuspend the cells again in Buffer B and centrifuge immediately.
10. Extract the histones by resuspending the pellet in 10 ml of cold 0.4 NH_2SO_4 , and holding in ice water for 30 minutes, vortexing occasionally. Remove debris from the solution by spinning for 10 minutes at 10K

Solutions

S buffer

- 1.2M Sorbitol
- 1mM DTT

Buffer 2

- 1.2M Sorbitol
- 20mM Hepes pH 7.4
- 1mM PMSF*
- 0.5 $\mu\text{g/ml}$ Leupeptin*
- 0.7 $\mu\text{g/ml}$ Pepstatin*

Buffer 3

- 1.2M Sorbitol
- 20mM Pipes
- 1mM MgCl_2 , pH 6.8
- 1mM PMSF*
- 0.5 $\mu\text{g/ml}$ Leupeptin*
- 0.7 $\mu\text{g/ml}$ Pepstatin*

NIB (Nuclei Isolation Buffer)

- 0.25M Sucrose
- 60mM KCl
- 14mM NaCl
- 5mM MgCl_2

- 1mM CaCl₂
- 15mM MES, pH 6.6
- 0.8% Triton X-100
- 0.7 µg/ml Pepstatin*
- 1mM PMSF*
- 0.5 µg/ml Leupeptin*

A wash

- 10mM Tris, pH 8.0
- 0.5% NP-40
- 75mM NaCl
- 30mM Sodium Butyrate*
- 1mM PMSF*
- 0.5 µg/ml Leupeptin*

B wash

- 10mM Tris, pH 8.0
- 0.4M NaCl
- 30mM Sodium Butyrate*
- 1mM PMSF*
- 0.5 µg/ml Leupeptin*
- 0.7 µg/ml Pepstatin*

***Add just before use!**

Notes:

1. Haploid yeast may be used. Use of diploid yeast is suggested to increase yield. Protease deficient strains may also increase yield.
2. Complete resuspension and washing is very important. It is easier to resuspend completely using a small volume, about 10 ml, and a pipet. Add the rest of the volume after the cell pellet is completely resuspended.
3. Solid sodium butyrate may be used directly. If liquid is used, the pH of the solution must be determined after addition, as butyric acid will change the pH dramatically.

Yeast Protein Extracts for SDS-PAGE

Method

1. Grow a 5-ml culture of yeast.

An OD₆₀₀ of about 1.0 is ideal, but this is quite flexible. Cultures that are very dense should be avoided because they are likely to be in stationary phase of growth. If the yeast are in stationary phase (not dividing), they become difficult to break and protein is difficult to recover. Often we grow an overnight culture, then pour most of it out in the morning, add another 4-5 mls of media to the same tube and let grow until late afternoon.

If it is important to balance the loads between samples, we take ODs, and then balance the amount of culture to use for extract so that the same total OD₆₀₀ is used; i.e., use 5 ml of a culture of OD₆₀₀ 0.8, and use 2.5 ml of a culture of OD₆₀₀ 1.6.

2. Spin yeast down (about 3000 rpm, 3-5 min) and aspirate supernatant.
3. Re-suspend pellet in 400 µl of 1.5X SDS-PAGE loading buffers containing leupeptin, pepstatin, PMSF. Transfer to an Eppendorf tube containing about 300-400 µl of glass beads on ice.

The inhibitors are really important for protein recovery.

- 4.
5. Vortex at top speed at 4°C for 5 minutes.

Use the vortex in the cold room.

6. To separate the extract from the glass beads, place each tube on top of another Eppendorf and poke a hole in the top and bottom of the tube using an 18-gauge needle. Give the tandem tubes a quick spin in the microfuge. The extract will now be in the bottom tube and the glass beads in the top tube. The extract is ready for heating and loading. Load 20-30 µl/lane.

Yeast RNA Isolation

Method

1. Grow 50 ml of yeast to mid-log phase (OD_{600} 0.8-2.0).

It is convenient to use a disposable 50-ml conical tube for steps 2-5.

2. Spin down and resuspend yeast pellet in lysis buffer (10mM Tris-HCl pH 7.4, 10 mM EDTA, 0.5% SDS).
3. Freeze by immersing the tube in liquid nitrogen, and store at -80°C until all samples are harvested.
4. Thaw samples and add an equal volume of hot phenol (65°C , either water equilibrated or equilibrated with low pH buffer) and vortex vigorously.
5. Incubate at 65°C for 30 minutes, vortexing every 5 minutes.
6. Transfer to a 15-ml round bottom polypropylene tube.
7. Spin in JA20 type rotor for 20 minutes at 10,000 rpm. Use appropriate bumpers.
8. Remove top aqueous phase to a new tubes and add 1/10 volume of 3M sodium acetate, pH 5.3, and vortex.
9. Add 2.5 volumes of 100% ethanol [or an equal (1.0) volume of isopropanol] and vortex.
10. Store 1 hour to overnight at -20°C .
11. Spin at 10,000 rpm for 20 minutes.
12. Pour off supernatant carefully and wash pellet with 75% ethanol.
13. Re-spin for 5 minutes. Carefully remove and discard supernatant.
14. Let air dry, and resuspend in TE. Usually about 1 ml will give an RNA concentration of about 1-2 $\mu\text{g}/\mu\text{l}$.

Yeast Smash 'n' Grab

(A 10-Minute DNA Preparation)

Method

1. Grow a 25 to 50 ml culture of yeast to log phase, OD₆₀₀ of 0.6 to 2.0. You need about 10-20 ml of culture for each transformation.

OD is fairly flexible, but log phase will give the highest transformation efficiency. If you are transforming a supercoiled plasmid – say from a maxi-prep – then even yeast scraped off a fresh plate will work ('fresh' means just grown, not stored in the refrigerator yet).

If you are trying to do a knock-out or have a small amount of DNA or impure DNA, then pay special attention to getting the yeast at the correct density. In this lab, we usually grow a small overnight culture (2-5 ml) and then seed a larger culture in the morning. Use 1-2 ml culture for each 50 ml media seeded. Let grow at least 4 hours.

2. Spin culture down in a 50-ml tube. Discard supernatant.
3. Wash yeast pellet in 10 ml of Li-TE. Spin. Discard supernatant.
4. Resuspend pellet in Li-TE. Use 100 µl per 10-ml culture. For example, use 1 ml for 100 ml of original culture.
5. Put 5 µl of DNA to be transformed and 5 µl of carrier DNA (calf thymus or salmon sperm DNA, 10 mg/ml) into a sterile microfuge tube. Add 100 µl of yeast and tap to mix.
6. Let sit at room temperature for 5 minutes.
7. Add 280 µl of 40% PEG in Li-TE to the transformation and invert to mix.
8. Incubate 45 minutes at 30°C.
9. Add 45 µl of DMSO, mix by inversion and heat shock for 5 minutes at 42°C.
10. Pellet yeast with short microfuge spin.
11. Aspirate supernatant. Change to a new sterile tip for each sample.
12. Resuspend yeast in 1 ml of sterile H₂O. Spin and discard supernatant as in step 11.
13. Resuspend yeast pellet in 0.5 ml of sterile H₂O and plate on appropriate selective media.
14. Incubate 2-4 days at 30°C.

Solutions

1 M Lithium Acetate

- Filter sterilize.

10X TE

(filter sterilized)

- 10 ml 1M Tris, pH 8.0
- 2 ml 0.5M EDTA
- 88 ml H₂O

Li-TE

- 10 ml 1M Lithium Acetate
- 10 ml 10X TE
- 80 ml H₂O
- Filter sterilize

40% PEG

- Make 50% PEG in H₂O
- Autoclave.
- Add 1/10 volume 1M lithium acetate
- Add 1/10 volume 10X TE

Yeast Transformation I

Method

1. Grow a 25 to 50 ml culture of yeast to log phase, OD_{600} of 0.6 to 2.0. You need about 10-20 ml of culture for each transformation.

OD is fairly flexible, but log phase will give the highest transformation efficiency. If you are transforming a supercoiled plasmid – say from a maxi-prep – then even yeast scraped off a fresh plate will work ('fresh' means just grown, not stored in the refrigerator yet).

If you are trying to do a knock-out or have a small amount of DNA or impure DNA, then pay special attention to getting the yeast at the correct density. In this lab, we usually grow a small overnight culture (2-5 ml) and then seed a larger culture in the morning. Use 1-2 ml culture for each 50 ml media seeded. Let grow at least 4 hours.

2. Spin culture down in a 50-ml tube. Discard supernatant.
3. Wash yeast pellet in 10 ml of Li-TE. Spin. Discard supernatant.
4. Resuspend pellet in Li-TE. Use 100 μ l per 10-ml culture. For example, use 1 ml for 100 ml of original culture.
5. Put 5 μ l of DNA to be transformed and 5 μ l of carrier DNA (calf thymus or salmon sperm DNA, 10 mg/ml) into a sterile microfuge tube. Add 100 μ l of yeast and tap to mix.
6. Let sit at room temperature for 5 minutes.
7. Add 280 μ l of 40% PEG in Li-TE to the transformation and invert to mix.
8. Incubate 45 minutes at 30°C.
9. Add 45 μ l of DMSO, mix by inversion and heat shock for 5 minutes at 42°C.
10. Pellet yeast with short microfuge spin.
11. Aspirate supernatant. Change to a new sterile tip for each sample.
12. Resuspend yeast in 1 ml of sterile H₂O. Spin and discard supernatant as in step 11.
13. Resuspend yeast pellet in 0.5 ml of sterile H₂O and plate on appropriate selective media.
14. Incubate 2-4 days at 30°C.

Solutions

1 M Lithium Acetate

- Filter sterilize.

10X TE

(filter sterilized)

- 10 ml 1M Tris, pH 8.0
- 2 ml 0.5M EDTA
- 88 ml H₂O

Li-TE

- 10 ml 1M Lithium Acetate
- 10 ml 10X TE
- 80 ml H₂O
- Filter sterilize

40% PEG

- Make 50% PEG in H₂O
- Autoclave.
- Add 1/10 volume 1M lithium acetate
- Add 1/10 volume 10X TE

Yeast Transformation II

Method

Low Efficiency Transformation (for Plasmids)

Inoculate 5 mls per transformation. Grow yeast overnight (use YPD if possible, selective media if necessary).

High Efficiency (for Linear DNA or Libraries)

Inoculate 5 mls. Grow yeast overnight. Inoculate 50 mls YPD to OD₆₀₀ 0.07. Grow for 4-5 doubles (OD₆₀₀ 0.6-1.0).

For Both

1. Harvest cells. Spin at 2500 rpm for 5 minutes, and discard media.
2. Wash once with sterile H₂O.
3. Resuspend in 1-3 mls sterile H₂O, transfer to Eppendorf tube, spin down, discard supernatant.
4. Resuspend in 1X lithium acetate/TE, spin down, discard supernatant.
5. Add ~1 µg (in 1-8 µl) transforming DNA and 10 µl of single stranded carrier DNA. Mix gently.
6. Add 600 µl PEG/lithium acetate/TE solution, and mix gently.
7. Incubate with gentle rotation at 30°C for 30 min.
8. Heat shock at 42°C for 15 min.
9. Add sterile H₂O, and wash yeast 2-3 times.
10. Resuspend in 500 µl of H₂O.
11. Plate on appropriate selective media.

Solutions

Carrier DNA

- 10 mg/ml
- Boil 5 min
- Place on ice for 5 min before use

10X Lithium Acetate

- 1M, pH 7.5

10X TE

- 100mM Tris, pH 7.5
- 10mM EDTA
- Filter sterilize

50% PEG (3500-4000)

- Dissolve in water
- May have to heat to dissolve

1X Lithium Acetate/TE

- 100 µl 10X TE
- 100 µl 10X LiAc
- 800 µl H₂O

PEG/Lithium Acetate/TE

- 960 µl 50% PEG
- 120 µl 10X LiAc
- 120 µl 10X TE

Mouse Protocols

Fixing and Embedding Embryos

Notes on timing

- For embryos E9.0 or younger, do all washes for 15-20 minutes
- For E10.5-11.5, 30 minutes
- Anything older than E12.5, at least 1 hour

Fixing and Storing Embryos

Fix embryos in fresh 4% PFA, 4°C, 6 hours to overnight.

Wash as follows, 15 minutes each at RT, rocking:

1. 2X PBS
2. 25% Methanol/PBS
3. 50% Methanol/PBS
4. 75% Methanol/PBS
5. 100% Methanol

Store embryos as long as you would like at -20°C in 100% methanol.

Embedding Embryos

15 minutes each at RT, rocking:

1. 2X 100% Ethanol
2. 2X 1:1 100%:Xylene
3. Xylene RT

Switch to 65°C for following steps:

4. Xylene
5. 2X 1:1 Xylene:Paraffin

Note: You can remove embryos to RT at this point (and only this point) and let sit O/N if you want to continue the next day.

6. 2X Paraffin
7. Embed

Once embedded, place molds at 4°C O/N, and remove from the molds the next day, since they come out much more easily when cool.

You will need

- Molds
- Forms/bases
- Fine forceps or 15g needles
- Dissecting scope
- Marking pen
- Alcohol burner (optional)
- Dissection scope for small embryos

If you are only planning on doing H&E staining, you can also fix in Bouin's solution and go directly into 95% ethanol; then proceed from step 8.

For embryos, make sure to mark down the "name" of embryo, age, genotype and orientation, if applicable, on the molds for ease in identification later.

Genotyping Protocols

Each genotyping reaction is a little bit different, requiring slightly different conditions for optimum PCR amplification.

The following worksheets show the various conditions that have been optimized for genotyping different mouse lines. Typically, more than one mouse is genotyped at a time. It is easiest to prepare a Master Mix (MM) that contains everything but the DNA for the PCR reactions.

The worksheets show the amount for one reaction.

- If you have five mice to genotype, then multiply the μL amounts for each reagent by nine:
 - Five genotyping reactions
 - One known positive control
 - One negative control with wild type mouse DNA
 - One negative control with water
 - One extra to compensate for pipetting errors
- Pipet 1 μL of mouse DNA (See the protocol for extracting DNA from mouse tails and yolk sacs.) into the bottom of PCR tubes – one tube per reaction
- Then, aliquot the well-mixed MM into each tube
- For 15 μL reactions, it is not necessary to vortex the reactions and spin them down before putting them into the PCR machine
- For 50 μL reactions, it is advisable to mix each tube thoroughly and spin them down before putting them in the PCR machine
- Primers are used at 10 μM concentration. dNTPs are used at 10 mM concentration
- The H_2O is endonuclease-free
- The buffer and MgCl_2 used are supplied with the different types of Taq polymerase. These are used without dilution
- All values in the worksheets are in μL
- The MM Volume is the amount to aliquot into each reaction tube

Gcn5 wt/flox

1X MM

- **H₂O**: 9.65 µL
- **Buffer**: 1.5 µL
- **MgCl₂**: 0.45 µL
- **DMSO**: 0.6 µL
- **Taq**,
Denville Chroma: 0.9 µL
- **dNTPs**: 0.3 µL
- **Primer 1**
WL20-EJ: 0.3 µL
- **Primer 2**
WL21-3-EJ: 0.3 µL
- **MM Volume**: 14 µL
- **DNA**: 1 µL

X 9

Five Mice to Genotype

- **H₂O**: 86.85 µL
- **Buffer**: 13.5 µL
- **MgCl₂**: 4.05 µL
- **DMSO**: 5.4 µL
- **Taq**,
Denville Chroma: 8.1 µL
- **dNTPs**: 2.7 µL
- **Primer 1**
WL20-EJ: 2.7 µL
- **Primer 2**
WL21-3-EJ: 2.7 µL
- **MM Volume**: 126 µL
- **DNA**: 1 µL

Gcn5 Δ 3-18 and Flox(neo)

Gcn5 Δ 3-18

1X MM

- **H₂O:** 9.65 μ L
- **Buffer:** 1.5 μ L
- **MgCl₂:** 0.45 μ L
- **DMSO:** 0.6 μ L
- **Taq,**
Denville Chroma: 0.9 μ L
- **dNTPs:** 0.3 μ L
- **Primer 1**
WL20-EJ: 0.3 μ L
- **Primer 2**
WL23-EJ: 0.3 μ L
- **MM Volume:** 14 μ L
- **DNA:** 1 μ L

Gcn5 Flox(neo)

1X MM

- **H₂O:** 9.65 μ L
- **Buffer:** 1.5 μ L
- **MgCl₂:** 0.45 μ L
- **DMSO:** 0.6 μ L
- **Taq,**
Denville Chroma: 0.9 μ L
- **dNTPs:** 0.3 μ L
- **Primer 1**
WL20-EJ: 0.3 μ L
- **Primer 2**
WL21-2-EJ: 0.3 μ L
- **MM Volume:** 14 μ L
- **DNA:** 1 μ L

Gcn5 HAT1 wt and mut (exon 12)

Gcn5 HAT1 wt (exon 12)

1X MM

- **H₂O**: 9.65 µL
- **Buffer**: 1.5 µL
- **MgCl₂**: 0.45 µL
- **DMSO**: 0.6 µL
- **Taq**,
Denville Chroma: 0.9 µL
- **dNTPs**: 0.3 µL
- **Primer 1**
JZ-HAT1-wt-F: 0.3 µL
- **Primer 2**
JZ-HAT1-R: 0.3 µL
- **MM Volume**: 14 µL
- **DNA**: 1 µL

Gcn5 HAT1 mut (exon 12)

1X MM

- **H₂O**: 9.65 µL
- **Buffer**: 1.5 µL
- **MgCl₂**: 0.45 µL
- **DMSO**: 0.6 µL
- **Taq**,
Denville Chroma: 0.9 µL
- **dNTPs**: 0.3 µL
- **Primer 1**
JZ-HAT1-m-F: 0.3 µL
- **Primer 2**
JZ-HAT1-R: 0.3 µL
- **MM Volume**: 14 µL
- **DNA**: 1 µL

Gcn5 HAT2 wt and mut (exon 13)

Gcn5 HAT2 wt (exon 13)

1X MM

- **H₂O:** 9.65 µL
- **Buffer:** 1.5 µL
- **MgCl₂:** 0.45 µL
- **DMSO:** 0.6 µL
- **Taq,**
Denville Chroma: 0.9 µL
- **dNTPs:** 0.3 µL
- **Primer 1**
JZ-HAT2-wt-R: 0.3 µL
- **Primer 2**
JZ-HAT2-F: 0.3 µL
- **MM Volume:** 14 µL
- **DNA:** 1 µL

Gnc5 HAT2 mut (exon 13)

1X MM

- **H₂O:** 9.65 µL
- **Buffer:** 1.5 µL
- **MgCl₂:** 0.45 µL
- **DMSO:** 0.6 µL
- **Taq,**
Denville Chroma: 0.9 µL
- **dNTPs:** 0.3 µL
- **Primer 1**
JZ-HAT2-m-R: 0.3 µL
- **Primer 2**
JZ-HAT2-F: 0.3 µL
- **MM Volume:** 14 µL
- **DNA:** 1 µL

Gcn5 Null

1X MM

- **H₂O**: 9.65 µL
- **Buffer**: 1.5 µL
- **MgCl₂**: 0.45 µL
- **DMSO**: 0.6 µL
- **Taq**,
Denville Chroma: 0.9 µL
- **dNTPs**: 0.3 µL
- **Primer 1**
Gcn5Df: 0.3 µL
- **Primer 2**
Gcn5Dr: 0.3 µL
- **MM Volume**: 14 µL
- **DNA**: 1 µL

PCAF - WT and Null

PCAF - WT

1X MM

- **H₂O:** 3.35 µL
- **Buffer D:** 7.5 µL
- **Taq**
Epicentre FailSafe: 0.15 µL
- **Primer 1**
PCAF-WT-F: 1.5 µL
- **Primer 2**
PCAF-WT-R: 1.5 µL
- **MM Volume:** 14 µL
- **DNA:** 1 µL

PCAF - Null

1X MM

- **H₂O:** 3.35 µL
- **Buffer D:** 7.5 µL
- **Taq**
Epicentre FailSafe: 0.15 µL
- **Primer 1**
PCAF-null-F: 1.5 µL
- **Primer 2**
PCAF-null-R: 1.5 µL
- **MM Volume:** 14 µL
- **DNA:** 1 µL

PCP2-Cre and P300

PCP2-Cre

1X MM

- **H₂O:** 4.29 µL
- **Buffer:** 1.2 µL
- **MgCl₂:** 0.96 µL
- **DMSO:** 1.0 µL
- **Taq,**
Denville Chroma: 0.9 µL
- **dNTPs:** 0.25 µL
- **Primer 1**
Cre-For: 0.6 µL
- **Primer 2**
Cre-Rev: 0.6 µL
- **Primer 3**
(IL2 internal control)
Pcp2Con-f: 0.6 µL
- **Primer 4**
(IL2 internal control)
Pcp2Con-R: 0.6 µL
- **MM Volume:** 11 µL
- **DNA:** 1 µL

P300

1X MM

- **H₂O:** 9.45 µL
- **Buffer:** 1.5 µL
- **MgCl₂:** 0.45 µL
- **DMSO:** 0.6 µL
- **Taq,**
Denville Chroma: 0.9 µL
- **dNTPs:** 0.3 µL
- **Primer 1**
CS-1: 0.3 µL

- **Primer 2**
PGK-1: 0.3 µL
- **Primer 3**
(Gcn5 internal control)
WL20-EJ: 0.3 µL
- **Primer 4**
(Gcn5 internal control)
WL21-3-EJ: 0.3 µL
- **MM Volume:** 14 µL
- **DNA:** 1 µL

Band Sizes

This chart shows the expected band sizes for each set of primers:

Genotype	Band Size, bp
GCN5 wt	210
GCN5 Δ 3-18	240
GCN5 Flox	310
GCN5 Flox(neo)	410
GCN5 HAT 1-wt	360
GCN5 HAT 1-mut	360
GCN5 HAT 2-wt	370
GCN5 HAT 2-mut	370
GCN5 Null	450
P300	400 [210-bp internal control (Gcn5)]
PCP2-Cre	100 [320-bp internal control (IL2)]
PCAF-wt	175
PCAF-null	390

Gene	Primer Name	Sequence
Gcn5 WT	WL20-EJ	CACAGAGCTTCTTGGAGACC
Gcn5 WT	W 21-3-EJ	GGCTTGATTCCTGTACCTCC
Gcn5 Δ 3-18	WL23-EJ	ATAGTAGCGACTGCGCAACC
Gcn5 Flox(neo)	WL21-2-EJ	CTGTGCCTTCTAGTTGCCAG
Gcn5 HAT1 WT exon 12	JZ-HAT1-wt-F	TTCCCACTCAGGGCTTCACGGA
Gcn5 HAT1 exon 12	JZ-HAT1-R	CTCTCACAGTAGACCAGGCTGG
Gcn5 HAT1 mut exon 12	JZ-HAT1-m-F	CCCACTCAGGGCTTCACGCG
Gcn5 WT exon 13	JZ-HAT2-wt-R	TGAAGTAGCCAATGGCGTACTCGT
Gcn5 exon 13	JZ-HAT2-F	GTGGTCCACTGACCTCAGACTG
Gcn5 mut exon 13	JZ-HAT2-m-R	TGAAGTAGCCAATGGCGTACTCTG
Gcn5 null	Gcn5Df	TCACTATCTCGGATGGCTT
Gcn5 null	Gcn5Dr	CCTCTTCGCTATTACGCCAG
PCAF WT	PCAF-WT-F	AAGCTGCTCACGTTTCTCACTTGG
PCAF WT	PCAF-WT-R	GGGTAAGCTCTCACCTTGAATAGG

Gene	Primer Name	Sequence
PCAF null	PCAF-Null-F	GGATGTGGAATGTGCGAG
PCAF null	PCAF-Null-R	TCCAACAGCCTCTCTAGCCT
IL2 WT	Pcp2Con-f	CTAGGCCACAGAATTGAAAGATCT
IL2 WT	Pcp2Con-r	GTAGGTGGAAATTCTAGCATCATCC
Pcp2	Cre-For	GCGGTCTGGCAGTAAAACTATC
Pcp2	Cre-Rev	GTGAAACAGCATTGCTGTCACTT
p300	CS-1	CATGCTCACAAGTGCCAGC
p300	PGK-1	CTGCACGAGACTAGTGAGACG

All the above protocols were optimized for the following PCR program:

Cycles	1	15				25				1	Hold
Temp (°C)	95	94	65	72	94	50	72	72	4		
Time	5:00	:30	:30	1:30	:30	:30	1:30	10:00	Hold		

Hematoxylin & Eosin Staining of Section for Histology

Place slides in slide holder and have all of solutions pre-made and in dipping trays prior to starting staining procedure. First two xylene washes are very important for complete de-paraffinization of the sections.

- **Xylene:** 3-5 minutes, 2X
- **100% Ethanol:** 3-5 minutes, 2X
- **95% Ethanol:** 3-5 minutes
- **85% Ethanol:** 3-5 minutes
- **70% Ethanol:** 3-5 minutes
- **Eosin*:** 15-30 seconds
- **50% Ethanol:** 3-5 minutes
- **30% Ethanol:** 3-5 minutes
- **H₂O:** 3-5 minutes
- **Hematoxylin:** 10 seconds to 2 minutes, depending on tissue
- **H₂O:** 3-5 minutes
- **30% Ethanol:** 3-5 minutes
- **50% Ethanol:** 3-5 minutes
- **70% Ethanol:** 3-5 minutes
- **85% Ethanol:** 3-5 minutes
- **95% Ethanol:** 3-5 minutes
- **100% Ethanol:** 3-5 minutes, 2X
- **Xylene:** 3-5 minutes, 2X

*Eosin

- 2.5 ml Acetic Acid
- 0.5 g Eosin
- 497.5 ml 70% Ethanol

Fixing Slides

- Blot away excess xylene, add 2-3 drops Permount and place coverslip on slide. Push out air bubbles

This should dry in ~10 min, but wait until the next day to place on microscope.

- You can also clean away extra Permount using xylene

Irradiating Mouse Embryos

Method

Note: E8.5-E10.5 embryos; ¹³⁷Cs irradiation chamber

1. Irradiate mice with 5 Grey.

p53 mRNA: Increased by 100 minutes

Protein expression: Increased by 6 hours

2. Place a single mouse in plastic cylinder and lock down with plastic lid so mouse lies flat. Place into irradiation chamber and cover with metal disc.
3. To determine timing for 5 Grey, it is helpful to have a chart for the chamber that gives rads/min by month and year.

For example: May 2008 = 151 rad/min. 5 Grey = 500 rads. So for May 2008, 3.31min exposure is needed.

4. To set wall timer, follow directions on side of metal casing.
5. Crank cover over irradiation chamber. Contact will cause timer to begin counting down.
6. When timer is done, open chamber and remove mouse.

Be sure to clean out cylinder when done.

Isolation of DNA from Paraffin-Embedded Tissue

Method

Note: In general, the yield is pretty low and variable.

1. Place 2 sections of tissue, 10 μm each, in a 1.5-ml tube.
2. Spin at 12,000 rpm for 5 minutes to get sections to bottom of tube.
3. Add 200 μl digestion buffer (50mM Tris, pH 8.2, 1mM EDTA, 0.5% Tween-20)
4. Add 2 μl 20 mg/ml proteinase K, for a final concentration of 200 $\mu\text{g/ml}$.
5. Incubate at 55°C for 3 hours O/N.
6. Spin at 12,000 rpm for 10 minutes. A ring of paraffin will form on the top of the liquid. Pierce the ring and transfer the supernatant to a fresh tube.

Make sure tubes are balanced to ensure that the paraffin ring forms well.

7. Heat-inactivate the proteinase K by heating to 95°C for 5 minutes.
8. Add 500 μl of 100% ethanol and 10 μl of 10M ammonium acetate. Incubate 30 minutes at -80°C.
9. Centrifuge at 12,000 rpm for 10 minutes. Decant supernatant.
10. Wash 2X with 70% ethanol, spinning at 12,000 rpm for 5 minutes each.
11. Drain tube and invert to dry.
12. Dissolve DNA in 50 μl TE buffer.
13. Determine concentration by spectrophotometry.

Preparing E8.5 Embryos for Karyotyping

Method

Pre-gelatinize a 24-well plate and pre-warm media.

Day 1: Culturing Embryos

1. Harvest embryos (Keep a portion for genotyping.). Transfer embryos into individual wells of a 24-well plate containing sterile PBS.
2. Move to sterile hood and transfer embryos into Eppendorf tubes with minimal PBS.
3. Add 50 μ l 0.25% trypsin and place in 37°C incubator for 3 minutes.
4. Immediately add 100 μ l media.
5. Mechanically disrupt embryos with Pipetman and transfer into individual wells containing 0.5 ml media in gelatinized 24-well plate.
6. Incubate overnight at 37°C with 5% CO₂.

Day 2: Fixing Embryos

1. Remove media.
2. Add 500 μ l fresh pre-warmed media containing 50 ng/ml colcemid.
2.5 μ l of a 20 μ g/ml stock per ml of media
3. Incubate 4 hours.
Note that time and concentration may vary, so you may want to test ahead of time with a wild-type litter.
4. Remove media and add 250 μ l 0.25% trypsin and place in 37°C incubator for 3 minutes.
5. Immediately add 1 ml media.
6. Pipette up and down to get into single cell suspension and transfer to Eppendorf.
7. Spin down at no more than 500 g for 2 minutes.
8. Pipet off media and add 500 μ l 0.56% KCl and incubate at 37°C for 20 minutes.
9. Spin down at no more than 500 g for 2 minutes.
10. Decant solution. Flick tube to resuspend cells in remaining solution.

This is important! If you don't do this, all your cells will clump together.

11. Add 1 ml fixative -- Methanol:Acetic Acid (3:1) -- and place at 4°C for 20 minutes.
12. Spin down at no more than 500 g for 2 minutes.
13. Add 200 μ l fresh fixative [Methanol:Acetic Acid (3:1)].

14. Keep at 4°C until ready for spreads.

Note: When you do spreads, if you see crystallization on the slide, either your Methanol:Acetic Acid solution is bad, or you still have KCl left in it and need to do an extra fix change.

Media

- 410 ml DMEM
- 75 ml FBS
- 5 ml 100X Pen/Strep

100X b-Mercaptoethanol (b-ME)

- 50 ml 1X PBS
- 36 µl 14M b-ME

Preparing Embryos for Telomere Restriction Fragment Analysis (TRF)

Method

- Use 50,000 cells per plug
- Use 1 E8.5 GCN5 null embryo per 2 agarose plugs (100 µl each)
- Use 1 E8.5 WT embryo for 4 agarose plugs (100 µl each)
- For Southern gel, use 4-5 null embryos and 5-6 WT embryos (10 lanes total)

1. One WT embryo + 200 µl PBS on ice.

In Eppendorf, use 1-ml syringe and 25g needle to make embryo "mush" on ice. Then add very hot 200 µl 2X Pulse Field Agarose (1.8%) to cells and pipet up and down to mix. Quickly pipet 100 µl per plug. Solution will congeal rapidly. Avoid pipetting bubbles into the plug.

2. One null embryo + 100 µl PBS on ice.

In Eppendorf, use 1-ml syringe and 25g needle to make embryo "mush" on ice. Then add very hot 100 µl 2X Pulse Field Agarose (1.8%) to cells and pipet up and down to mix. Quickly pipet 100 µl per plug. Solution will congeal rapidly. Avoid pipetting bubbles into the plug.

3. Pour agarose plugs in small wells with bottom taped. Once set, remove tape and transfer plugs from each genotype to 15-ml tube with 5 ml lysis buffer**.
4. Incubate all plugs at 55°C for 2 days.
5. Wash plug 5X, 4 hours each, in 15 ml TE at 4°C to dialyze.

You do not want to see any bubbles when you shake the plugs after your last wash. Then, you can store plugs indefinitely at 4°C.

6. **Restriction enzyme digestion.**

Digest one plug per genotype overnight at 37°C as follows:

174 µl H₂O

20 µl 10X NEB buffer #2

2 µl HinfI (NEB)

2 µl RsaI (NEB)

2 µl BSA

7. Pulse field gel electrophoresis.

The next day cut the plug in half, using a razor blade and ruler to make sure sizes are uniform. Save the other half of plug in 0.5X TBE at 4°C. It will keep for several months.

Keeping gel on benchtop, fill each well with 0.5X TBE and load 1/2 plug into well of 0.8% TBE gel (1.29 g PF agarose + 160 ml 0.5X TBE) made with pulse field agarose. It is easy to load if you use a metal spatula and pipette tip.

Seal the plug into the well using melted agarose. Leave one well open so you can load molecular weight marker after gel is set up in buffer.

8. Run gel with pulse field electrophoresis in 0.5X TBE using BioRad FIGEMapper power supply with circulation on. Gel run = 16 hrs.
9. Stain gel with EtBr and take picture of gel with ruler for marking molecular weight standards. Make sure all loaded lanes have DNA that is well digested.
10. Dry down gel at 75°C for 1.5 hr on two sheets Whatman paper, with gel covered with Saran Wrap. Mark the orientation of the gel and location of marker lane on Saran Wrap. Put nothing else on gel. Gel can be stored like this in a drawer.
11. **Rehydrate gel** in 2X SSC.

Carefully remove Whatman paper from bottom of gel. Cut the corner of gel nearest the marker lane. Flip gel over with gloved hands and carefully rub off any adhering paper.

Be careful -- gel is fragile.

12. C-rich oligo hybridization.

Roll gel and place in hybridization tube with ~40-50 ml prehyb for 4 hours to O/N at 58°C.

13. Label C-rich oligo to detect G-rich single strand overhang.

To phosphorylate oligo:

4 µl C-rich oligo (1 µg/µl) (5'-CCCTAACCCCTAACCCCTAACCC-3')

2 µl T4 PNK forward reaction buffer

8 µl H₂O

5 µl gamma-³²P-ATP

1 µl T4 polynucleotide kinase

Mix oligo and water together and boil for 1 min. Place on ice for quick chill to ensure that there is no secondary structure. Then add rest of reaction. Incubate 15 minutes to 1 hour at 37°C. Add 0.5mM EDTA to stop reaction.

14. Purify oligo on G-25 spin column. Count with scintillation counter.
15. Use 500,000 cpm/ml hyb solution (Prehyb solution** + kinased oligo). Use a minimum 5 ml hyb solution. Hybridize at 58°C O/N.
16. Washes:

Quick wash 4X in SSC 0.1% SDS

3X for 20 minutes in 4X SSC 0.1% SDS, 80 mls each at RT

3X for 20 minutes in 4X SSC 0.1% SDS, 80 mls each at 55°C

17. Wrap gel in plastic wrap and expose on MS film for 3-5 days at -80°C.
18. Gel can be stored in desk drawer until ready for next hybridization. Rehydrate in 2X SSC before hybridizing.
19. **G-rich oligo hybridization** to detect length of double stranded telomeric DNA.

You need to denature the DNA in the gel prior to hybridization!!!

Denature for 1 hour at RT in 0.6M NaCl/0.2M NaOH

Neutralize for 1 hour at RT in 1.5M NaCl/0.5M Tris, pH 7.4

Rinse gel in H₂O for 30 minutes at RT

20. Repeat steps 12-17 with G-rich oligo (5'-GGTTAGGGTTAGGGTTAGGG-3').

****Solutions**

Lysis Buffer

- 2% N-lauryl sarkosine
- 400mM EDTA
- 1 mg/ml proteinase K
- Store at 4°C

50 ml

- 1 g
- 40 ml 0.5M
- 2.5 ml 20 mg/ml

Prehyb [4 L]

Heat and stir until SDS dissolves. Warm to 60°C before use.

- 0.5M EDTA [8 ml]
- SDS Powder [280 g]
- 1M Sodium Phosphate, pH 7.2 [2000 ml]
- H₂O [1200 ml]

100 ml

Heat and stir until SDS dissolves. Warm to 60°C before use.

- 0.2 ml
- 7 g
- 50 ml
- 30 ml

1M Sodium Phosphate, pH 7.2

- 1M Na₂HPO₄
- 1M NaH₂PO₄-H₂O

100 ml

- 68.4 ml
- 31.6 ml

Making Mouse Embryonic Fibroblasts (MEFs)

Method

1. Dissect E12.5-14.5 mouse embryos into a 10-cm dish with PBS. To ensure a purer fibroblastic population, remove the brain and limbs, and scoop out the internal organs (You can use some of this for genotyping.).
2. Place each individual embryo in a well of a 6-well plate in PBS and transfer plate to tissue culture hood. Transfer to new sterile 6-well plate with 1 ml trypsin per well.
3. Mince embryos up with a sterile razor blade (ethanol and flamed) into very small pieces, with a consistency similar to sludge. Then use 1-ml pipette to help homogenize the embryos further. Place in 37°C incubator for 10 min.
4. Remove from incubator and once again use 1-ml pipette to pipet up and down to further breakup the cells. There will be some viscous material on the bottom of the plates. Immediately add 2 ml MEF media to each well.
5. Split volume between 3 10-cm dishes which have been gelatinized with 0.1% gelatin and incubate at 37°C. Cells should attach within next two days. Change media once the cells have attached.
6. Aspirate remaining cartilaginous chunks (very obvious when you change media).
7. Grow to confluency, then freeze two 10-cm dishes as p0 and continue to grow the other plate, splitting 1:10.

Gelatinizing plates

1. Make 0.1% gelatin solution (bovine skin type I collagen) by dissolving 0.5 g collagen into 500 ml PBS and autoclaving.
2. Cover surface of tissue culture dish with gelatin and let sit at RT 1-2 hours.
3. Pipet off excess solution and let dry min 15 minutes. These dishes can now be stored at 4°C indefinitely.

Solutions

MEF Media

- 440 ml DMEM
- 50 ml FBS (heat-inactivated)
- 5 ml Pen/Strep
- 5 ml L-glutamine
- Filter sterilize

Beta-Mercaptoethanol (BME)

- 72 µl 14M BME
- 100 ml 1x PBS

RT-PCR Primer Sets

RT-PCR Set	Primer 1	Primer 2	Organism	Reference	Temperature / Size	Expression
Activin b	GTCAATTTGACGTGGTTTCC	GCAAGAATGTGCTGATCAAC	Mouse	Keller <i>et al.</i> , Jan 1993 MCB 13(1) p473-486	Annealing temp = 50 Size = 426	Expressed in ES cells / primitive ectoderm. Disappears by 3rd differentiation.
ATM (NM_007499)*	ATCGCAGAGCGCCTCCATGTC	GAAGAACATGATCTGTGGGTG	mouse	Heyer <i>et al.</i> , G&D 2000 14:2072	443 bp Primers span 3 introns(2.2 kb)	Upregulated following DNA damage
AFP	GCTCACACCAAAGCGTCAAC	CCTGTGAACCTCTGGTATCAG	Mouse	Keller <i>et al.</i> , Jan 1993 MCB 13(1) p473-486	Annealing temp = 50 Size = 1400	Exp yolk sac E8.5 / visceral endoderm. Not expressed in ES cells.
AIB1	CGTCCTCCATATAACCGAGC	TCATAGGTTCCATTCTGCCG	Human	Kurebayashi J <i>et al.</i> , Clin Canc Res 6:512-518: 2000	Annealing temp = 57 Size = 256	-
b-Actin	TGACGGGGTCACCCACACTGTGCCATCTA	CTAGAAGCATTTGCGGTGGACGATGGAGGG	Human	Kurebayashi J <i>et al.</i> , Clin Canc Res 6:512-518: 2000	Size = 610	-
b-Major Globin	CTGACAGATGCTCTCTTGGG	CACAACCCAGAAACAGACA	Mouse	Keller <i>et al.</i> , Jan 1993 MCB 13(1) p473-486	Annealing temp = 55 Size = 1296;	Exp Fetal liver E14. Exp by D4 ES differentiation.
bH1	AGTCCCATGGAGTCAAAGA	CTCAAGGAGACCTTTGCTCA	Mouse	Keller <i>et al.</i> , Jan 1993 MCB 13(1) p473-486	Annealing temp = 55 Size = 1053	Exp yolk sac E8.5. Exp by D4 ES differentiation.
Brachyury	TCCAGGTGCTATATATTGCC	TGCTGCCTGTGAGTCATAAC	Mouse	Keller <i>et al.</i> , Jan 1993 MCB 13(1) p473-486; Heyer <i>et al.</i> , G&D 2000 14:2072	Annealing temp = 55 Size = >>947	Gastrulation marker. Exp embryo E8.5/mesoderm. Exp by D4 ES differentiation, then declines at D8.
c-kit	TGTCTCTCCAGTTTCCCTGC	TTCAGGGACTCATGGGCTCA	Mouse	Keller <i>et al.</i> , Jan 1993 MCB 13(1) p473-486	Annealing temp = 55 Size = 765	Exp bone marrow
CBP	TCAGTCAACATCTCCTTCGC	TGTTGAACATGAGCCAGACG	Human	Kurebayashi J <i>et al.</i> , Clin Canc Res 6:512-518: 2000	Annealing temp = 58 Size = 272	-
CBP**	CAAGCACTGAATCCACAAGCACAAA	TATTCTTGATATCTGTAGGAAAAGC	Mouse	MC designed in lab	-	-
Collagen IV	CAAGCATAGTGGTCCGAGTC	AGGCAGGTCAAGTTCTAGCG	Mouse	Keller <i>et al.</i> , Jan 1993 MCB 13(1) p473-486	Annealing temp = 55 Size = 463	Exp PYS cell line / primitive endoderm. Low levels in undifferentiated ES cells with inc at 24

						hrs, then high levels at 4 days post differentiation.
ER-a	agacatgagagctgccaacc	gccaggcacattctagaagg	Human	Kurebayashi J <i>et al.</i> , Clin Canc Res 6:512-518: 2000	Annealing temp = 55 Size = 299	-
ER-b	TCACATCTGTATGCGGAACC	CGTAACACTTCCGAAGTCGG	Human	Kurebayashi J <i>et al.</i> , Clin Canc Res 6:512-518: 2000	Annealing temp = 58 Size = 346	-
GAPDH (NM_008084)**	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGCCATGTAGGCCAT	Mouse	Heyer <i>et al.</i> , G&D 2000 14:2072	229 bp	-
GAPDH (NM_008084)**	CCATGACAACCTTGGCATTG	CCTGCTTCACCACCTTCTTG	Mouse	MC designed in our lab	~300 bp	-
GAPDH	CCAATGTGTCCGTCGTGGATCT	GTTGAAGTCGCAGGAGACAACC	Mouse	Poleskaya <i>et al.</i> , EMBO J 2001 Dec 3;20(23):6816-25	-	-
GATA-1	ATGCCTGTAATCCAGCACT	TCATGGTGGTAGCTGGTAGC	Mouse	Keller <i>et al.</i> , Jan 1993 MCB 13(1) p473-486	Annealing temp = 55 Size = 581+smear	Exp Fetal liver E13. Exp by D5 ES differentiation.
GATA-3	CCTTAAACTCTTGGCGTCC	AGACACATGTCATCCCTGAG	Mouse	Keller <i>et al.</i> , Jan 1993 MCB 13(1) p473-486	Annealing temp = 55 Size = 532	Exp fetal thymus E18. Occasionally detected in undifferentiated ES. Exp by D4 differentiating.
HPRT	CACAGGACTAGAACCTGC	GCTGGTGAAAAGGACCTCT	Mouse	Keller <i>et al.</i> , Jan 1993 MCB 13(1) p473-486	Annealing temp = 50 Size = ~1100	Housekeeping gene
KU70 (NM_010247)**	CTTCATTTTCTGGTTGATG	TGGTGATGATGTCCTGTAG	Mouse	MC designed in our lab	656 bp Primers span 2 introns.	-
KU80/86**	ATTCCTTTTCTAAAGTGGATGAG	AATCAGATCATCAATAGCGCT	Mouse	MC designed in our lab	415 bp Primers span 4 introns.	-
MCK	CACCATGCCGT TCGCAACA	CACCATGCCGTTT GCACAACA	Mouse	Poleskaya <i>et al.</i> , EMBO J 2001 Dec 3;20(23):6816-25	-	-
MDM2 C- terminus	GTAGAATTTGAAGTTGAATCT	CTAGGGGAAATAAGTTAGCAC	Human	Kawai H <i>et al.</i> , JBC Vol. 276(49):45928-45932	-	-
N-CoR	GCTGATGAGGATGTGGATGG	TTGGAATCTTGGATGTGCC	Human	Kurebayashi J <i>et al.</i> , Clin Canc Res 6:512-518: 2000	Annealing temp = 58 Size = 349	-

p300 bromodomain (U10877)	AGCCAAAGAAAAAGATTTCA	ACATCACTGGGTCAATTTCTT	Human	Kawai H <i>et al.</i> , JBC Vol. 276(49):45928- 45932	-	-
p300**	GTGGTGATTAATCAGCTTCAGA	CCCTGCTGAAGAGGCTCAGTCAGTA	Mouse	MC designed in lab	-	-
p53 (NM_011640)* MGSC	GGATAGCAAAGAGCACAGAGC exon 1 or just upstream according to blast, 472bp from 2nd primer. One bp discrepancy w/ MGSC seq: GGATAGGAAAGAGCACAGAGC	CCAGTCTTCGGACAAGCGTGAC at 3' end of exon 1 according to blast of MGSC. One bp discrepancy w/ MGSC seq: CCAGTCTTCGGAGAAGCGTGAC	Mouse	Heyer <i>et al.</i> , G&D 2000 14:2072	-	Upregulated following DNA damage.
p53 N-terminus	GCCACCATTGGACTACAAGGAC	GCGGAGATTCTTCTCTCTGT	Human	Kawai H <i>et al.</i> , JBC Vol. 276(49):45928- 45932	-	Upregulated following DNA damage.
P/CAF (Q92831)	AGAACATTGCTTCGCTCGG	TGCCTCAAGTCCAGAAGAGG	Human	Kurebayashi J <i>et al.</i> , Clin Canc Res 6:512-518: 2000	Annealing temp = 57 Size = 345	-
SMRT	TGTGGTTCATAAGCCACTCTGC	CGGAATCTCCCTCCTCCC	Human	Kurebayashi J <i>et al.</i> , Clin Canc Res 6:512-518: 2000	Annealing temp = 58 Size = 179	-
SRC-1	GCTCGTTCATCCATTGCC	ACTACTTGTCATGCCAACGG	Human	Kurebayashi J <i>et al.</i> , Clin Canc Res 6:512-518: 2000	Annealing temp = 57 Size = 372	-
TIF2	TAATGCACAGATGCTGGCC	TCTGTGTATGTGCCATTCGG	Human	Kurebayashi J <i>et al.</i> , Clin Canc Res 6:512-518: 2000	Annealing temp = 58 Size = 314	-
TRF2 (AF003000)	GCCCAAAGCATCCAAGAC	ACTCCATCCTTCACCCACTC	Mouse	MC designed in our lab	173 bp Spans 2 introns.	-

*These have not yet worked in Dent lab.

**This has worked in Dent lab.

Tail and Yolk Digests for PCR

Method

Tail Digests

1. Cut tails or toes into 2-3 small pieces and add 750 μ l tail digest buffer containing 0.2 mg/ml proteinase K. Heat 55°C for 3 hours to O/N. If rocking, vortex briefly after 1 hour and return to 55°C.
2. Extract with 500 μ l P:C 1:1, vortex and spin 2 minutes at high speed. Transfer 600 μ l aqueous to new tube.
3. Add 550 μ l isopropanol, vortex and let stand 2-5 minutes to precipitate DNA.
4. Spin at high speed 5 minutes, remove liquid and wash pellet with 2X 70% ethanol. Dry pellet at RT for ~1hour.
5. Resuspend in 100 μ l TE or H₂O. It may take O/N at 4°C to resuspend.
6. Use 1 μ l for PCR genotyping.

Tail Digest Buffer Stock Solution

Final

- 10mM Tris, pH 8.0
 - 25mM EDTA, pH 8.0
 - 100mM NaCl
 - 1% SDS
 - 0.2 mg/ml Proteinase K
- Add proteinase K right before use*

For 100 ml

- 1.0 ml 1.0M Tris, pH 8.0
 - 5.0 ml 0.5M EDTA, pH 8.0
 - 2.0 ml 5.0M NaCl
 - 10 ml 10% SDS
 - 10 l/ml 20 mg/ml Proteinase K
- Add proteinase K right before use*

Yolk Sac Digest for PCR Genotyping

E8.5-E11.5

1. Put yolk sac in 50 µl PBT/0.2% Tween® 20 with 1 µl fresh 10 mg/ml proteinase K.
2. Incubate 55°C for 3 hours to O/N.
3. Boil samples for 5 minutes to inactivate proteinase K.
4. Spin briefly to collect pellet cell debris at bottom of tube.
5. Take 1 µl for PCR.

For E12.5-15.5, only use part of amniotic sac.

Alternative Yolk Sac Digest for PCR Genotyping

E8.5-E9.5

Based on ES DNA prep, this has worked really well with embryo yolk sacs.

1. Put yolk sac in 50 µl ES cell DNA digest buffer and digest O/N at 55-60°C.
2. Boil samples for 5 minutes to inactivate proteinase K.
3. Precipitate DNA by adding 100 µl of ice-cold ethanol + 15 µl 5M NaCl. Keep on ice for 15 minutes.
4. Spin down 4°C for 15 minutes.
5. Wash pellet with 70% ethanol.
6. Resuspend in 25 µl H₂O.

ES Cell DNA Digest Buffer

- 10mM Tris, pH 7.5
- 10mM EDTA
- 10mM NaCl
- 0.5% Sarkosyl

Generally, 1/10-µl dilution of DNA for PCR is used.

On those for which this doesn't work, increase the concentration to a full 1 µl per 25-µl PCR reaction.

Whole Mount Immunohistochemistry

Notes

- Throughout the entire protocol, the embryos should be gently agitated to improve penetration of the tissue
- At E10-11, pinch hole in telencephalon and at base of head so that neural tube will flush easily. This will help prevent Ab trapping.

Method

1. Fix embryos in 4% paraformaldehyde/PBS at 4°C O/N.
2. Bleach embryos in 5:1 H₂O₂:PBS at RT for 3-5 hours.

You can then run through methanol series and store in 100% methanol at -20°C if desired. You will then need to rehydrate to PBS before proceeding with protocol.

3. Incubate 2X in PBSMT at RT for 1 hour each.
4. Incubate at 4°C O/N with primary antibody in PBMST. A 1:100-250 dilution is generally used.
5. Wash 2X in PBMST at 4°C, and 3X in PBMST at RT for 1 hour.

Note: Increase volume and number of changes if possible.

6. Incubate 4°C O/N with secondary antibody diluted in PBSMT. Peroxidase-coupled secondary generally used at a 1:200-500 dilution.
7. Repeat wash steps a second day, adding a final wash in PBT for 20 minutes.
8. Incubate embryos in 0.3 mg/ml DAB and 0.5% NiCl₂ in PBT at RT for at least 20 minutes.

NiCl₂ may precipitate a little, so add extra water.

9. Add 0.00003% H₂O₂ (yes, really) and incubate at 4°C O/N.
10. Rinse in PBT for 2 days or longer.
11. You can dehydrate and store in 100% methanol.

12. Alternatively, embryos can be cleared in benzyl alcohol:benzyl benzoate (1:2 BABB).

Note: Use glass containers with BABB!!

Solutions

PBSMT

- 2% Milk
- 0.1% Triton X-100 in PBS

PBT

- 0.2% BSA (Sigma A-4378)
- 0.1% Triton X-100 in PBS
- Add List Text Here

DAB

- Diaminobenzidine [*Carcinogenic*]

Whole Mount *In Situ*

Method

1. Dissect embryos in PBS, remove membranes and dissect as necessary.

For older embryos use the forceps and make a hole in the roof of the hind brain and open the forebrain.

2. Fix in 4% paraformaldehyde 4°C O/N, rocking in 5-10 ml volume.
3. Wash in PBT 2X for 5 minutes at 4°C, gently rocking.
4. Wash in sequence in 25%, 50%, 75% methanol:PBT for 10 minutes at RT, rocking.
5. Wash 2X in 100% methanol for 10 min, rocking.
6. Store in 100% methanol at -20°C.

DIG-Labeled Probe Synthesis

Linearizing Plasmid

Reaction Mix

- 10X EZ Buffer™: 2.0 µl
 - Reaction EZ (20 U): 1.5 µl
 - Plasmid DNA 1 µg/µl: 5.0 µl
 - H₂O: 12.5 µl
1. Incubate 37°C for 1-3 hours. Run 1 µl on 1% TBE gel to check for complete digestion.
 2. Add 80 µl TE8 buffer, extract with 100 µl phenol:chloroform, spin 5 minutes at RT and transfer top aqueous layer to new tube.
 3. Repeat extraction. Then extract once with 100 µl chloroform and save aqueous layer.
 4. Add 1/10 volume 3M sodium acetate, 2 volumes ethanol, 1 µl glycogen or yeast tRNA. Incubate for 1 hour at -20°C.
 5. Spin 15 minutes at 4°C, maximum speed. Remove supernatant and wash pellet with 70% ethanol. Air dry.
 6. Resuspend pellet in 10 µl TE8 (0.5 µg/µl) buffer and store at 4°C.

Reaction

- DEPC-H₂O: 12.5 µl
- 10X Transcription Buffer: 2.0 µl
- 2.0 µl DIG Nucleotide Mix:

10mM G, A, CTP
6.5mM UTP
3.5mM DIG-UTP:

- Linearized Plasmid 0.5 µg/µl: 2.0 µl
- Polymerase (T7, T3): 1.0 µl
- RNase Inhibitor: 0.5 µl
- **Exception:**
When using SP6 polymerase, adjust reaction to:

4 µl 5X Transcription Buffer
2.0 µl 100mM DTT
9.5 µl DEPC-H₂O

Run-Off Transcription

1. Incubate 37°C for 2 hours.
2. Check transcription by running 1 µl on 1% TBE gel.

A single predominant band should be seen at a greater intensity than the plasmid band. Additional fainter higher MW bands may be present.

3. Add 1 µl DNase (RNase-free) and incubate at 37°C for 15 minutes.
4. Add 100 µl TE8, 10 µl 4M LiCl, 300 µl ethanol and incubate at -20°C for 1 hour to O/N.
5. Spin 10 minutes in microfuge at 4°C, maximum speed. Wash pellet with 70% ethanol and air dry pellet.
6. Redissolve pellet in 50 µl TE8, and add 50 µl hybridization buffer. This can be stored at -20°C for several months.

Notes

- CsCl DNA works best, or miniprep DNA that has not been RNased
- Enzymes which leave 3' overhangs should not be used for run-off transcription

Day 1: Pretreatment and Hybridization -- ~5 hours

- All steps are performed on nutator
 - All washes are performed in glass scintillation vials in volumes of 5-10 mls, unless otherwise specified
 - Make all solutions fresh with DEPC-H₂O and filter
1. Transfer embryos to clean scintillation vials and rehydrate in 75%, 50%, 25% methanol:PBT-DEPC.
 2. Wash 2X in PBT-DEPC, 5 minutes each.
 3. Bleach embryos with 6% H₂O₂ in PBT for 1 hour at RT.
 4. Wash 3X PBT for 5 minutes at RT.
 5. Treat with proteinase K in PBT for 15 minutes at RT.

For E8.0-9.0, use 0.333-0.25 µg/ml (1/30-1/40 dilution of 10 µg/ml)

E9.5-11.5, use 0.5-2.0 µg/ml (1/20-1/5 dilution of 10 µg/ml)

6. Wash with 2 mg/ml glycine in PBT for 10 minutes at RT.

Make fresh and filter.

7. Wash 2X PBT for 3 minutes at RT.
8. Postfix with 4% paraformaldehyde and 0.2% glutaraldehyde for 20 minutes at RT.
9. Wash 2X with PBT at RT.

Transfer to 1.5-ml o-ring screw-top tubes. For larger embryos, leave in scintillation vials and adjust volumes as necessary, but be sure not to invert tubes, as embryos may stick to the cap after this point.

10. Add 1.0 ml prehyb to tubes and mix gently by inversion. Remove and add another ml. Prehyb at 70°C for 1 hour.

At this point, while still in prehyb, you can store the embryos at -20°C, either before or after heating.

11. Make up hyb solution (1 ml prehyb + approx 1 µg/µl DIG-probe [10-25 µl usually]), and keep liquid at 37°C until ready to add to embryo.

12. Remove prehyb and add 0.5 ml hyb solution. Invert gently several times, and then remove liquid. Add final 0.5 ml hyb solution and hybridize O/N at 70°C.

Note:

If you keep your formamide frozen, take it out of freezer to thaw O/N so can be used to make solutions for Day 2.

Day 2: Posthybridization and Antibody (Ab) Hybridization -- ~7 hours

Embryos are extremely sticky in Solutions I and III. Be very careful not to touch the embryos with pipette tip or plastic pipette once in these solutions!!

1. Add 5 ml Solution I to clean glass scintillation vials. Transfer embryos from Eppendorf tubes into pre-warmed Solution I in scintillation vials.
2. Wash 3X for 30 minutes in Solution I at 70°C.
3. Switch to pre-warmed Solution III and wash 2X for 30 minutes at 65°C.
4. Wash with fresh TBST (0.1% Tween 20 and 2mM levimasole) 3X for 5 minutes each at RT. Then transfer embryos to o-ring capped screw tubes during last wash.
5. Preblock with 1 ml 10% sheep serum (heat-inactivated) in TBST for 2.5 hour at RT, rocking.
6. While preblocking, adsorb **secondary antibody***
7. Remove blocking serum and add 1 ml Ab mixture. Invert gently several times then remove. Add other ml of Ab mixture and rock gently O/N at 4°C.

***Adsorb Secondary Antibody**

Per embryo (2 ml final volume per embryo final):

- Put approx 3 mg (per embryo) embryo powder in a screw capped tube
Scale up per embryo
- Add 0.5 ml TBST (per 2 ml final) and rock at 70°C for 30 minutes
- Vortex for 10 minutes
- Cool on ice for 5 minutes, and add 5 µl sheep serum (heat-inactivated) and 1 µl mouse β-DIG (per 2 ml final)
- Shake gently at 4°C for 1 hour.
- Spin at 4000 rpm for 10 minutes at 4°C
- Collect supernatant into a common 15-ml tube
- Dilute with 1% sheep serum (heat-inactivated) in TBST to a final volume of 2 ml/embryo (~1.5 ml per embryo)
- Spin mixture again at 4000 rpm for 10 minutes at 4°C

Day 3: Post-Ab Hybridization -- ~6 hours

1. Transfer embryos from o-ring tubes into 5 ml fresh TBST with 2mM levimasole in glass scintillation vials. Wash 3X for 5 minutes each at RT.
2. Wash 5X 1-1.5 hour each in TBST with 2mM levimasole at RT, rocking.
3. Wash O/N in TBST with 2mM levimasole, rocking, at 4°C.

Day 4: Detecting -- ~1-2 hours

1. Wash 3X in NTMT 1 minute at RT.
2. Transfer embryos to o-ring tubes during last NTMT wash.
3. Prepare **Reaction Mix****.
4. Remove NTMT.
5. Add 1 ml Reaction Mix, and mix gently. Remove and add new ml of Reaction Mix.
6. Cover tubes with foil and place on rocker for 20 minutes at RT. Check embryos to see if you are getting a signal.

If not developed by 20 minutes, monitor ~once an hour, or as you feel needed. After each check, add fresh reaction mix.

7. When the reaction is judged complete, transfer to scintillation vials and wash 2X with NTMT for 10 minutes each at RT, covered in foil.
8. Wash 2X in PBT for 10 minutes at RT.

If probe gives you high background, you can extend this step at 4°C for several days.

9. Post fix with 4% paraformaldehyde/0.1% glutaraldehyde for 1 hour at RT. Wash 2X PBT. Store at 4°C in Eppendorf tubes for several months. Take pictures as soon as possible after development since stain will fade.
10. To clear embryos to make visualization of light staining easier:

Wash embryos with increasing concentrations of glycerol in PBT: 30%, 50%, 70%, 80% for 15 minutes to 1 hour, depending on embryo size. You can add 0.05% azide for long term storage at 4°C.

****Reaction Mix**

- 6.75 µl NBT
- 5.25 µl BCIP
- 2 ml NTMT

Use fresh stocks of NBT [0.075 g/ml 70% dimethylformamide (DMF)] and BCIP-Na salt (0.05 g/ml H₂O [or BCIP-toluidine salt (0.05 g/ml DMF)]).

Solutions

BCIP

- 0.05 g per 1 ml H₂O for sodium salt
or
- 1 ml DMF for toluidine salt

DEPC-H₂O

- 0.1% DEPC in H₂O
- Let sit at 37°C O/N
- Autoclave

Embryo Powder

- Sacrifice 1-2 litters of E12.5-E14.5 mouse embryos
One E13.5 litter should yield 200-400 mg embryo powder
- Remove membranes, and homogenize in minimum volume ice-cold PBS. It is preferable to mush embryos up in PBS using mortar and pestle, then homogenizing by pulling back and forth through an 18g needle
- Add 4 volumes ice-cold acetone
- Vortex and put on ice for 30 minutes
- Spin down in Oak Ridge tubes at 10,000 g for 10 minutes at 4°C
- Discard floating debris and rinse pellet in ice-cold acetone
- Discard floating debris and rinse pellet in ice-cold acetone
- Allow pellet to completely dry on filter paper
- Grind to powder with mortar and pestle
- Store in Eppendorf tubes at -20°C

NBT

- 0.075 g in 1 ml dimethylformamide (DMF)

NTMT

Make up fresh and filter before use.

Final Concentration

- 100mM NaCl
- 100mM Tris, pH 9.5
- 50mM MgCl₂
- 0.1% Tween-20
- 2mM Levamisole

For 100 ml

- 2 ml 5M NaCl
- 5 ml 2M Tris, pH 9.5
- 5 ml 1M MgCl₂
- 0.1 g Tween-20
- 0.048 g Levamisole
- 88 ml H₂O

4% Paraformaldehyde (PFA)

This is good for ~1 week, although fresh PFA is always best.

- 4 g Paraformaldehyde
- 90 ml H₂O
- 20 µl 10N NaOH
- Mix and heat at 65°C for ~10 minutes. Swirl to ensure all of paraformaldehyde is in solution.
- Add 10 ml 10X PBS for a final volume of 100 ml.
- Filter and store at 4°C.

PBT

- Make 10X PBS in DEPC-H₂O
- Autoclave.
- Dilute to 1X with DEPC-H₂O
- Add 0.1% Tween-20
- Filter before use

Prehybridization (Prehyb) and Hybridization (Hyb) Solutions

All components should be made with DEPC-H₂O.

Heat to 65°C once made, then filter and store aliquoted at -20°C.

For hybridization solution, add approx 1 µg probe per ml prehyb solution.

Final Concentration

- 50% Formamide
- 5X SSC, pH 4.5
- 50 µg/ml Yeast RNA
- 1% SDS
- 50 µg/ml Heparin

For 100 ml

- 5 ml Formamide
- 25 ml 20X SSC, pH 4.5
- 0.5 ml 10 mg/ml Yeast RNA in DEPC-H₂O
(Extracted 2X with phenol:chloroform)
- 10 ml 10% SDS
- 0.1 ml 50 mg/ml in DEPC-H₂O
- 14.8 ml DEPC-H₂O

Sheep Serum

- Inactivate by heating to 55°C for 60 minutes
- Can be stored in aliquots at -20°C

Solution I

Make up fresh. Preheat before filtering and before use.

Use approximately 25 ml/embryo.

Final Concentration

- 50% Formamide
- 5X SSC, pH 4.5
- 1% SDS

For 100 ml

- 50 ml Formamide
- 25 ml 20X SSC, pH 4.5
- 10 ml 10% SDS
- 15 ml H₂O

Solution III

Make up fresh. Preheat before filtering and before use.

Use approximately 25 ml/embryo.

Final Concentration

- 50% Formamide
- 2X SSC, pH 4.5

For 100 ml

- 50 ml Formamide
- 10 ml 20X SSC, pH 4.5
- 40 ml H₂O

20X SSC, pH 4.5

Final Concentration

- 3M NaCl
- 0.3M Na₃-citrate
- Adjust pH with 5M citric acid.

For 1 Liter

- 175.3 g
- 88.2 g
- 800 ml H₂O
- Bring to 1 liter with H₂O

TBST

Make from stock of 10X TBS, dilute to 1X and add Tween-20 and levamisole.

Does not require DEPC-H₂O.

100 ml 10X TBS

- 8 g NaCl
- 0.2 g KCl
- 25 ml 1M Tris, pH 7.5
- Bring to 100 ml with H₂O

100 ml TBST

- 10 ml 10X TBS
- 0.1% Tween-20
- 2mM Levamisole
- Bring to 100 ml with H₂O

Bacterial Protocols

Bacterial Media Recipes

LB

Per liter

- 10 g Bactotryptone
- 5 g Yeast Extract
- 10 g NaCl
- Adjust pH to 7.5 with 1N NaOH 0.5 g NaCl
- Autoclave
- *Plates:*

Add 20 g/L bactoagar prior to autoclaving, and pour when media has cooled so it is still warm to the touch.

M9

Per liter

- 50 ml 20X M9 salts*
- 20 ml 20% Glucose
- 1 ml 1M MgSO₄
- Add 100 ml 10X M9 salts
- 930 ml Autoclaved Deionized H₂O

TB**

**For protein expression in LB media, due to presence of lactose in tryptone.
(Sambrook *et al.*, 1989)

Per liter

- 900 ml Deionized H₂O
- Autoclave, cool to 60°C, and add 100 ml sterile potassium phosphate solution
- 12 g Bactotryptone
- Add glucose to 1% final concentration using an autoclaved 20% stock when dealing with toxic genes, or with promoters very sensitive to T7 concentration
- 24 g Yeast Extract
- 4 ml Glycerol

***M9 Salts**

10X M9 Salts

Per liter

- 10 g NH_4Cl
- 30 g KH_2PO_4
- 60 g $\text{Na}_2\text{HPO}_4 \cdot 7 \text{H}_2\text{O}$
- Autoclave

20X M9 Salts

- 20 g NH_4Cl
- 60 g KH_2PO_4
- 120 g $\text{Na}_2\text{HPO}_4 \cdot 7 \text{H}_2\text{O}$
- Autoclave

Potassium Phosphate

- 23.1 g KH_2PO_4
- 125.4 g K_2HPO_4
- Autoclave

Colony Hybridization

Method

Allow plates to grow O/N. Fairly large sized colonies, not very dense with no satellites, are easiest for picking.

1. Lift colonies onto a dry nylon or nitrocellulose filter. Allow to lie in place until filter is completely wetted, usually only 20 seconds or so.
2. Mark the filter and plate with an 18g needle three times around edge, not evenly spaced for orientation. Lift filter off plate carefully with a forceps. Entire colonies should be lifted with filter. Let plate sit 6 hours at 37°C, or O/N at RT to regrow colonies.

For next steps have prewetted Whatman paper already set-up.

3. Place filter colony side up on Whatman paper prewetted with Solution A for 3 minutes.
4. Blot underside of filter on paper towel and repeat step 3.
5. Blot underside of filter on paper towel. Place filter on Whatman prewetted in Solution B for 5 minutes.
6. Blot underside of filter on paper towel. Place filter on Whatman prewetted in Solution C for 3 minutes.
7. Blot underside of filter on paper towel. Place filter on Whatman prewetted in 2X SSC for 30 seconds.
8. Blot underside of filter on paper towel. Dry filters in oven at 80°C for 30 minutes or until dry. Alternatively, dry under a heating lamp.
9. Crosslink membranes in UV crosslinker.
10. Prehybridize in Rapid Hyb™ Buffer if using nylon filters or in prehyb solution below if using nitrocellulose, 65°C for 10 minutes to 1 hour.
11. Hybridize in Rapid Hyb™ Buffer if using nylon filters or in hyb solution below if using nitrocellulose, 65°C for O/N. Use 2 X 10⁵ cpm/ml probe.
12. Wash 2X at RT, 10 minutes, and 2X at 65°C, 30 minutes each, in Wash Buffer I.
13. Wash 1X at 65°C, 20 minutes, in 0.2X SSC/0.2% SDS.

Monitor all washes with Geiger counter!!

14. Wrap damp blots in plastic wrap and expose to film, no longer than 2 hours at RT.
15. Using pinpricks to match filter to film to plate, pick positive colonies for minipreps.

Solutions

Solution A

- 10% SDS

Solution B

- 0.5M NaOH
- 1.5M NaCl

Solution C

- 0.5M Tris, pH 8
- 1.5M NaCl

Prehyb (Nitrocellulose Membranes)

Add Paragraph Text

- 0.4 g BSA
- 0.08 ml 0.5M EDTA
- 21.0 ml NaPi pH 7.2
- 14 ml 20% SDS
- 4.9 ml H₂O

Hyb (Nitrocellulose Membranes)

Add Paragraph Text

- 0.4 g BSA
- 0.08 ml 0.5M EDTA
- 21.0 ml NaPi, pH 7.2
- 2 ml 20% SDS
- 17 ml H₂O

Wash Buffer I

Add Paragraph Text

- 182 ml H₂O
- 8 ml 1M NaPi
- 0.4 ml 0.5M EDTA
- 10 ml 20% SDS

NaPi for 1 L

Add Paragraph Text

- 134 g Na₂HPO₄
- 4 ml H₃PO₄
- Check pH

Notes

1. Always have a negative control, i.e., empty vector ligation to determine hybridization background.
2. After lifts, never allow plates to overgrow. Satellite colonies will always form around a clone and can confuse results. O/N at RT works best for regrowing.
3. There is no need to overwet Whatman papers during lysis steps.
4. Any normal Southern blot protocol will work. Remember, when using nitrocellulose filters, to not use formamide-based solutions, since the filters will become brittle and fall apart.
5. Positive colonies should be quite obvious after 1- to 2-hour exposure. If it takes longer to see a signal, you likely do not have any inserts on your plate.

*Adapted from protocols originating in the laboratories of Dr. Sharon Dent and Dr. William Klein at MD Anderson Cancer Center

Making Electrocompetent Cells

Method

1. Grow 5 ml O/N in LB.
2. Inoculate 400-500 ml LB with 1/100 volume bacteria from O/N culture.
3. Grow to $OD_{600} = 0.6$.
4. Chill cells on ice for 15 minutes to several hours.
5. Pellet cells at 4°C, 2600 rpm, GSA rotor.
6. Wash 2X in 200 ml pre-chilled, unused, sterile H₂O.
7. Pellet as above.
8. Resuspend in final volume of 2 ml **GYT***.
9. Freeze 50- μ l aliquots immediately in dry ice/ethanol.
10. Store -80°C.

*GYT

- 10% Glycerol
- 0.125% Yeast Extract
- 0.25% Tryptone
- Q with H₂O
- Autoclave and store at 4°C

Making Chemically Competent (heat shockable) Cells

Method

1. Inoculate 500 ml LB with 5 ml of a 25-ml O/N culture.
2. Grow at 37°C until $OD_{600} = 0.45-0.55$
3. Chill cells on ice water for 2 hours.
4. Pellet cells at 4°C, 2500 g (3700 rpm in GSA rotor) for 15-20 minutes.
5. Resuspend cells in 10 to 20 ml ice-cold **Ca/Mg buffer****.
6. Then bring volume up to 500 ml with the Ca/Mg buffer.
7. Incubate on ice for 45 minutes.
8. Centrifuge cells 1800 g (3200 rpm GSA rotor) for 10 minutes at 4°C.
9. Gently resuspend pellet in 50 ml ice-cold Mg/Cl buffer.
10. Pool cells if you have split them up and add 80% glycerol drop by drop, on ice with gentle swirling, to a final concentration of 15% v/v.
11. Aliquot cells (0.2-1 ml), freeze on dry ice and store at -80°C.

****Ca/Mg Buffer**

- 100mM $CaCl_2$
- 70mM $MgCl_2$
- 40mM Sodium Acetate pH 5.5
- Filter sterilize

Bacterial Plasmid Mini-prep

Method

1. Pick one colony into 5 ml LB + antibiotic selection (50 µg/ml ampicillin -- If plasmid is larger than ~12 Kb, double the amount of antibiotic) and grow O/N at 37°C.
2. Spin down 1.5 ml of overnight culture in Eppendorf for 1 minute on High.
3. Aspirate supernatant and resuspend cell pellet in 100 µl Solution I.
4. Add 200 µl Solution II mix gently by inversion 10-15 times.
5. Add 150 µl Solution III, vortex briefly to mix and spin for 5 minutes on High.
6. Transfer supernatant to fresh tube and add 500 µl phenol:chloroform, vortex and spin for 5 minutes on High.
7. Transfer aqueous layer to fresh tube and add 1 ml ethanol, mix well by inversion and spin for 5 minutes on High.
8. Remove supernatant and wash pellet with 100 µl 70% ethanol. Spin for 1 minute, maximum speed.
9. Remove as much of the ethanol as possible and dry tubes on bench for 5-10 minutes.
10. Resuspend DNA in 40 µl of H₂O containing 20 µg/ml RNase A.
11. Determine DNA by spectrophotometry and test for proper plasmid recovery by restriction digest.

Solutions

Solution I

- 25mM Tris-HCl, pH 8.0
- 10mM EDTA

Solution II

- 0.2N NaOH
- 1.0% SDS

Solution III 3M Potassium Acetate, pH 4.8

- 60 ml 5M Potassium Acetate
- 11.5 ml Glacial Acetic Acid
- 28.5 ml H₂O

E. Coli Transformation and Blue/White Selection

Heat Shock Transformation

Items to have ready ahead of time:

- LB media
- 1.5-ml microfuge tubes
- 42°C waterbath
- Ice
- 37°C waterbath and incubator
- LB agarose plates with appropriate antibiotic

Protocol

1. Thaw competent cells on ice, usually 100–200 µl per tube

We most often use DH5α and XL-1 blue bacteria.

2. Add a maximum of 20 µl of a ligation reaction, or 10-200 ng plasmid DNA.
3. *Mix very gently!*
4. Incubate the tubes on ice for 30 minutes.
5. Heat shock the cells for 45 seconds at 42°C.

The length of time may differ, depending on bacterial strain.

6. Place the tubes immediately on ice for at least 2 minutes.
7. Add 800 µl of LB medium (no antibiotics) to each tube.
8. Incubate for 1 hour at 37°C, preferably shaking vigorously.
9. Spin down briefly and remove most of the supernatant.
10. Resuspend cell pellet with the 200 µl medium in the tube by pipetting.
11. For ligations, plate the entire suspension on an LB plate containing antibiotic. For a plasmid transformation, plate out 10-20 µl on one LB plate and the remainder on a second plate containing the appropriate antibiotic.
12. Incubate the plates overnight at 37°C.

Electro-Transformation

1. Pre-chill cuvettes and LB or SOC media.
2. Thaw competent cells on ice.

Again, we most often use DH5α and XL-1 blue bacteria.

3. Add 100 μ l cells, plus 1 μ l DNA solution, or no more than 1.5 μ l of ligation mix, for electroporation, and electroporate at 2.5 kV/400 ohms (varies with size of cuvette).
4. Immediately resuspend cells in chilled 800 μ l LB and let recover at 37°C for 30 minutes.
5. Plate on selective agar.

Blue/White Selection for XL-1 Blue Cells

- Use this selection method when cloning into a vector (i.e., pBKS/pBSK), where you will disrupt a LacZ gene if an insertion occurs
- Thirty minutes to 1 hour prior to spreading of transformed cells on plate, spread 100 μ l 2% X-gal (in DMSO or dimethyl formamide) and 50 μ l 0.1M IPTG (238 μ g/ml H₂O) on each plate.
- The next day, colonies that have an insertion disrupting the LacZ gene will be white, and those with no insertion will be blue. You only need to screen the white colonies for proper insertion.

Cell Culture Protocols

Isolating RNA, DNA and Protein from ES Cells

Methods

RNA

1. From a single 3-day confluent well of a 6-well dish, scrape cells off bottom and pellet.
2. Add 0.5 ml TRIzol® Reagent, let sit at RT for 10 min and follow rest of procedure from TRIzol protocol, with volumes cut in half (since protocol starts with 1 ml TRIzol Reagent).
3. Resuspend final pellet in 20 µl formamide and store at -80°C.
4. Determine RNA concentration by spectrophotometry, making sure the blank cuvette has 1 µl formamide in it.

****Always run sample of RNA on agarose gel to check for degradation.****

DNA

1. From a single 3-day confluent well of a 6-well dish, scrape cells off bottom and pellet.
2. Add 0.5 ml **ES cell DNA digest buffer*** and digest O/N at 55-60°C.
3. Precipitate DNA by adding 1 ml of [ice-cold ethanol + 15 µl 5M NaCl]. Keep on ice for 15 minutes.
4. Spin down 4°C for 15 minutes.
5. Wash pellet with 70% ethanol.
6. Resuspend in 50-100 µl H₂O.
7. Determine concentration by spectrophotometry.

***ES Cell DNA Digest Buffer**

- 10mM Tris, pH 7.5
- 10mM EDTA
- 10mM NaCl
- 0.5% Sarkosyl
- 1 mg/ml Proteinase K

Protein

1. From a single 3-day confluent well of a 6-well dish, scrape cells off bottom and pellet.
2. Add 0.25 ml RIPA with protease inhibitors and, if you are looking at histones, Na butyrate added.
3. Let lyse on ice for 20 minutes.
4. Sonicate for 10 pulses at 30% power.
5. Spin out debris for 10 minutes at 4°C.
6. Transfer aqueous to new tube. You can store at -20°C.
7. Use Bradford assay to determine concentration of total protein.

To Precipitate Protein

1. Add 8 volumes acetone: 2 volumes protein solution.
2. Place on ice for 30 minutes.
3. Spin for 10 minutes, 4°C, at maximum speed.
4. Discard supernatant.
5. Use SpeedVac to finish drying pellet.
6. Resuspend in RIPA + loading dye.

Solutions

RIPA

- 1x PBS
- 1% Nonidet® P40
- 0.5% Sodium Deoxycholate
- 0.1% SDS

500 ml

- 50 ml 10x PBS
- 5 ml
- 2.5 g
- 5 ml 10%

Protease Inhibitors

- 10 µg/ml PMSF
- 1 µM Pepstatin
- 1 µM Leupeptin

Propidium Iodide Staining For FACS

Method

1. Collect 2×10^6 cells.
2. Resuspend cell pellet in 2 ml PBS.
3. Vortex gently while adding 5 ml of 95% ethanol.
4. Fix at least 30 minutes at RT.
5. Store at 4°C (or -20°C) until ready to stain.

Staining

1. Spin cells out of fix (3000x 5 minutes).
2. Resuspend pellet in 1 ml of PI Working Solution.
3. Add 100 µl of 1 mg/ml DNase-free Rnase.
4. Incubate at 37°C for 30 minutes.
5. Samples are ready for flow cytometry (FCM) analysis. They may be stored in refrigerator O/N.

Solutions

Note: For a single FCM run, use the same PI working solution to reduce variation between samples.

Stock Propidium Iodide (PI)

- 500 µg/ml made in H₂O

Working Solution

- 1:10 dilution with PBS or Tris buffer

General Protocols for DNA

Making Blunt DNA Ends

Klenow Repairs 5' Overhangs to Generate Blunt Ends

- 5'ATGCATGC_{TACG}
 - 3'TACGTACGTACG
 - 5'ATGCATGC ~~ATGC~~
 - 3'TACGTACG
-
- Fill-in recessed 3' termini
 - 3'=>5' exonuclease
 -
 - **Very weak** -- Use T4 DNA polymerase
-
1. In a 20-μl reaction, digest 0.1 to 4 μg DNA with reaction enzyme.
 2. Add 1 μl of 0.5mM dNTP. It is unnecessary to inactivate the restriction endonuclease, to change buffers or to repurify the DNA prior to adding the Klenow fragment.
 3. Add 1 to 5 units of the Klenow fragment and incubate at 30°C for 15 minutes.
 4. Stop the reaction by heating to 75°C for 10 minutes or by adding 1 μl of 0.5M EDTA.

For restriction fragments produced by cleavage with different endonucleases, it is possible to repair one end selectively. This is done by cleaving with enzyme 1, repairing the ends, inactivating the Klenow fragment by heat (75°C for 10 minutes), and cleaving with enzyme 2.

T4 DNA Polymerase Trims/Fills in 3' Overhangs

This protocol employs the activity of T4 DNA polymerase, which catalyzes 3'=>5' synthesis from primed single-stranded DNA. The enzyme has a 3'=>5' exonuclease activity, but lacks 5'=>3' exonuclease activity.

5' ATGCATGCTACG
3' TACGTACGATGC (Fill-in of recessed 5' terminus)

5' ATGCATGC ~~ATGC~~ (3'-5' exonuclease removes overhang)
3' TACGTACG

For fill-in, set up the same and incubate at 12-16°C for 15 minutes.

Note: At higher temperatures the exonuclease activity is very active.

Protocol 1: Blunting DNA with T4 DNA Polymerase

Using digested DNA which has been precipitated and resuspended in water...

1. Prepare the 5X Reaction Buffer*
2. Incubate the mixture at 11°C for 20 minutes, or at room temperature for 5 minutes.
3. Stop the reaction by adding EDTA to 10mM final concentration, then heat at 70°C for 10 minutes.
4. Extract with phenol:chloroform and precipitate DNA for further use.

*5X Reaction Buffer

- Digested DNA
- 2mM dNTP Mix
- T4 DNA Polymerase
- Water, Nuclease-Free

4 µl

- 1 µg
- 1 µl
(0.1mM final concentration)
- 1 unit
- Q to 20 µl

Protocol 2: T4 fill-in with larger amounts of DNA

Using digested DNA which has been precipitated and resuspended in ddH₂O to a final concentration of approximately 500 µg/ml.

1. Set up the following reaction(s)**.
2. Stop the reaction by adding EDTA to 10mM final concentration, then heat at 70°C for 10 minutes.
3. Extract with phenol:chloroform and precipitate DNA for further use.

****Reaction Mixtures**

Small Reaction (1 to 10 µg DNA)

Final reaction volume is 50 µl.

- 5 µl of 10X T4 DNA Polymerase Buffer
- 2.5 µl 2mM dNTPs
- 2.5 µl 2 mg/ml BSA
- 2 to 20 µl DNA (1 to 10 µg of DNA)
- 5 units T4 DNA Polymerase
- 20 to 38 µl ddH₂O

Large Reaction (10 to 30 µg)

Final reaction volume is 100 µl.

- 10 µl 10X T4 DNA Polymerase Buffer
- 5 µl 2mM dNTPs
- 5 µl 2 mg/ml BSA
- 20 to 60 µl DNA (10 to 30 µg of DNA)
- 10 units T4 DNA Polymerase
- 20 to 60 ddH₂O
- Incubate at 12°C for 20 minutes or 37°C for 5 minutes
- Inactivate at 65°C for 10 minutes

Recipes for DNA Loading Dye

Solutions

6X Dye II

Store at RT.

- Bromophenol Blue
- Xylene Cyanol
- Ficoll Type 400
- H₂O

10 ml

Store at RT.

- 25 mg
- 25 mg
- 1.5 g
- Q to 10 ml

6X Dye IV

Store at 4°C so that mold doesn't grow in the sucrose.

- Bromophenol Blue
- Sucrose
- H₂O

10 ml

Store at 4°C so that mold doesn't grow in the sucrose.

- 25 mg
- 4 g
- Q to 10 ml

6X Dye for Cloning

Store at RT. This works well for cloning since BMB is left out and won't obscure fragments in the 200-600 bp range.

- Orange G
- Xylene Cyanol
- Ficoll Type 400
- H₂O

10 ml

Store at RT. This works well for cloning since BMB is left out and won't obscure fragments in the 200-600 bp range.

- 25 mg
- 25 mg
- 1.5 g
- Q to 10 ml

Notes

1. Depending on percentage of agarose, Xylene Cyanol runs ~4 Kb; Bromophenol Blue ~200-400 bp; Orange G at bottom of gel.
2. 10 ml of 6X should last you several years.
3. You can use any combination of dyes for your loading buffer -- just choose the ones that won't obscure the bands you want to see. An Orange G alone buffer, for instance, will never have a dye front that obscures your bands.

Oligonucleotide Purification

Method

Pre-siliconize plates and one Eppendorf tube per oligo with Sigmacote®.

1. Resuspend (or dry down) oligo to 33 µg/µl (OD₂₆₀) in H₂O.
2. Take 8 µl 33 µg/µl oligo + 8 µl formamide and heat at 90°C for 2 minutes.
If you need more than 33 µg of oligo, run more than one lane.
3. Make a medium-sized 15% PAGE sequencing gel. Pre-run gel for ~15 minutes, and then flush wells. Run a separate lane with Sequencing Stop Buffer, which contains bromophenol blue (BMB) and xylene cyanol (XC) for sizes. BMB runs approximately 10 bases and XC runs approximately 55 bases.
4. Run at 300V for ~2-4 hours.
5. Remove top gel plate and cover gel with plastic wrap, then flip and coax gel off of bottom plate. Then cover other side with plastic wrap.
6. Place gel on intensifying screen and look from the top with shortwave UV (minimal time). You should see an intense band corresponding to your oligo plus a smear of smaller, incompletely synthesized DNA.

Notes: If you overload the lanes, you will not get good separation and will end up having a lot of contamination in the "intense band" that you see in an overloaded lane.

Use a marker to circle area on one side of plastic wrap.

7. Remove from UV, flip gel over and remove plastic wrap from other side. Cut out indicated area of gel, dice up gel fragment and place in siliconized Eppendorf.
8. Add 1ml 0.5M NH₄Ac/0.01M Mg(Ac)₂. Vortex and incubate 6 hours O/N at 37°C.
9. Spin down gel pieces and withdraw supernatant. Rinse gel slices with an additional 0.5 ml same buffer and add to other supernatant.
10. Dry down in SpeedVac to about 200 µl. Then precipitate DNA with 1 ml 100% ethanol (The salts will probably precipitate but should dissolve during the ethanol washes.).
11. Wash pellet (and salts) with 70% ethanol and spin down DNA.
12. Resuspend DNA in 200 µl H₂O.
13. Extract with phenol:chloroform twice.
14. Read at OD₂₆₀ of 5 µl in 100 µl for concentration.

15% Gel (0.5-mm thickness)

- 31.5 g Urea
- 29.5 ml Acrylamide (40%, 19:1)
- 15 ml 5X TBE
- 8 ml H₂O

*From S. Y. Roth Dent: Personal notes 1999

Single Stranded DNA Isolation and Mutagenesis

Getting ssDNA

1. Transform plasmid into dut-ung-F^o cells (CJ236) on LB/Ab selection plate.
2. Inoculate a single colony into 2X TY media + 100 µg/µl amp + 0.1% glucose. Grow O/N 37°C.
3. Take 100 µl of O/N culture and add 3 µl M13K07 helper phage and incubate 20 minutes 37°C.
4. Add 3 ml 2X TY + 100 µg/ml amp + 20 µg/ml kanamycin + 0.25 µg/ml uridine + 0.1% glucose. Incubate at 30°C for 20-24 hours or 37°C for 8-12 hours.

Some researchers take colony into this, plus phage, and incubate 37°C.

For 3 ml, add 6 µl 50 mg/ml amp, 6 µl 10 mg/ml kan, 0.3 µl 2.5 mg/ml uridine, 30 µl 10% glucose.

5. Transfer to Eppendorf tubes and spin bacteria down at 12K for 2 minutes. Save supernatant (phage).
6. Add 1/4 volume 20% PEG-8000/3.5M ammonium acetate. Incubate at RT for 15 minutes.
7. Harvest phage by spinning at 12K at 4°C for 20 minutes. Thoroughly drain supernatant.

You should have a white pellet at this point.

8. Resuspend pellet in 1/10 volume of fresh **digestion buffer**.*
9. Heat at 55°C for 20 minutes.
10. Extract DNA 1X with phenol:chloroform, then 1X with chloroform.
11. Precipitate DNA with 1/10 volume 3M sodium acetate, then with 2 volumes ethanol.
12. Dissolve DNA in 20 µl H₂O, and check 1 µl on gel. ssDNA with uracil incorporation should run similarly to undigested plasmid DNA -- it is about the same size.

*Digestion Buffer

-
- 1mM EDTA
- 10mM Tris, pH 8.0
- 0.2% Sarkosyl
- 50 µg/ml Proteinase K

1 ml

- 965.5 μl H_2O
- 2 μl 0.5M EDTA
- 10 μl 1M Tris
- 20 μl 10% Sarkosyl
- 2.5 μl 20 mg/ml Proteinase K

Mutagenesis

Phosphorylation of Oligonucleotides

1. Set up **phosphorylation reaction****.
2. Incubate at 37°C for 30-45 minutes.
3. Heat-inactivate kinase at 65°C for 10 minutes.

**Phosphorylation Reaction

- 20 pmol Mutagenizing Oligo
- 2 μl 10mM dATP
- 4 μl 5x Kinase Buffer
- 1 μl T4 Polynucleotide Kinase (NEB)
- 12 μl H_2O
- Total volume: 20 μl

Annealing

1. Set up **annealing reaction†**.
Run one tube with no oligo, as a control.
2. Heat at 70°C for 5 minutes in a beaker of water in hybridization oven.
3. Take beaker of water out on benchtop with thermometer and let cool gradually to 30°C (~40 minutes to 1 hour).

†Annealing Reaction

- 1.2 μl ssDNA (50-100 ng)
- 7 μl Phosphorylated Oligo (7 pmol)
- 1 μl 10x Annealing Buffer
- 0.8 μl H_2O
- Total Volume: 20 μl

10x Annealing buffer

- 200mM Tris, pH 7.4
- 20mM MgCl₂
- 500mM NaCl

Second Strand Synthesis

1. Set up **synthesis reaction**‡.
2. Incubate for 5 minutes at RT.
3. Incubate for 90 minutes to 3 hours at 37°C
4. Run 5 µl on gel.

You should see an upward shift compared to ssDNA.

‡Synthesis Reaction

- 1 µl 10x Synthesis Buffer
- 1 µl 10mM dATP
- 0.2 µl 0.1M DTT
- 0.5 µl T4 DNA ligase
- 0.5 µl T4 DNA polymerase

10x Synthesis Buffer

- 175mM Tris, pH 7.4
- 37.5mM MgCl₂
- 4mM dNTPs

Transformation

1. For heat-competent XL-1 blues, use 7 µl of reaction.
2. Plate out on LB/Amp.
3. Expect significantly fewer colonies on mutated plates compared to control.

Southern Blot

Genomic DNA Digest

- 10 μ l DNA (10 μ g)
- 3 μ l 10X Buffer
- 1-2 μ l Restriction Enzyme (40 units)
- 3 μ l 10mM Spermidine-HCl
- 3 μ l 1 mg/ml BSA
- 10 μ l H₂O
- Total volume: 30 μ l

Note: Use DNA from a standard tail prep resuspended in 100 μ l. You may want to spec the DNA pf 2-3 samples to determine an average volume for 10 μ g DNA rather than just using 10 μ l.

1. Incubate O/N at 37°C in 96-well plate (or small PCR tubes). Seal edges of plate with Parafilm®. Place damp paper towel on top of plate (or tubes) and wrap the whole thing in plastic wrap. Place in 37°C chamber. (This should prevent condensation in the caps of the tubes.)
2. Run entire reaction mix on 0.8% TAE gel. (Medium-sized gel: 150V ~2.5hrs)
3. Take a picture of the gel with a ruler along the side. Place the 0-cm mark at the bottom of the wells so you can determine where molecular weight markers are via ruler.
4. Soak gel in 0.1N HCl (stock = 14.4N) for at least 20 minutes on shaker.
5. Soak gel in 0.4M NaOH for 10-20 minutes on shaker.
6. **Set Up Capillary Transfer**

Precut GeneScreen® membrane and 4 pieces of Whatman® paper.

Fill container with 0.4M NaOH, place glass plate across it and drape a long piece of Whatman across it so its ends are in the NaOH in the container.

Set up transfer, beginning at the bottom, as follows (Leave at RT O/N.):

Top of transfer
Wrap all edges with plastic wrap to prevent bypass of the capillary, and stack paper towels on top to pull the NaOH through the gel.
Place book or weight on top.
2 pieces Whatman paper
Membrane (mark wells with 25g needle)
Gel, bottom side up
2 pieces Whatman paper, cut to size of gel, prewet in NaOH
Bottom of transfer

7. Next day, remove paper towel and top 2 pieces of Whatman paper. Make sure wells are marked with needle and clip edge of what is the front top left corner for orientation.
8. Wash membrane in 0.2M Tris, pH 7-8 / 2X SSC for 5 minutes. Then crosslink membrane with UV crosslinker.
9. Blot off extra liquid, then roll membrane and place in hybridization tube with DNA side inward. Prehybridize with 15 ml Rapid Hyb™ 15-30 minutes at 65°C.

Hybridize

Note: Make probe either that morning or the day before.

1. Pour off prehyb solution and add 20 ml fresh Rapid Hyb™ Buffer with $2-3 \times 10^5$ cpm/ml probe.
2. Boil probe for 5 minutes, then cool on ice for 5 minutes before adding to hyb solution.
3. Hybridize 1.5 - 3 hours at 65°C (You can let it continue O/N).
4. Discard hyb and rinse tube with 10 ml 2X SSC, and discard. Then wash in 25 ml 2X SSC for 10 minutes at RT.
5. Wash 2X with 25 ml 15 minutes each at 65°C 1X SSC/0.1% SDS, and discard.
6. Wash 2X with 25 ml 15 minutes 65°C 0.5X SSC/0.1% SDS, and discard.
7. Wash 2X with 25 ml SSC 10 minutes at RT.
8. Blot off extra liquid, wrap in plastic wrap and put in phosphoimaging cassette (1 hour) or film (3 days to 1 week).

Strip Southern

Pour boiling 0.1% SDS onto membrane, then let cool to RT on benchtop.

Let cool to room temperature while shaking.

Check on phosphoimager.

General Protocols for RNA

Northern Blot

Prepare Gel

1. For standard medium-sized gel, dissolve 1.5 g agarose in 108 ml DEPC H₂O. Boil then cool to 60°C in a water bath.
2. Place flask in fume hood once at 60°C and add 27 ml formaldehyde and 15 ml 10X MOPS. Swirl to mix, then pour gel and allow to set.
3. Keep gel tank in fume hood due to presence of formaldehyde. Pour running buffer (1X MOPS in DEPC H₂O) in amount sufficient to cover gel.

Prepare Sample and Run Gel

1. Adjust volume of RNA sample (10 µg) to a total of 5.5 ml with DEPC H₂O, then add 2.5 ml 10X MOPS, 4.5 ml 12.3M formaldehyde (stock concentration) and 12.5 ml formamide.

Prepare RNA marker at same time.

2. Mix by vortexing and centrifuge briefly. Incubate at 15 minutes at 55°C.
3. Add 4 ml formaldehyde loading buffer and 1 µl 10 mg/ml ethidium bromide, vortex and quick spin to collect liquid and load into gel.
4. Run the gel at 5 V/cm (for a medium gel: ~70 V) until the bromophenol blue dye has migrated halfway to 2/3 way down the gel. This usually takes ~3 hours.

Note: People in this lab have run medium gels up to 120 V without problems, thus far.

5. Photograph gel with a ruler on the side to measure placement of markers.

Transfer of RNA

1. Soak gel in 5 volumes distilled DEPC H₂O for 5 minutes, shaking. Repeat 4X.
2. Place GeneScreen® membrane, cut to size, in DEPC H₂O for a few seconds to hydrate.
3. Soak membrane in 10X SSC for 15 minutes.
4. Set up capillary blot using 10X SSC as the transfer solution. Be sure to remove all bubbles between the layers.

<p style="text-align: center;">Top of transfer</p> <p style="text-align: center;">Stack paper towels on top to pull the SSC through the gel. Place book or weight on top. 3 pieces Whatman® paper Membrane (Mark wells and what will be top left corner of membrane with needle or pen.) Gel, bottom side up 3 pieces Whatman paper, cut to size of gel, prewet in SSC</p> <p style="text-align: center;">Bottom of transfer</p>
--

5. Transfer 16-24 hours.
6. Carefully remove filter paper without disturbing membrane.
7. Lift membrane away from gel with plastic forceps.
8. Rinse the membrane briefly in 2X SSC to remove residual agarose. Fix RNA to the membrane using UV crosslinker.
9. Place the membrane RNA side up on filter paper to dry. You can rehydrate it in 2X SSC.

Hybridization of Probe

1. Prehybridize membrane in a minimum volume at 42°C for 2-4 hours with agitation.

Pre-heat prehyb solution before placing on membrane.

2. Remove prehyb solution and add fresh pre-heated hybridization solution and ~5 X 10⁵ dpm denatured probe.
3. Hybridize O/N at 42°C with agitation.

Washes

1. Wash blot 1X for 5 minutes at RT with 2X SSC.
2. Wash 3X for 20 minutes each at 65°C with 0.2X SSC/0.1%SDS.
3. Wash 1X for 5 minutes at RT with 2X SSC.
4. Wrap in plastic wrap and place on phosphorimager or film.

Stripping Northern

1. Pour boiling stripping solution onto membrane, then keep at 80-90°C for 5 minutes.
2. Pour off this solution. You can repeat as necessary. Check blot on phosphoimager to ensure successful removal of probe.

Solutions

DEPC H₂O

- 0.1% DEPC in H₂O
- Let sit O/N at 37°C
- Autoclave

10X MOPS in DEPC H₂O

- 0.4M MOPS, pH 7.0
- 0.1M Sodium Acetate
- 0.01M EDTA
- Store covered in foil up to 3 months at 4°C.

500 ml

- Add 20.8 g MOPS to 350 ml DEPC H₂O
- pH to 7 with 10N NaOH before adding remaining ingredients
- 25 ml of 2M stock made with DEPC H₂O
- 10 ml of 0.5M stock, preferably made with DEPC H₂O
- Store covered in foil up to 3 months at 4°C.

Formaldehyde Loading Buffer

- 1mM EDTA
- 0.25% (w/v) Bromophenol Blue
- 0.25% (w/v) Xylene Cyanol
- 50% (v/v) Glycerol
- Store up to 3 months at RT

10 ml

- 20 ml 0.5M EDTA
- 0.25 g

- 0.25 g
- 5 ml
- Store up to 3 months at RT

10X SSC

- 3M NaCl
- 0.3M Sodium Citrate
- Autoclave.

100X Denhardt's

- 1 g Polyvinylpyrrolidone (PVP; MW=40.000)
- 1 g BSA
- 1 g Ficoll 400
- Add dH₂O to 50 ml and filter sterilize
- Store at 4°C or aliquot and store frozen

Prehybridization Solution

- 5X SSC
- 50% (w/v) Deionized Formamide
- 5X Denhardt's Solution
- 1% SDS
- 10% Dextran Sulfate, Sodium Salt (MW=500,000)

100 ml

- 20 ml 20X
- 50 ml
- 5 ml 100X
- 10 ml 10%
- 10 g
- 100 mg/ml denatured sheared, nonhomologous DNA, i.e., salmon sperm DNA
- Shear by pushing through syringe and then boil before adding to prehyb solution

Hybridization Solution

- 5X SSC
- 50% (w/v) Deionized Formamide

- 5X Denhardt's Solution
- 1% SDS
- 10% Dextran Sulfate, Sodium Salt (MW=500,000)

100 ml

- 20 ml 20X
- 50 ml
- 5 ml 100X
- 10 ml 10%
- 10 g

Stripping Solution

- 1% SDS
- 0.1X SSC
- 40 mM Tris, pH 7.5
- Store up to 1 year at RT

*Combination of protocols from *Current Protocols in Molecular Biology* and *GeneScreen® Plus Protocols*

Large and Small Scale RNA Isolation

Small Scale Isolation

1. Place up to 20 mg tissue in a 1.5-ml Eppendorf tube.
2. Add 200 µl denaturing solution.
3. If there is lots of tissue, use autoclaved glass homogenizer which has been treated with RNaseZap®.
4. Vortex vigorously until all tissue is broken up and exposed to solution, with no chunks left in solution.
5. Add 20 µl 2M sodium acetate, pH 4.0, and vortex.
6. Add 200 µl water-saturated phenol, and vortex.
7. Add 80 µl chloroform:isoamyl alcohol (49:1), and mix well.
8. Centrifuge 4°C, maximum speed, 15 minutes.
9. Transfer aqueous layer to new tube.
10. Precipitate with 200 µl isopropanol, vortex and let sit on ice for ~10 minutes.
11. Centrifuge 4°C, maximum speed, 15 minutes.
12. Pour off supernatant and pipette off extra.
13. Wash with 75% ethanol, vortex and spin, pour off supernatant and pipette off extra.
14. Dry, inverted, for 10 minutes at RT.
15. Resuspend in 20-200 µl 100% formamide. You can also resuspend in DEPC H₂O.
16. Check 1 µl 1:100 or 1:200 by spectrophotometry, being sure to use 1 µl formamide in the blank.

If you are selecting for poly(A), do it here, then...

17. DNase if you are using this for RT-PCR.
18. Resuspend in 200 µl denaturing solution.
19. Extract with phenol:chloroform, then with chloroform:isoamyl alcohol.
20. Precipitate with 200 µl isopropanol.
21. Wash with 500 µl 75% ethanol, vortexing well.
22. Centrifuge 4°C, maximum, 5 minutes.
23. Dry, inverted, 10 minutes at RT.
24. Resuspend in 20-200 µl 100% formamide.
25. Check 1 µl 1:100 or 1:200 by spectrophotometry, being sure to use 1 µl formamide in the blank.

Large Scale RNA Isolation

1. Flash freeze embryos or tissue in liquid nitrogen. Weigh frozen sample.
2. Grind samples to fine powder using mortar and pestle (which has been treated with RNaseZap or rinsed with 1% SDS and then DEPC H₂O), sitting on ice .

Keep adding liquid nitrogen as you grind, to keep tissue frozen.

3. Use pre-chilled, cleaned, metal spatula to scrape/scoop powdered sample into 15/50 ml conical with appropriate volume of denaturing solution = 1 mg/10 ml. You can keep adding liquid nitrogen to help collect sample in pestle and scoop out once nitrogen has evaporated. Vortex occasionally to make sure the tissue is fully covered in guanidine isothiocyanate solution as quickly as possible.
4. Vortex sample to completely expose to denaturing solution.

Follow steps 5 - 14 above, scaling up volumes as appropriate.

For centrifugation, use either a glass conical or plastic capped Sorvall tube which has been autoclaved or treated with RNaseZap and washed with DEPC H₂O. Spin in Sorvall SA600 at 10K for 15 minutes at 4°C.

5. Add 20 µl 2M sodium acetate, pH 4.0, and vortex.
6. Add 200 µl water-saturated phenol, and vortex.
7. Add 80 µl chloroform:isoamyl alcohol (49:1), and mix well.
8. Centrifuge 4°C, maximum speed, 15 minutes.
9. Transfer aqueous layer to new tube.
10. Precipitate with 200 µl isopropanol, vortex and let sit on ice for ~10 minutes.
11. Centrifuge 4°C, maximum speed, 15 minutes.
12. Pour off supernatant and pipette off extra.
13. Wash with 75% ethanol, vortex and spin, pour off supernatant and pipette off extra.
14. Dry, inverted, for 10 minutes at RT.
15. Once pellet has been dried, resuspend in 100% formamide at 4°C in 500-1000 µl. You can also resuspend in DEPC H₂O. Let pellet dissolve at RT for 15 minutes or at 4°C O/N.
16. Check 1:500 sample in H₂O by spectrophotometry. Be sure to use 1 µl formamide in the blank.

Denaturing Solution

- 4M Guanidine Isothiocyanate
- 25mM Sodium Citrate, pH 7
- 1M β -Mercaptoethanol (β -ME)
- 0.5% N-Laurylsarcosine (Sarkosyl)
- Prepare by dissolving 250 g Guanidine Isothiocyanate in 293 ml H₂O, 17.6 ml 0.75M sodium citrate, pH 7, and 26.4 ml 10% Sarkosyl at 65°C with stirring. Keep at RT for up to 3 months.
- For a working solution, add 0.35 ml β -ME to 50 ml stock solution at time of RNA isolation.

Note: We have found that resuspending the RNA in formamide has increased the longevity of the RNA when stored at 4°C, -20°C and -80°C. RNA resuspended in formamide can be used for northern, RT-PCR and nuclease protection assays.

RNA Precipitation

Note: In general, overnight precipitation gives better yields for all of these protocols.

Isopropanol Precipitation

1. Bring up volume to 135 μ l and add 15 μ l ammonium acetate solution (5M NH_4OAc , 100mM EDTA).
2. Extract 1X with phenol:chloroform.
3. Extract 1X with chloroform.
4. Precipitate RNA with 1 volume isopropanol for 30 minutes at -20°C .
5. Spin down precipitate 15 minutes at 14,000 rpm at 4°C .
6. Wash 1X with 70% ethanol/DEPC
7. Resuspend in RNase-free water or formamide.

Lithium Chloride (LiCl) Precipitation

Notes:

LiCl precipitation does not efficiently precipitate RNA < 300 nts. Use isopropanol precipitation instead. Efficient LiCl precipitation requires >0.2 $\mu\text{g}/\mu\text{l}$ RNA

1. Bring volume up to 50 μ l with RNase-free water.
2. Add 1/2 volume (25 μ l) LiCl solution.
3. Incubate >1 hour at -20°C .
4. Spin down 15 minutes at 14,000 rpm to pellet RNA.
5. Wash pellet with 70% ethanol/DEPC.
6. Resuspend RNA in DEPC water (0.1%).

Precipitation from Formamide Resuspension

Note: Suspension in formamide protects the RNA from degradation by RNases.

1. RNA can be precipitated from formamide by adding 4 volumes of 100% ethanol at -20°C for 30 minutes to 1 hour.
2. Centrifuging at 14,000 rpm for 10 minutes at 4°C .
3. Wash pellet with 70% ethanol/DEPC.
4. Resuspend RNA in DEPC water (0.1%).

General Protocols for Protein

Bradford Assay

Method

Standard	200 µg/ml BSA Stock	Final Protein Concentration	Wells
1	0 µl	0 µg/200 µl	A1,B1
2	2 µl	0.4 µg/200 µl	C1, D1
3	3 µl	0.6 µg/200 µl	E1, F1
4	4 µl	0.8 µg/200 µl	G1, H1
5	5 µl	1.0 µg/200 µl	A2, B2
6	7.5 µl	1.5 µg/200 µl	C2, D2
7	10 µl	2.0 µg/200 µl	E2, F2
8	12.5 µl	2.5 µg/200 µl	G2, H2
Bring to 10 µl with dH ₂ O.		Add protein to 200 µl Bradford dye solution to final concentrations listed above for each standard. Enter these numbers into plate counter.	

1. Prepare in a 96-well plate for reading in a plate reader.
2. All samples and standards are prepared in duplicate.
3. For each standard -- *except for standard 8* -- add enough water to bring to 10 µl.
4. Dilute samples 1:10 or 1:100 in water in duplicate and load 10 µl per lane in row 3.
5. Dilute 1 part Bradford Reagent to 4 parts dH₂O and mix well.
6. Be sure to make more than enough dilute reagent for all the samples.

If you run out of reagent part way through the plate, you will have to discard everything with the old reagent and start over. Samples done with a different dye dilution than your standards will not be accurate.

7. Add 200 µl dilute Bradford reagent to each well. Use the dye soon after diluting and read the plate immediately.

The reagent is only good for ~20 minutes.

8. After the plate has been read, make sure your standards are linear. If not, try reading it one more time. Otherwise, you will have to start over from the beginning.
9. Determine concentration:

10 µl of a 1:100 dilution = [(concentration as read *100)/10] µg/µl

Far Western*

Method

1. Separate the protein sample of interest on an SDS-PAGE or acid urea gel. Transfer the gel to a solid support membrane. Either nitrocellulose or PVDF membranes yield good results. However, the PVDF membranes are easier to handle and give somewhat stronger signals, probably due to increased protein binding capacity.
2. After transfer, blots may be stained for 5 minutes in freshly diluted Ponceau S (Sambrook, 1989).
3. Destain the membrane in water until the proteins are clearly visible. Light pencil marks can be placed adjacent to markers or identifiable bands to mark them for future reference. The stain fades quickly so the marks must be placed immediately.
4. Following Ponceau S staining, block the blots for 2 hours in 0.05% Tween-20 in PBS, followed by 2 hours in 1% BSA in PBS.

The blocking of non-specific binding sites on the membranes is a critical step. Too little blocking results in high backgrounds while extended time in blocking solutions results in weakened or lost signal. The reason for the diminished signal is unclear, but protein renaturation apparently takes place during the blocking step, so an optimal renaturation may require limited blocking.

5. After rinsing in PBS, the blots may be used directly for probing or may be wrapped in plastic wrap and stored for up to 2 weeks at 4°C.
6. To make the probe, translate the protein of interest in vitro in the presence of ³⁵S-methionine. Dilute the IVT in PBS containing 1% goat serum, 0.3% BSA, in a volume sufficient to cover the membranes to be probed (typically 3 ml). Pre-incubate the blots for 10 minutes in dilution buffer without probe, and then incubate for 2 hours at room temperature in a 50-ml conical tube containing the diluted probe. Rotate the tubes mechanically throughout the binding reaction.
7. Wash the membrane 4X in 200 ml PBS for 5 minutes per wash. Background is generally quite low and extended washing does not reduce background substantially.*
8. Allow the membranes to air dry, and then expose to film or to phosphoimaging screens. Overnight exposure to X-ray film is usually sufficient to detect positive interactions.

Probes for Far Westerns can be generated by other methods. A recombinant protein can be biotinylated by commercially available reagents and then detected with streptavidin-

horseradish peroxidase, or labeled with ^{125}I . Alternately, an unlabeled recombinant protein can be used and then detected by traditional western.

*If there is a background problem, it is generally best to try to purify the probe on a microspin column such as Amicon. After translation, the probe is diluted with translation buffer or with 40mM Hepes, pH 7.4, 40mM DTT, and purified by centrifugation at 10, at room temperature.

Solutions

Ponceau S Stain

- 2 g Ponceau S
- 30 g Trichloroacetic Acid
- 30 g Sulfosalicylic Acid
- Bring to 100 ml with water
- Dilute 1:10 with water prior to use

PBS-Tween-20

- 137mM NaCl
- 3mM KCl
- 7mM Na_2HPO_4
- 15mM K_2HPO_4 , pH 7.9-8.0

*From Edmondson DG and Roth SY, 1998, Methods, 15:355.

In Vitro Kinase Assay (for ATM/ATR)

Method

Note: One 10-cm dish per antibody (Ab) immunoprecipitation (IP) will give three kinase reactions per IP.

1. Irradiate two 10-cm plates of HEK293 cells with 15 Gy and let recover for 30 minutes. Then lyse in 800 μ l TGN buffer, scrape plates and transfer to an Eppendorf tube.
2. Incubate on ice 20 minutes, then spin down cell debris, 10 minutes, 4°C, maximum speed. Pool all aliquots together to ensure equal concentration of protein.
3. Aliquot 750 μ l extract into one tube with no Ab. Aliquot another tube with 750 μ l extract with 15 μ l ATM/ATR Ab. Incubate 1 hour at 4°C, with rotation.
4. At the same time, incubate two tubes with 45 μ l Protein A beads/5% BSA in 45 μ l TGN buffer each, for 1 hour at 4°C, to block.
5. Quick-spin all tubes, low speed, quick pulse.
6. After 1 hour, combine one tube lysate/Ab with one tube BSA/beads and incubate for another hour at 4°C.
7. Resuspend each tube in 90 μ l kinase buffer + 30 μ Ci β -³²P-ATP. Then split into three separate tubes.
8. Add 1 μ g substrate (small volume).
9. Incubate 30 minutes at 30°C.
10. Add protein loading dye and run on gel (For GCN5, run 6-7% gel at 150 V.).
11. Stain gel with Coomassie, destain (dispose of everything in radioactive waste), then dry on gel dryer and expose to film 1-4 hours. You can scan gel for a record of Coomassie.

Washes

- Wash 2X TGN
- Wash 1X RIPA
- Wash 2X Kinase Buffer
- Wash volume = 750 μ l
- Spin each at 5000g for 1-2 minutes

Solutions

TGN Buffer

- 50mM Tris, pH 7.5
- 50mM Glycerophosphate
- 150mM NaCl
- 10% Glycerol
- 1% Tween-20
- 1mM NaF
- 1mM NaVO₄
- 1mM Phenylmethylsulfonylfluoride
- 2 µg/ml Pepstatin A
- 5 µg/ml Leupeptin
- 10 µg/ml Aprotinin
- 1mM DTT

Kinase Buffer

- 10mM HEPES, pH 7.5
- 50mM Glycerophosphate
- 50mM NaCl
- 10mM MgCl₂
- 10mM MnCl₂
- 5 µM ATP
- 1mM DTT

Silver Staining of SDS-PAGE Gels

Notes:

- This method is 100 to 1000 times more sensitive than Coomassie
- You can detect as little as 0.1-1 ng protein
- Wear gloves and handle gels carefully, since pressing on the gel can cause staining artifacts
- Use clean glassware and ddH₂O

Method:

1. Run SDS-PAGE gel.
2. Fix proteins in gel by incubating 4-12 hours at RT, shaking gently in ethanol:glacial acetic acid:H₂O (30:10:60) with at least 5 gel volumes of solution.
Be wary of fumes.
3. Discard fixing solution, add 5 volumes 30% ethanol and incubate 30 minutes at RT.
4. Repeat.
5. Wash with 10 volumes H₂O for 10 minutes at RT.
6. Repeat.
7. Wearing gloves, add 5 volumes 0.1% AgNO₃ (freshly diluted from 20% stock, which is stored in a dark container). Incubate 30 minutes at RT.
8. Discard solution and rinse both sides of gel for ~20 seconds each under stream of ddH₂O.

Be careful in handling the gel.

9. Add 5 volumes freshly made 2.5% sodium bicarbonate/0.02% formaldehyde. Incubate at RT.

Formaldehyde solution is generally provided as a 37% stock solution.

Watch carefully. The stained bands should appear within a few minutes.

*While watching the gel, **be wary of the formaldehyde fumes!!** These are toxic!!*

If you overdevelop the gel, it will begin to turn yellowish-brown all over.

10. Quench the reaction by washing the gel in 1% acetic acid for a few minutes -- again, be wary of fumes. Then wash the gel for several 10-minute periods in H₂O.

Western Blot

Warning: Acrylamide is a neurotoxin. Wear gloves at all time while handling gels!

Pouring the Gel

1. Pour resolving gel about 3/4 of the way up the plate. Be sure there will be space between the bottom of the comb and the top of the resolving gel. Carefully overlay the resolving gel with H₂O and let gel polymerize in a vertical position at RT for 30 minutes.
2. Drain the H₂O off and pour the stacking gel on top of the resolving gel and insert comb. Add extra gel if necessary to eliminate any bubbles at the top. Let polymerize for about 30 minutes at RT.
3. Remove comb and bottom spacer. Rinse with H₂O from tap and set up gel apparatus. Pour 1X TGS in top and bottom reservoirs of gel apparatus. Use syringe to flush out wells of any unpolymerized acrylamide. Also use bent needle syringe along bottom of gel to be sure no bubbles are stuck along the bottom of the gel.

Loading Samples

1. Heat samples (in Laemmli sample buffer) at 100°C for 3 minutes. Quick spin and load. Be sure to load marker as well.
2. Run gel. Voltage and time will vary according to the size of the protein in question and the percentage of the gel. Ask for advice from experienced colleagues.
3. When gel has completed its run, take down gel. Remove top glass plate carefully, and, using a paper towel, remove the stacker layer of the gel. Often, you can cut the top left corner of the gel for orientation.

Transferring the Gel (for semi-dry blotter)

1. Pre-equilibrate gel in 10% methanol:1X TGS on a slow shaker while you set up the rest for transfer:

Cut 12 pieces of Whatman® paper and 1 PVDF in the same size as the gel. Make sure to wear gloves while handling these.

Wet PVDF in methanol for ~2 minutes, then put in H₂O for 5 minutes on shaker to hydrate.

Switch PVDF into transfer buffer (10% methanol:1X TGS)

2. Take out the blotting plates and individually place 6 pieces of Whatman paper, pre-wet in transfer buffer. Be sure there are no bubbles.
3. Lay gel on Whatman papers face down.

You can use the last Whatman and float the gel on to it to transfer to the stack.

4. Place PVDF on top of gel and use an ink pen to mark the sizes of the marker lanes onto the blot. You can also mark lanes if want to.
5. Place the last 6 pre-wet Whatman papers on top of the PVDF. Then place top plate of blotter and screw down to finger-tight.
6. Run transfer at 300 mA for 45 minutes to 1 hour. Again, this may vary depending on the size of proteins of interest and the percentage of the gel. In addition, the percentage of methanol in the transfer buffer can range anywhere from 5%-15%. This will affect how long you transfer.

Blocking, Incubating and Developing Blot

1. While the gel is transferring, make up 1% instant milk/TBST blocking solution.

This can go up to 5% milk for blocking, depending on antibody.

2. Transfer blot with tweezers and gloved hands into blocking solution and let block for 1 hour at RT, shaking. You can do this in a Tupperware® container or Seal-a-Meal® bag.
3. Incubate with primary antibody (Ab) diluted in blocking serum O/N shaking at RT. (Some people do this at 4°C as well). If the blot is in a Seal-a-Meal bag, try to keep as few bubbles as possible and use a minimum volume to conserve Ab.
4. Wash blot 5X for 5 minutes in TBST at RT, shaking.
5. Incubate in secondary Ab conjugated to horseradish peroxidase (HRP), diluted in blocking serum, for 1-3 hours at RT, shaking. A common dilution of secondary Ab is 1:20,000-50,000. Remember to confirm what the primary Ab was made in so that you use the correct secondary Ab.
6. Wash blot 5X for 5 minutes in TBST at RT, shaking
7. Process blot using SuperSignal® or Amersham's ECL detection kits.

Recipes

Regular (30:0.8) Laemlli SDS-PAGE Resolving Gel

	7%	8%	10%	12%	15%
30:0.8 Acrylamide:Bis (ml)	5.1	5.87	7.33	8.81	11
1M Tris, pH 8.8 (ml)	8.24	8.24	8.24	8.24	8.24
10% SDS (ml)	0.2	0.2	0.2	0.2	0.2
50% Glycerol (ml)	1.12	1.12	1.12	1.12	1.12
H ₂ O (ml)	7.3	6.53	5.11	3.59	1.4
10% Ammonium Persulfate (ml)	70	70	70	70	70
TEMED (ml)	7	7	7	7	7

Westerns Extras

Alkaline Phosphatase (AP) Developing

1. Use secondary antibody conjugated to AP and incubate 2 hours.
2. Wash 6X for 5 minutes in Tris buffered saline-Tween-20 (TBS-T).
3. Put in 10 ml AP buffer + 66 μ l NBT and 66 μ l BCIP and develop.
4. Place in stop buffer (PBS + 20mM EDTA) for 10 minutes to stop reaction.
5. Lay on glass and dry blot in dark place.

AP Buffer

- 100mM Tris, pH 9.5
- 100mM NaCl
- 5mM MgCl_2

NBT

- 1 g/20 ml 70% DMF
- Store at -20°C

BCIP

- 0.5 g Disodium Salt/20 ml
- Store at -20°C

Stripping Blot for Reprobing: Stringent

1. Incubate in stripping buffer for 30 minutes at 50-70°C.
2. Wash membrane 2X in TBS-T.
3. Incubate with SuperSignal® to verify it has been stripped completely.
4. Wash 2X for 10 minutes in TBS-T.
5. Block for 2-4 hours, then place into primary antibody O/N.

Stripping Buffer

- 2% SDS
- 62.5M Tris, pH 6.8
- 100mM β -mercaptoethanol

50 ml

- 10 ml 10% SDS
- 3.1ml 1M Tris, pH 6.8
- 347 μ l 14.4M β -mercaptoethanol

Stripping Blot for Reprobing: Mild

Note: This has been used on 22% histone gels and reprobed 6-8 times before any loss of signal was noted.

1. Incubate in mild stripping buffer for 1 hour at 37°C.
2. Wash membrane 2X in TBS-T.
3. Incubate with SuperSignal to verify it has been stripped completely. Expose to film for at least 1 hour, longer if it took longer to get the original signal.
4. Wash 2X for 10 minutes in TBS-T.
5. Block for 2-4 hours, then place into primary antibody O/N.

Mild Stripping Buffer

- 2% SDS
- 100mM Tris, pH 7.5
- 14 mM β -mercaptoethanol

Staining Gels/Blots

Note: Stain blot with 1-2X Ponceau S, and destain with water. The stain will be very light, but you can then wrap the blot in Saran Wrap® and scan the stained blot into photo-editing software on the computer. Use the contrast function to darken the image to see the stain.

Coommassie Staining Gel

1. Take gel and place in Coommassie for 2 hours to O/N.
2. Destain gel until bands are clearly apparent. Add Kimwipes to destain to help absorb the dye without altering the make-up of the destain. Change Kimwipes® as they become full of dye.
3. To prevent cracking, you can add 1% glycerol to the last hour prior to drying the blot.

Coommassie Dye (2.5 L)

- 6.25 g Coommassie® Brilliant Blue G-250
- 1.12 L Methanol
- 250 ml Glacial Acetic Acid
- 1.12 L H₂O
- Store at room temperature

Destain (10 L)

- 300 ml Glycerol
- 2.5 L Methanol
- 1 L Glacial Acetic Acid
- 6.2 L H₂O
- Store at room temperature

Histone Acetyltransferase Protocols

In-Gel HAT Assay

Method

1. Pour an 8% SDS-PAGE gel containing 1 mg/ml calf thymus histones (Sigma type IIA).

Stacking gel can contain histones or not.

Use a 10 mg/ml stock of histones dissolved in water, and replace part of the water portion of the gel with the histones. Add SDS last (after histones but before accelerants). The gel suspension should be slightly cloudy.

2. Dissolve samples (potential HATs) in SDS-PAGE sample buffer with protease inhibitors, but do not boil.
3. Following electrophoresis, wash gel for 1 hour in Buffer B with 20% isopropanol. Then wash for 30 minutes in Buffer B alone.
4. Incubate gels in Buffer B containing 8M urea for 1 hour at RT, with agitation. The gel will swell slightly.
5. Renature: Change to 50 ml Buffer B with 0.04% Tween-40, swish and incubate at 4°C, with agitation, for 30 minutes.
6. Change to 50 ml fresh Buffer B with 0.04% Tween-40 and incubate O/N 4°C, with agitation.
7. Wash in Buffer A for 30 minutes at 30°C. Then incubate with 10 μCi ^3H -acetyl CoA in 10 ml Buffer A for 30 minutes at 30°C, with agitation. This works well if you use a Seal-a-Meal® bag.
8. Wash gel extensively (5X, 1hour each) in 5% TCA, then wash O/N in 5% TCA, to remove unbound label.
9. Fluorograph and expose to film 2 days to 2 weeks.

Solutions

Buffer A (100 ml)

- 5 ml 1M Tris, pH 8.0
- 0.1 ml 1M DTT
- 0.02 ml 0.5M EDTA
- 10 ml Glycerol
- 0.1 ml 1M PMSF

Buffer B (100 ml)

- 5 ml 1M Tris, pH 8.0
- 0.1 ml 1M DTT
- 0.02 ml EDTA

Liquid HAT Assay

Method

20- μ l Reaction

- 4 μ l 5X HAT Buffer
- 13.2 μ l Water
- 1 μ l Enzyme
- 0.8 μ l ^3H -acetyl CoA
- 1 μ l 10 mg/ml Histones or Substrate

1. Set up reaction *in order* listed.
2. Incubate 30 minutes 30°C. Spin down briefly.
3. Spot half of reaction on Whatman® P81 phosphocellulose filter paper circle and air dry.

Note: Prelabel filter papers with pencil on top of Saran Wrap®.

4. Wash filters for 30 minutes at 4°C in ice-cold 10% trichloroacetic acid (TCA).
 5. Wash 2X, 5 minutes each, RT, in 10% TCA.
 6. Wash 1X 5 minutes in 100% ethanol. Let filters dry under heat lamps.
 7. Place in scintillation fluid and count ^3H .
 8. When appropriate, you can electrophorese other half of reaction.
- Add 2X SDS-PAGE buffer
 - Boil
 - Load on 22% PAGE gel (60:0.4 acrylamide:bis) resolver with standard stacker. Run medium sized gel approximately 1700 V/hr. Stain with Coomassie and destain. Then fluorograph by placing in EN 3 HANCE for 30 minutes, then water, for 30 minutes. Dry on heated gel dryer. Develop at -80°C for 3-5 days to see which histones/proteins have been labeled.

5X HAT Buffer

- 75mM Tris, pH 7.8
- 1.25mM EDTA, pH 8.0
- 0.25% Tween-20
- 12.5mM DTT
- 25% Glycerol

Protease Inhibitor Cocktail

- 10 µg/ml PMSF
- 1µM Pepstatin
- 1µM Leupeptin
- Add 1 µl per ml protein sample

*This procedure is basically as described by Brownell and Allis, PNAS 92, 6364-6368, 1995.