Recipes

10mM Acetyl CoA
- Acetyl CoA tri lithium salt: MW- 809.6
- 10mg/1.23mL or 50mg/6.17mL
- Dissolve in 0.1M Tris Cl: pH 7.8

5% Acrylamide
- 50 mL 10x Sangers
- 25 g Acrylamide (crystal)
- 0.83 g Bis- acrylamide
- 240 g Urea
- Dilute to 500mL with Milli Q H₂O
- Filter sterilize with a .45 micron filter

Actinomycin D.
- Dissolve at 10mg/mL in 100% MeOH or 1:1 MeOH: EtOH
- Treat cells with 10µg/mL to achieve 100% inhibition
- Transcription blocked within 5 min

0.7% Agarose
- Use a 500 mL autoclaved flask
- For smalls gels make 100 mL, for large gels make 300 mL
- Per 100 mL:
  - 98 mL Milli-Q H₂O
  - 2 mL 50x TAE
  - 0.7 g agarose
- Microwave solution until all agarose is dissolved
- Wait until it is cool enough to handle (55°C) and add 4 uL of Ethidium Bromide (4µL/100mL) then pour

1.5% Agarose
- Use a 500 mL autoclaved bottle
- For small gels make 100 mL, for large gels make 300 mL
- Per 100 mL:
  - 98 mL Milli-Q H₂O
  - 2 mL 50x TAE
  - 1.5g agarose
- Microwave solution until all agarose is dissolved
- Wait until it is cool enough to handle (55°C) and add 4 uL of Ethidium Bromide (4µL/100mL) then pour
10x Ammonium Persulphate
  • 1 g ammonium persulphate
  • Dissolve in 10 mL Milli-Q H₂O
  • Vortex until fully dissolved and store at -4°C

1000x Ampicillin stock solution (100 mg/ml)
  • 5 g of ampicillin
  • 50 mL of Milli-Q H₂O
  • Filter sterilize using a .22µm steriflip under cell culture hood
  • Aliquot 1 mL into 1.5 mL tubes
  • Store in appropriate box in the -20°C

Beta-Gal Rxn Buffer 250 ml
  • 50 mL 500mM KH₂PO₄, pH 8.0
  • 250 µL 1M MgCl₂
  • Bring final volume to 250 mL using Milli-Q H₂O
  • Before Use: Add 1% Galacton right before

50mM Calcium Chloride
  • .735 g Calcium Chloride
  • Dissolved in Milli-Q H₂O for 100mL total volume
  • Change recipe amounts based on ratio

Cesium Chloride for RNA preparation
  • 479.9 g CsCl (final M should be 5.7 M)
  • 18.6 g Na₂EDTA (final M should be 0.1 M)
  • Dissolve and bring volume up to 500 mL
  • pH to 7.0 with NaOH
  • Autoclave (with or without DEPC)
  • Readjust to 1.40 refractory units with DEPC H₂O

Chloramphenicol
  • 60mg/mL dissolved in 100% ethanol

Coomasie Destain
  • 50% MeOH and 10% Acetic Acid in H₂O

Coomasie Stain
  • 2 g Coomasie stain
  • 100 mL Methanol
  • 400 mL glacial acetic acid
  • Bring volume up to 4 L with Milli-Q H₂O
Cycloheximide
- 10mg/mL
- Dissolve in Milli-Q H₂O
- Treat cells with 10µg/mL to achieve 100% inhibition
- Protein synthesis blocked within 5 minutes

Denaturation Solution
- 175 g 1.5M NaCl
- 40 g 0.5M NaOH
- Dissolve to a total volume of 2 liters with Milli-Q H₂O

50x Denhardt’s
- BSA (sigma A-6003)
- Ficoll
- Polyvinylpyrrolidine
- Bring volume to 1 Liter with Mill-Q H₂O
- Filter sterilize
- Aliquot 25 mL into 50 mL tubes
- Store in -20°C freezer

.05% DEPC H₂O (solution for deproteination)
- .5 mL of diethylpyrocarbonate (in cold room) per L of H₂O
- Sit at room temperature over night and then autoclave

Dexamethasone Stock Solution
- (.03925 g) Dexamethasone 10mM in 100% absolute ethanol (10 mL)
- CALCULATION:
  - 9.81 mg / 25 mL = 0.00981 g / 25 mL = 10 mM Dexam.

Dexamethasone Working Solution
- 10mL stock solution in 90 mL PBS

dH5α aliquots
  - **Work on ice next to the flame**
  - Aliquot 50 µL of stock dH5a (in -80°C) into 1.5mL tubes
  - Label, date and initial
  - Store in -80°C, A-1
Dialysis Tubing
- Place 2 L of H₂O in a large beaker
- Cut tubing in 2-3 foot strips and submerge in beaker
- Cover with foil and autoclave tape and put in autoclave
- Autoclave separately: 4 liters of both H₂O and TE
- When finished, rinse tubing in H₂O and then in TE
- Store in cold room in a capped jar of TE

Dithiothreitol (DTT)
- 15.45 g DDT into 100 mL of Milli-Q H₂O
- Aliquot and store in appropriate rack in -20°C

DMEM (Dulbecco’s Modified Eagles Medium)
- 2 g Streptomycin
- 1.25 g Penicillin
- 0.16 g Biotin
- 0.16 g Pantothentic acid
- 74 g Sodium Bicarbonate
- Total volume of 20 L

DNA Ladder
- 250 µL DNA (1g/µL) Invitrogen: 10787-018
- 420 µL 6x DNA Loading Buffer
- 1830 µL Milli-Q H₂O
- Mix well, aliquot 250 µL in 1.5 mL tubes, store in -20°C
- Label “kb+ Ladder”

10x DNA Loading Buffer
- .25 g 0.25% bromophenol blue
- .25 g 0.25% xylene cyanol
- 25 g 25% Ficoll (type 400) in H₂O
- Bring total volume to 100 mL
- Aliquot into small sterile bottles and autoclave

6x DNA Loading Buffer (with xc only)
- 20 g Sucrose
- Dissolve in 50mL Milli-Q H₂O
- Vortex and put in water bath to help dissolve
- Add xylene cyanole until the color becomes dark blue

From core store
CAT#LC5925 (Invitrogen)
10 mM dNTPs
- 500 µL dNTP master mix (located in -20°C freezer)
- 4.5 mL Milli-Q H₂O
- Mix gently in conical
- Aliquot 250 µL into 1.5 mL tubes
- Label “dNTP 10mM” with date and initials

0.25M EDTA
- 186.26 g Na₂EDTA
- Mix in 1 L of Milli-Q H₂O
- Adjust pH to 8.0 with appropriate NaOH (must be pH 8 for it to dissolve)
- Autoclave

Ethidium Bromide (10mg/ml)
- Use a 15 mL tube
- .100 g EtBr
- Dissolve in 10 mL Milli-Q H₂O
- Wrap in foil and store in appropriate cabinet

Fluorescein
- Use AMBER colored tubes, if none are available wrap regular tubes in foil)
- 1: 5000 dilution in dd H₂O
  - Ex: For 20 tubes use 4µl Fluorescein and 19,996µl (19.996ml) H₂O
- Aliquot 1 mL into 1.5 mL amber tubes
- Store in qPCR box in -20C

Formamide (deionized)
- 20 g Dowex MR-3 resin
- Mix in 500 mL formamide
- Stir in bottle for 30 minutes
- Filter through Whatman paper (2 to 3x)
- Aliquot 35 ml into 50 mL tubes and freeze

Geneticin
- Make up in H₂O in 2 mL tubes that have been sterilized and put under UV light
- Use microbiological potency in calculation (usu. ~.7 mg/mg)

GET Buffer
- 25 mL 1M Tris; pH 8
- 40 mL 0.25M EDTA; pH 8
- Add together and autoclave
- 45 mL 20% glucose is added post-autoclaving
20% Glucose
- 20 g Glucose
- Bring total volume up to 100 mL with Milli-Q H₂O
- Filter sterilize with a .22 µm filter in the hood
- Store at room temp

40% Glucose
- 200 g (D) glucose
- Dissolve to a final volume of 500 mL in Milli-Q H₂O
- Filter sterilize with a .22 µm filter in the hood
- Store at room temp

5M Guanidine-isothiocyanate
- 295.4 g Guanidine thiocyanate
- 14.7 g Sodium Citrate-2H₂O
- Add DEPC H₂O and adjust pH to 7 with NaOH
- Bring the volume up to 500 mL
- Filter sterilize through Nalgene filter (Corning won’t work)

0.02 M and 6 M Hydrochloric Acid
** Do in hood **
- CALCULATIONS for other desired molarities
  \[ M_1 \times V_1 = M_2 \times V_2 \]\n  HCl Stock is 12.1 M (M₁)
  \[ 12.1M(x) = (\text{desired molarity})(\text{desired total volume}) \]
- 0.02 M
  - 413 µl HCl and 249 ml H₂O
- 6 M
  - 124 ml HCl and 126 ml H₂O

Hypotonic Lysis Buffer
- 10 mL 1M Tris-Cl
- 1 mL 5M NaCl
- 1.5 mL 1M MgCl₂
- Dissolve in 500 mL Milli-Q H₂O
Insulin
** Do in hood **
- Use 50 mL tube
- 100 mg insulin
- 100 mL 0.02 M HCl
  - 98 mL Milli-Q H₂O + 2 mL HCl if starting with 1M (1N) HCl
  - 99.667 mL Milli-Q H₂O + 333 μL HCl if using 6N HCl
- Filter sterilize in the hood using a 0.22 μm sterile filter under the cell culture hood
  - 2 x 50 mL, attach 2nd 50 mL tube on bottom and filter again
- Aliquot 1 mL into 1.5 mL tubes
- Store in -20°C in appropriate rack

500x Kanamycin Stock
- Use a 50 mL tube
- 2.5 g of kanamycin
- Dissolve in 50 mL Milli-Q H₂O
- Use a 0.22 μm sterile filter under the hood
- Aliquot 1 mL into 1.5 mL tube
- Store in appropriate box in -20°C

1x Krebs-Ringer-HEPES Buffer without BSA (KRH)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>g/500ml</th>
<th>g/1000ml</th>
<th>g/2000ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>3.5</td>
<td>7.0</td>
<td>14.0</td>
</tr>
<tr>
<td>KCl</td>
<td>0.074</td>
<td>0.149</td>
<td>0.298</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.068</td>
<td>0.136</td>
<td>0.272</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.036</td>
<td>0.072</td>
<td>0.144</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.060</td>
<td>0.120</td>
<td>0.240</td>
</tr>
<tr>
<td>HEPES</td>
<td>2.38</td>
<td>4.77</td>
<td>9.52</td>
</tr>
</tbody>
</table>

- pH to 7.5
- Filter sterilize under the hood and aliquot into 500mL bottles
- 10% BSA stock/H₂O (filter sterilized)

<table>
<thead>
<tr>
<th></th>
<th>Total KRH</th>
<th>0.5% final BSA</th>
<th>0.5% final BSA</th>
<th>0.5% final BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25ml</td>
<td>50ml</td>
<td>100ml</td>
<td>500ml</td>
</tr>
<tr>
<td>0.5% final BSA</td>
<td>1.25ml</td>
<td>2.5ml</td>
<td>5ml</td>
<td>25ml</td>
</tr>
<tr>
<td>KRH</td>
<td>23.75ml</td>
<td>47.5ml</td>
<td>95ml</td>
<td>475ml</td>
</tr>
</tbody>
</table>
LB Agar Plates
- 32 g LB Agar
- Dissolve in 1 L of Milli-Q H₂O in 2000 mL Erlenmeyer with a large stir bar (leave stir bar in during autoclaving)
- Cover with tin foil and autoclave tape. Autoclave on slow exhaust in a small autoclave tub containing water
- Cool Agar to around 60°C with gentle stirring on a stir plate.
- Add appropriate antibiotic (1 mL of 100mg/mL AMP or 2 mL Kan) and stir gently.
- Using aseptic technique, pour agar into 10 cm dishes (Fisher cat# 08-757-12). Pour just enough to cover the bottom of the plate. Cover plate and allow to solidify and cool at room temp for 1 hour
- Stack plates, wrap in parafilm, and store inverted @ 4°C

2x LB Broth
- 80 g LB Broth Base
- Dissolve into 1.5 L Milli-Q H₂O
- pH to 8 by adding 10M NaOH
- Bring the total volume up to 2 L
- Distribute 200 mL into 1000 mL Erlenmeyer flasks (makes 10 flasks)
- Cover top with foil and autoclave tape, autoclave for 30 min on slow exhaust, allow to cool before use.
- To use: add appropriate antibiotic (200µL AMP or 400 µL KAN), 2 mL 40% D-Glucose, and 1 mL bacterial culture

1x MGB (40 mL)
- 35.4 mL sterile Milli-Q H₂O
- 4 mL 10x MGB
- 400 µL 2-mereaptoethanol
- 200 µL Triton X-100

50mM MgCl₂
- 0.19042 g MgCl₂
- 40 mL Milli-Q H₂O
- Mix in 50 mL conical using vortex
- In Cell Culture hood, use 0.22µL filter to filter sterilize
- Aliquot 1 mL into 1.5 mL tubes
- Label tubes “50mM MgCl₂” with date and initials
- Store in appropriate box in -20C Freezer
5x MOPS Buffer
- 183.1 g of (250 mM) MOPS
- 105.0 g of Tris-Base
- 6.51 g of EDTA
- 17.5 g SDS (use 175 mL of 10% SDS)
- Bring total volume up to 3.5 L
- pH to 7.7 and store in appropriate container

5x High MW Running Buffer
- Use for separating proteins >20 kDa
- 250 mM Mops
- 250 mM Tris
- 5 mM EDTA
- 0.5% SDS (No need to pH)

5x Low MW Running Buffer
- Use for separating proteins 2-50 kDa
- 250 mM Mops
- 250 mM Tris
- 5 mM EDTA
- 0.5% SDS (No need to pH)

10% Neutral Buffered Formalin
*IN A WELL VENTILATED AREA*
- 4 g Sodium Phosphate, monobasic
- 6.5 g Sodium Phosphate, dibasic
- 100 ml Formaldehyde, 37%
- 900 ml DI H₂O
- Adjust pH to 6.8

Neutralization Solution
- 175 g of 1.5 M NaCl
- 242 g of 1 M Tris Base
- Adjust pH to 8
- Bring total volume up to 2 L with Milli-Q H₂O
NZTM Agar Plates
- 10 g NZ amine
- 5 g NaCl
- 5 g Yeast extract
- 2 g MgSO$_4 - 7$H$_2$O
- Dissolve in 1 L of Milli-Q H$_2$O
- Adjust pH to 7.5 with NaOH
- Add 15 g Bacto agar
- Autoclave
  *for top agar- add 7 g of agarose instead of bacto agar

NUN Buffer 50mL
- 3.3 g Urea
- 3.3 mL 5M NaCl
- 5.5 mL Hepes, pH 7.5
- Bring volume up to 50 mL with Milli-Q H$_2$O
- Before Use: Add Na OrthoVanadate 1mM, DDT 1.1mM, Beta glycerol phosphate 30mM and PIC 1 and 2

10x PBS
- 80 g NaCl
- 2 g KCl
- 11.5 g Na$_2$HPO$_4 - 7$H$_2$O (bottle labeled “for PBS”)
- 2 g KH$_2$PO$_4$
- Dissolve in Milli-Q H$_2$O to a final volume of 1 L
- Adjust pH to 7.4
- Autoclave and store at room temperature in the glass cabinet

1x PBS
- Use a 500 mL autoclaved bottle
- 50 mL premade 10x PBS
- 450 mL Milli-Q H$_2$O
- Autoclave and store at room temperature in the glass cabinet

100x Penicillin/Streptomycin/Biotin Tissue Culture
- For 500 mL:
  - 5 g Penicillin G
  - 3.12 g Streptomycin Sulfate
  - 0.4 g Biotin
Phenol
- Thaw 250 g of phenol in a water bath at 60-65°C
- Add 1M Tris (pH 8) until the bottle is nearly full
- Pour into bottle so the 2 phases can be viewed
- Separate O/N suction off top layer and add more Tris (pH 8)
- Allow to settle and suction of top phase
- Add 0.1M Tris (pH 7.8) repeat suction
- Add 0.1M Tris (pH 7.8), 0.4 mL 2-mercaptoethanol (.1%) and 0.25 g 8-hydroxyquinoline (.2%)

Phenyl-Chloro IAA
- Use glass pipets under the hood
- 25 mL phenol
- 24 mL chloroform
- 1 mL Isoamyl alcohol
- Mix in a 50 mL tube and store in the fridge

1x Phosphate Buffer Saline for tissue culture
- 91.6 g of Sodium Chloride (NaCl)
- 1 g Potassium Chloride (KCl)
- 2 g Potassium Phosphate monobasic (KH₂PO₄)
- 14.8 g Na₂HPO₄-7H₂O
- Bring total volume to 10 L with Milli-Q H₂O
- When dissolved, pH should be between 7.2 and 7.4
- Pour 400 mL into 500 mL bottles
- Autoclave
- Cool prior to tightening caps

1x Phosphate Buffer Saline with antibiotics
- 91.6 g of Sodium Chloride (NaCl)
- 1 g Potassium Chloride (KCl)
- 2 g Potassium Phosphate monobasic (KH₂PO₄)
- 14.8 g Na₂HPO₄-7H₂O
- 1 g Streptomycin
- 0.625 g Penicillin
- Bring total volume to 10 L using Milli-Q H₂O
- When dissolved, pH should be between 7.2 and 7.4
- Pour 400 mL into 500 mL bottles
- Autoclave
- Cool prior to tightening caps
Physiological NaCl
• 9 g NaCl
• Dissolve in 1 L Milli-Q H₂O

PIC I
• 1mg/mL Leupeptin
• 1 mg/mL Antipain
• 10 mg/mL Benzamidine
• Dissolve in Aprotinin
• (Make solution in desired amount)

PIC II
• 1 mg/mL Chymostatin
• 1 mg/mL Pepstatin-A
• Dissolve in DMSO
• (Make solution at desired amount)

PMSF
• 200 mM of PMSF stock in isopropanol

Polybrene (Hexadimethrine Bromide)
• Dissolve Polybrene (in cold room dessicator) in ddH₂O to 8mg/mL
• In cell culture hood, use syringe and 0.22µL filter to filter sterilize
• Aliquot 500 µL into 1.5 mL tubes
• Label tubes “Polybrene (8mg/mL)”
• Store in appropriate box in -20°C Freezer

Proteinase K Buffer
• 1 ml 10 mM Tris HCl pH 7.5
• 294 mg 20 mM CaCl₂
• 50 ml 50% glycerol
• 49 ml Milli-Q H₂O
• Autoclave and store at 4°C
• Add 5 ml buffer to 100 mg Proteinase K and store at -20°C

Puromycin
• 10 mg/mL in PBS
• Filter sterilize in the hood and aliquot 1 mL into 1.5 mL tubes
• Store in appropriate place in -20°C freezer
Q Solution
• Use a 50 mL tube
• 23.42 g of betaine
• 40 mL of Milli-Q H₂O
• When dissolved filter sterilize using .22µm steriflip in the cell culture room
• Aliquot 1 mL into 1.5 mL tubes
• Store in -20°C freezer in appropriate box

QBT Solution (for Qiagen system)
• 43.83 g NaCl
• 10.46 g Mops
• Dissolve in 800 mL Milli-Q H₂O
• Adjust pH to 7
• Add 150 mL of 100% ethanol and 15 mL of 1% Triton X-100
• Bring final volume to 1000 mL with Milli-Q H₂O

QC Solution
• 58.44 g NaCl
• 10.46 g MOPS (free acid)
• Dissolve in 800 mL of Milli-Q H₂O
• Adjust pH to 7
• Add 150 mL of 100% ethanol and bring total volume up to 1 L with more Milli-Q H₂O

QF Solution
• 73.05 g NaCl
• 6.06 g Tris base
• Dissolve in 800 mL of Milli-Q H₂O
• Adjust pH to 8.5
• Bring final volume up to 1 L with Milli-Q H₂O

RNase A (10mg/ml)
• 100 mg RNase A
• 10 mL of 10 mM Tris (pH 7.5)
• 10 mL of 15 mM NaCl
• Dissolve
• Heat to 95 °C for 15 minutes
• Aliquot 2 mL and place in the freezer in the appropriate rack
10x Sangers Buffer
• 648 g Tris Base
• 220 g Boric Acid
• 38 g EDTA
• Fill up to 4 liters and mix
• Store at room temperature

10% SDS
• 200 g of SDS into a beaker of Milli-Q H₂O
• Dissolve and bring up to 2 liters
• Aliquot 400 mL into 500 mL bottles
• Label and store at room temperature

4x SDS Loading Buffer
• 1.38 mL Beta-Mercaptoethanol
• 12 mL Tris (1 M, pH 6.8)
• 20 mL Glycerol
• 10 mL 20% SDS
• Add a pinch of Bromophenol Blue
• Bring volume to 50 mL with Milli-Q H₂O

4x SDS Running Buffer
• 192 g Tris Base
• 920 g of Glycine
• 64 g SDS
• 24 g EDTA
• Mix and bring volume up to 16L with Milli-Q H₂O

3M Sodium Acetate
• 492.18 g Sodium Acetate
• 400 mL Glacial Acetic Acid
• Dissolve and bring total volume up to 2 L with Milli-Q H₂O
• Check pH (should be between 5.0 and 5.2)
• Autoclave

Sodium Phosphate Buffer (pH 6.8)
• 765 mL monobasic Sodium Phosphate
• 735 mL of dibasic Sodium Phosphate
• pH solution after fully mixed

1M Sodium Phosphate Dibasic from Na₂HPO₄·7H₂O
• 268 g Sodium Phosphate dibasic
• Mix and dissolve in a glass beaker
• Bring total volume up to 1 L and autoclave in bottles
1M Sodium Phosphate Monobasic from Na$_2$HPO$_4$·H$_2$O
- 138 g Sodium Phosphate
- Bring total volume to 1 L with Milli-Q H$_2$O
- Autoclave in 500 mL bottles

Sodium Pyrophosphate
- 25 g Sodium Pyrophosphate (MW 446.06)
- Dissolve in 1 L of H$_2$O
- Autoclave

100x Sodium Pyruvate
- 5.5 g in 500 mL DMEM
- Filter Sterilize and aliquot
- Store at 21°C

3M NaOAc (pH 5.2)
- 246.09 g NaOAc
- 200 mL Glacial acetic acid
- Bring total volume to 1000 mL with Milli-Q H$_2$O
- Adjust pH to 5.2
- Autoclave and store at RT

20x SSC
- 700.8 g NaCl
- 352.8 g Sodium Citrate
- Fill up to 4 liters and mix
- pH to 7.0 using HCl
- Store at room temperature

Super broth
- For 3L:
  - 96g Tryptone
  - 60g Yeast
  - 15g NaCl
- For 1L:
  - 32 Tryptone
  - 20g Yeast
  - 5g NaCl

Sybergreen
- 1:100 dilution with Milli-Q H$_2$O
- Store in Amber colored tubes (or wrap in foil if none are available)
1x TAE
- 80 mL 50x TAE
- 3920 mL Milli-Q H₂O
- Store in 4 Liter container, room temperature

50x TAE
- 242 g Tris Base
- 1.86 g Na₂EDTA-2H₂O
- 57.1 mL glacial acetic acid
- Dissolve and bring total volume up to 1 L
- Autoclave in 500 mL bottles and store in the glass cabinet

10x TBE
- 432 g Tris Base
- 220 g Boric Acid
- 2.96 g EDTA
- Mix well and dissolve in 3 L of Milli-Q H₂O
- Adjust pH to 8.0 with concentrated HCl
- Bring total volume up to 4 L and store at room temperature

TBS (Tris buffered saline)
- 100 mL Tris-Cl, pH 8.0
- 9 g NaCl
- Bring to final volume of 1 L with Milli-Q H₂O and store at -4°C

TE
- 40 mL 1 M Tris (pH 8.0)
- 16 mL 0.25 M EDTA (pH 8.0)
- Bring final volume up to 4 L with Milli-Q H₂O
- Pour 400 mL into 500 mL bottles
- Autoclave and store at room temperature

1x Transfer Buffer
- 400 mL 10x Transfer Buffer
- 800 mL Methanol (UNDER FUME HOOD)
- 2800 mL Milli-Q H₂O
- Mix and transfer to a 4L container and store at 4°C.

0.1M Tris (pH 7.8)
- 24.2 g Trisma Base
- Dissolve in 2 L of Milli-Q H₂O
- Autoclave and store at room temperature
1M Tris (pH 8.0)
- 242 g Trisma Base
- Bring total volume up to 2 L with Milli-Q H₂O
- Autoclave and store at room temperature

tRNA (50 mg/mL)
- Make up in Ultra Pure water
- Aliquot into 1.5 mL tubes
- Boil for 15 minutes
- Store at -20 °C

Troglitazone (TZD)
- 0.11 g Troglitazone
- In cell culture hood add 5 mL DMSO
- Mix well
- Aliquot 100 µL in 1.5 mL tubes
- Wrap in Aluminum Foil
- Store in -20°C freezer
- Label tubes “TZD 50mM”

Trypsin
- 2.5 g Trypsin
- Using a “fleaker” add 500 mL PBS
- Add trypsin and mix until dissolved
- Add 400 mL of PBS for final volume of 900 mL
- Filter sterilize using a 500 mL steriflip under the hood
- Aliquot 1 mL into 1.5 mL tubes

10x TTBS (TBST)
- 630.4 g Tris-HCl
- 350.4 g NaCl
- 40 mL Tween 20
- Bring volume up to 4 L with Milli-Q H₂O and stir
- pH to 7.4
- Store in 4 °C
- (Dilute 1:10 for working solution)

1x Western Lysis Buffer
- 5 mL 10% SDS
- 3 mL 1M Tris pH 6.8
- .186 g EDTA
- Bring the volume up to 50mL with Milli-Q H₂O
**Oil Red-O Staining of Adipocytes**

1) Prepare Oil Red-O: Stock is 0.5% in isopropanol. To make working solution, add 60 mL stock to 40 mL H$_2$O (or anything with that ratio), remove precipitate with Whatman 40 filter

2) Wash plate 1 time with 1x PBS

3) Fix cells with 3.7% Formaldehyde for 2 minutes.

4) Wash 1 time with H$_2$O

5) Add Oil Red-O Working Solution (3mL/10cm plate; 1mL/6cm plate) and incubate for 1 hr at room temperature

6) Aspirate Oil Red-O and wash with H$_2$O; store in H$_2$O and Sodium Azide

**Skin Sample Preparation**

1) Cut a strip of skin that is approximately 0.5-1 cm wide and approximately 3 cm long. Be consistent with what part of the body you select from.

2) Curl the skin into a circle around a marble with the hair outside.

3) Place the skin wrapped around the marble into a well to keep it standing upright. Use either a 12-well or a 24-well plate.

4) In a fume hood, add 10% buffered formalin (or a different fixative if desired) into the well until the skin is submerged.

5) Let it fix for 24-48 hours before removing the skin, which should be rigid, and placing it into a cassette. The cassette should be stored in buffered formalin or 70% ethanol until further processing.

*Make sure the width of the strip is not too wide or it will get compressed when you seal the cassette. The goal is to have a skin prep that is perpendicular to the 'ground', thereby minimizing error in the apparent thickness of the skin when sectioning.
Ken’s Lysis Buffer

19.5 mL L-RIPA
100 µL 100mM NaVO3
200 µL 100X PMSF
100 µL PIC I
100 µL PIC II

Total volume of 20 mL

1. Rinse cells with cold PBS, add 1 mL of the lysis buffer, scrape plates and transfer cells to a new tube.

2. Vortex on high for 10 seconds and then incubate on ice for 15 minutes.

3. Boil tube at 100°C for 5 minutes, put on ice.

4. Spin in the cold room for 5 minutes on high (use this supernatant for Bradford assay)

5. Add 4x loading buffer to the supernatant, boil for 10 minutes and place and ice.

6. Spin again for 5 minutes on high.

**Use this supernatant to load the gel**
3T3-L1 Differentiation Protocol

Materials

- Dulbecco’s Modified Eagles Medium (DMEM, GibcoBRL-Cat# 11965-084: high glucose, with L-glutamine, with pyroxidine HCl, without sodium pyruvate)

- Calf Serum (GibcoBRL-Cat# 10437-028/Lot # 1026566)- filter sterilize (0.22µL filter) before mixing into DMEM

- Isobutylmethylxanthine (IBMX; Sigma I-7018)

- Dexamethasone (Sigma D-4902)

- Insulin (Bovine; Sigma I-5500)

- MEM Sodium Pyruvate (100mM; GibcoBRL-Cat# 11360-070)

- Pen/Strep/Glutamine (100x P/S/G; GibcoBRL-Cat# 10378-016)

Solutions

- 10% Calf Serum/DMEM
  - 60 mL Calf Serum
  - 6 mL MEM Sodium Pyruvate
  - 6 mL 100x P/S/G
  - 500 mL DMEM

- 10% FBS/DMEM
  - 60 mL Fetal Bovine Serum
  - 6 mL 100 mM MEM Sodium Pyruvate
  - 6 mL 100x P/S/G
  - 500 mL DMEM

- IBMX Solution
  - Dissolve IBMX in a solution made if 0.5N KOH to a final concentration of 0.0115g/mL
  - Filter Sterilize with a .22 mm syringe filter

- MDI Induction Media
  - To required volume
  - 1:100 IBMX
  - 1:100 Insulin
  - 1:1000 Dexamethasone
Method

Preadipocyte maintenance and passage:

Plate the cells in 10% CS/DMEM on treated polysteren culture dishes from Corning and incubate them at 37 °C in 10% CO2. It is important to feed the preadipocytes every couple of days to avoid letting them get to confluent (>70%), if you want to continue to passage them and differentiate them at a later date. Take care to split them appropriately. They can be split as far as 1:15, though it is usually done 1:10 or less depending on need.

Adipocyte Differentiation Protocol

1) Grow preadipocytes to confluency on 10% calf serum/DMEM

2) Two days post confluency (Day 0) stimulate the cells with MDI induction media. You will notice a distinct change in the morphology of the cells (become more spindly) in the next two days.

3) After MDI (Day 2) change the media to insulin media. The media will begin to get more viscous as free fatty acids are produced by the cells and secreted into the media.

4) Two days later (Day 4) change the media to 10% FBS/DMEM. Feed cells with 10% FBS/DMEM every two days. Full differentiation is usually achieved by day 8.
Transient Transfection of 3T3-L1 Cells

Preparation of DNA

Mix appropriate amount of plasmid to be transfected and carrier DNA (phenol/chloroform extracted salmon sperm DNA)

Per 10 cm dish: 35-45μg DNA
Per 6 cm dish: 12-15 μg DNA

(Pool DNA for dishes transfected with the same plasmids)

Preparation of cells

Before transfection change media to DMEM + 10% CS of a batch known to work well for transfection. Return to incubator for 30 minutes or more.

Preparation of CaPO₄-DNA Precipitate

- In the hood

1) Mix DNA and CaCl₂/HEPES (0.5 ml/10 cm dish, 0.167 ml/6 cm dish)

2) Add NaCl/HEPES/Na₂PO₄ (0.5 ml/10 cm dish, 0.167 ml/6 cm dish) to a sterile 15 ml tube.

3) Add the DNA/CaCl₂/HEPES dropwise to the tube while bubbling air into the mixture using a 1 mL pipet and an automatic pipettor.

4) Let the mixture stand for 30 minutes at room temperature. A cloudy precipitate will form during this period.

Transfection

Remove cells from incubator and add the CaPO₄/DNA precipitate slowly to each dish (1 ml/10 cm dish, 0.33 ml/6 cm dish). Rock the dishes gently to prevent local changes of pH. Put cells back in incubator for 4-5 hours.
Glycerol Stock

1) Remove the dishes from the incubator and remove the medium carefully.

2) Add 12.5% glycerol/PBS solution (4 ml/10 cm dish, 2 ml/6 cm dish), rock gently and let it stand for 3 minutes.

3) Carefully remove the glycerol/PBS solution and gently add medium (DMEM + 10% of the appropriate serum).

4) Treat no more than 4 dishes at a time, but the treatment of another set of 3 can easily be initiated 1 minute after the first set.

Solutions

All solutions are filter sterilized (.22μm filter) and containers should only be opened in the hood.

The CaCl$_2$/HEPES and the NaCl/HEPES/NaHPO$_4$ solutions are adjusted to a pH of exactly 7.12, aliquoted into 50 ml tubes and kept frozen.

<table>
<thead>
<tr>
<th>CaCl$_2$/HEPES</th>
<th>g/mol</th>
<th>g/500ml</th>
<th>g/1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 M CaCl$_2$*2H$_2$O</td>
<td>147.02</td>
<td>18.38</td>
<td>36.76</td>
</tr>
<tr>
<td>0.025 M HEPES</td>
<td>238.3</td>
<td>2.98</td>
<td>5.96</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NaCl/HEPES/NaHPO$_4$</th>
<th>g/mol</th>
<th>g/500ml</th>
<th>g/1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.28 M NaCl</td>
<td>58.44</td>
<td>8.18</td>
<td>16.36</td>
</tr>
<tr>
<td>0.025 M HEPES</td>
<td>238.3</td>
<td>2.98</td>
<td>5.96</td>
</tr>
<tr>
<td>0.0015 M NaHPO$_4$</td>
<td>141.96</td>
<td>0.1065</td>
<td>0.213</td>
</tr>
</tbody>
</table>

12.5% glycerol/PBS
62.5 ml glycerol
437.5 ml PBS
**General Cell and Tissue Lysis**

Choose 1st step depending on what you are lysing and proceed to 2nd step

1: [Cell Lysis]
   1) Set Plates on Ice
   2) Wash plates with cold PBS two times
   3) Add fresh lysis buffer onto plates (on a 100mm plate: 0.5ml for fibroblasts and 1 ml for adipocytes)
   4) Scrape cells from plates

1: [Tissue Lysis]
   1) Completely thaw out tissue from the -80. Chop tissues into small pieces.
   2) Mix enough lysis buffer to cover the tissue. Homogenize tissue with dounce homogenized 20 times.

2:
   - Rotate lysate at 4°C or 30 minutes.
   - Spin down cells by 14K rpm for 20 minutes.
   - Carefully remove fat cake from top using a needle and syringe, transfer supernatant into clean tubes and discard the pellet of unbroken cells and debris
   - Measure Protein Concentration
   - Mix 20-60 µg of lysate with 1/4V of 4x LDS sample buffer
   - Boil samples for 5 minutes
Cocktail for Adipocyte Differentiation

**IBMX (M)**
- MAKE FRESH
- 3-isobutyl-1-methylxanthine (sigma
- Make 0.0115 g/ml, dissolve in 500µl H2O and 500µl KOH (Filter sterilize)
- Use 1:100 Dilution

**Insulin Stock (I)**
- 167 µl (1mg/ml) in 0.02M HCl (Filter sterilize)
- Use 1:1000 dilution

**Dexamethasone (D)**
1 st Stock: 10mM/100% Absolute Ethanol
   add a drop of NaOH if not soluble
2 nd Stock: 1mM/PBS (Filter Sterilize)
   Use 1:1000 dilution

**FBS (Filter Sterilize)**
- 10% in DMEM
  Make the mix of IBMX, Insulin and Dexamethasone in the desired volume of 10% FBS in DMEM add to the confluent preadipocyte plates
  10ml/10cm  5ml/6cm  2ml/3.5cm plates
- Incubate for two days
- Give insulin (1:1000) in 10% FBS/DMEM
  Incubate for two days
- Feed with 10% FBS in DMEM
  Every other day
C/EBPα Blot

1) Run gel @ 100-125V for 90-120 minutes until dye front has reached the bottom

2) Transfer to shiny side of PVDF membrane
   a. 100V for 90 minutes using ice
   b. 30V overnight

3) Block membrane with 5% milk in TTBS for at least 2 hours @ room temp, or overnight at 4°C on the rotator.

4) Briefly rinse the blot with TTBS

5) Dilute Int. C/EBPα A.P. Ab 1:1000 in TTBS with 1% γ-globulins. Shake membrane with Ab. @ room temp for 2 hours.

6) Wash blot in ~20 ml volumes of TTBS 3 times for 5 minutes, and once for 15 minutes.

7) Prepare secondary Ab α-rabbit HRP dilute 1:4000 in TTBS with 5% milk, shake blot with secondary Ab 1 hour at room temperature.

8) Repeat washing (step 6)

9) Mix the ECL using 3 ml each of the Pico and 300μl each of the Femto, incubate the blot in ECL for ~5 minutes.

10) Wrap blot in plastic and expose to the film.
**Growing Up Plasmids**

I) Transformation of E. Coli

**Materials needed:**
- Chemically competent DH5α aliquots (50 μl)
- LB Broth (or SOC)
- Plasmid
- LB agar plate containing appropriate antibiotic

1. Thaw appropriate number of chemically competent DH5α by placing on ice
2. Add 1-5 μl plasmid (depending on concentration) to thawed cells and mix gently.
3. Place on ice for 5-30 minutes.
4. Heat shock cells by placing in 42°C bath for 30 seconds or 37°C for 2 minutes.
5. Place immediately on ice for 2 minutes (Place SOC in 37°C water bath to warm up).
6. Add 1 ml room temperature LB Broth (or 300 μl23o00- SOC) to cells and place in 37°C shaker for an hour. Place at an angle to increase surface area.
7. Plate out 100-200 μl of culture on plate containing appropriate antibiotic and incubate overnight at 37°C upside down, with agar on top (to prevent accumulation of condensation on the agar). Store remaining bacteria in 4°C cold room in case more is needed to replate.
8. Next morning check for colonies. Wrap edges of plate with parafilm to prevent desiccation and place in 4°C cold room until needed. If there are very few colonies (or none), one can concentrate the remaining transformed bacteria by spinning 1 min/1000rpm, resuspending pellet in 100 μl and plating entire volume.

2. Preparing mini culture

**Materials needed:**
- LB plate containing distinct colonies
- LB broth containing proper antibiotic (Ampicillin stock is 1000x, Kanamycin is 500x)
- Filtered 20μl/200μl tips or inoculation loop
- 14 ml round bottom tubes

**Options**

--- Prepare mini culture the day before and let it grow overnight

1) Aliquot 3-5 ml of LB broth with antibiotic into round bottom tubes

2) Carefully pick a bacteria colony using either the inoculation loop (touch the colony) or the tip by stabbing the agar (do not touch more than one colony)

3) Shake inoculation loop inside the LB broth to detach the colony from the loop. If a tip is being used release it into LB broth. Replace cap on tube but leave it loose.

4) Place tubes in 37°C shaker overnight.

5) Next day, remove tubes from shaker, tighten caps and place at 4°C until needed.

--- Prepare mini culture in the morning and removing 8 hours later checking for cloudiness in the broth with same techniques as listed above.

**C. Preparing Maxi culture**

**Materials needed:**
- Mini culture
- LB broth containing antibiotic (Ampicillin stock is 1000x, Kanamycin is 500x)
- Flask

1) Add proper antibiotics to 200 ml of LB broth in a flask (200 μl Amp)

2) Add 1 ml of day mini culture or 500 μl of overnight mini culture to flask

3) Place culture in 37°C overnight

4) After 14-16 hours of growth, store at 4°C or use immediately.

**D. Maxi prep of DNA**

**Materials needed:**
- Maxi Culture
- Qiagen Maxi prep kit
- Sodium acetate
- 100% ethanol
-70% ethanol (made by diluting 100% ethanol)

Follow directions in the kit except:

Following elution add 1.5 ml sodium acetate and fill tube to 50 ml with 100% ethanol. Place overnight in -20°C freezer. Next day spin down at 4°C at maximum centrifuge speed. Wash by removing supernatant and adding 5 ml 70% ethanol to the pellet. Vortex to remove pellet from the bottom of the tube, then spin for 5 minutes. Remove supernatant and dry upside down for 10 minutes. Carefully resuspend DNA in 500 μl TE.

E. Quantification if DNA using UV Spec

Materials needed:
- 1x TE
- Plasmid to be quantified diluted 1:50 in TE (2 μl plasmid: 98 μl TE)
- Two quartz cuvettes

1) Rinse out cuvettes with ddH₂O and dry by gently taping on kim-wipe as well as carefully removing remaining liquid with a micropipette.

2) Add 100 μl 1x TE to chipped cuvette to serve as a blank, place blank in first slot of spec

3) Add diluted plasmid to other cuvette, place cuvette in the second slot of spec

4) Using program 4 on spec, measure absorbance at 260 and 280.

5) 260/280 should be around 1.7-2

6) Take absorbance at 260 and multiply by 1.25 to get the concentration (μg/μl). If diluted 1:100, multiply by 2.5. If diluted 1.35 multiply by 0.75 etc.

F. Verify identity by restriction enzyme analysis

Materials needed:
-Plasmid(s) to be analyzed
-Appropriate enzymes (always kept on ice)
-10 X buffer and 10x BSA (if needed)
- ddH₂O
1) Determine the appropriate enzymes to best verify the identity of your plasmids.

2) If using more than one enzyme in a digestion, check NEB chart to identify the best buffer to use for the reaction. Also, if one enzyme requires 10x BSA, you must use it in the reaction even if the other doesn’t (BSA won’t affect the activity of the other enzyme.

3) If performing the same digestion on more than one plasmid, make up a master mix as in the following example. In this example there are 10 plasmids to test and the digest is 1 μl of each with BamHI and HindIII together.

<table>
<thead>
<tr>
<th>Item</th>
<th>μl/sample</th>
<th># samples +1</th>
<th>Total μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x BamHI buffer</td>
<td>2</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>10x BSA</td>
<td>2</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>BamHI</td>
<td>0.25</td>
<td>11</td>
<td>2.75</td>
</tr>
<tr>
<td>HindIII</td>
<td>0.25</td>
<td>11</td>
<td>2.75</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>14.5</td>
<td>11</td>
<td>159.5</td>
</tr>
</tbody>
</table>

4) Once the master mix is made, aliquot appropriate amount of mix into a clean 1.5 ml tube. In the previous example, the added amount of the mix would be 19 μl. Then add the appropriate amount of plasmid. In the previous example, the added amount of the plasmid would be 1μl. Place reactions in a 37°C water bath for 1 hour.

5) Pour 0.7% agarose gel with the appropriate amount of lanes. Let it cool until solid (about 30 minutes). Add 6x DNA LB (in the previous example the amount would be 4 μl) to samples and load the gel along with a ladder. When ran take a picture of the gel.

6) If identity of plasmid is verified, label and place in appropriate plasmid box slot.
**Biorad Protein Assay: Bradford**

Standards: 1mg/ml BSA stock

-dilute 1:100 to get 1 mg/ml BSA

(Add to get H-20)

20μl = 2μl/ml 780 μl
40μl = 4μl/ml 760 μl
60μl = 6μl/ml 740 μl
80μl = 8μl/ml 720 μl
100μl = 10μl/ml 700 μl (optional)
120μl = 12μl/ml 680 μl

**Protocol**

1) Add 1, 2, 3, or 4 μl of concentrated unknown, and bring volume up to 795μl with water.

2) Add 200μl concentrated Biorad reagent and incubate at room temperature for 5 minutes.

3) Assay absorbance at 595 nm.
**Tail Digest Protocol (all except sFRP5 mice)**
*most commonly used digest protocol at OAM lab*

10x Modified Gitschier Buffer (10x MGB)

- 223 mL Tris (1.5 M, pH 8.8) = 670 mM Tris
- 10.97 g Ammonium Sulfate = 166 mM Ammonium Sulfate
- 3 g Magnesium Chloride = 65 mM Magnesium Chloride

Fill to 500 ml with Milli-Q H$_2$O, mix well and autoclave

1x MGB (40 ml)

- 354. ml Milli-Q H$_2$O
- 4 ml 10x MGB
- 400 μl 2- Mercaptoethanol (final 1%)
- 200 ul Triton X-100 (final 0.5%)

Pro K Mastermix (Per Sample)

- 1 ml 1x MGB
- 50 μl Pro K solution (20 mg/ml)

[Ex: for 15 samples, 1500 μl 1x MGB + 75 μl Pro K Solution]

**METHOD:**

1. Clip 2-5 mm mouse tail with a clean razor. Place in labeled 1.5 ml tube and store at -20°C until further processing.

2. Add 100 μl 1x MGB and heat at 95°C for 10 minutes.

3. Allow to cool for 5-10 minutes to about 55-65°C. Vortex and centrifuge

4. Add 100 μl Pro K mastermix. Vortex and spin.

5. Incubate at 55°C for 30 minutes. *See step 8*

6. Vortex on high for one second.

7. Repeat steps 5 and 6 four or five times.

8. **Step 5 may be extended to an overnight digest. If this is done, ignore step 7.**

9. When tail is fully digested, vortex and the pulse the tubes in the centrifuge.

10. Heat at 95°C for 10 minutes to deactivate the Pro K.

11. Centrifuge at maximum speed for 5 minutes. Store the digested tail DNA at -20°C.

12. Use 1 μl of tail prep for 50 μl PCR reaction.
**EMSC (Ears Mesenchymal Stem Cells) Isolation and Culture**

**Prepare:**

- HBSS (Invitrogen #14025-092)
  
  Add 100 μl Primocin (Invivogen #ant-pm-1) per 50 ml HBSS
  
  Filter with 0.22 μm

- Collagenase I (Worthington Biomedical Corporation #LS004196)
  
  2mg/ml Collagenase in sterile HBSS
  
  Filter with 0.22 μm

- Red Blood Cell Lysing Buffer (Sigma #R7757)

- Autoclave all needed tools

**Isolate Cells:**

1) Sacrifice mice and submerge collected ears only into 6 well plates with 70% ethanol

2) Collect external ears and put in sterile HBSS + antibiotics

**Perform remaining steps under the hood:**

3) Wash ears with sterile HBSS + antibiotics in the 6 well plates. 3 ml per well

4) Using sterile scissors and razor blades, cut ears into tiny pieces in collagenase solution. It will be easier to cut if you use a small amount of collagenase to cut the ears and then add the minced ears to the remaining collagenase.

5) Digest for 1 hour at 37°C in a water bath. Shake every 15 minutes.

6) Filter through 70 μm cell strainer to remove extra unwanted pieces. (BD Biosciences #352350)

7) Centrifuge at 1350 rpm for 8-9 minutes

8) Discard supernatant

9) Resuspend the pellet in red blood cell lysing buffer following Sigma's instructions. Be gentle! Add 1 ml of buffer, pipette to mix, let sit for 1 minute, add 10 ml cell culture medium and pipette it again to mix.

10) Centrifuge at 1350 rpm for 8-9 minutes
11) Discard supernatant

12) Resuspend the pellet in culture medium (EMSC + Primocin) and seed

   Seed cells from 6 animals/12 ears in one 100mm dish

13) Change medium every 2-3 days.

**Split Cells:**

1) Remove medium from wells

2) Wash subconfluent primary cultures with PBS (Invitrogen #10010-023)

3) Remove PBS

4) Add 0.25% Trypsin (Invitrogen #25300-054)

   2-3 ml to a 100mm dish

5) Place in incubator for about 3 minutes until cells detach

6) Add culture medium to neutralize the trypsin

   6-8 ml to a 100mm dish

7) Collect and centrifuge at 1350 rpm for 8-9 minutes

8) Discard supernatant

9) Resuspend the pellet in culture medium and seed

   Cells are typically diluted 1:5 or 1:6 at each passage

**Culture Medium:**

DMEM/F12 (Invitrogen #11330-032) + 15% FBS (Invitrogen #10082-147) + 100 μl Primocin (Invivogen #ant-pm-1) per 50ml Media
Western Blot Protocol

Sample Preparation: Cell Lysate and Conditioned Media

**Cells** from 10 cm plates are collected at 100% confluence, Day 0, in 1 ml of Western Lysis Buffer (WLB) and stored at -20°C.

Cells from 6 well plates should be lysed in 750μl to 1 ml of WLB with protease tablets

**Conditioned media** is collected in 15 ml tubes, spun down at 5000 rpm for 5 minutes to form a pellet.

1) (Cells can be treated with serum free (SF) conditions by washing with 1x PBS and adding 4-7 ml DMEM with 0.7% FBS or BSA.

2) 3T3L1 cells at Day 0 should not be kept under these conditions for more than 6 hours, as cells begin to die at a rapid rate without serum.

3) Spin down the media collected from these cells since there will be cells suspended in it that must be cleared before proceeding.

4) Samples are boiled on a 95°C heat block for 5 minutes (preadipocytes should be boiled for 15 minutes to fully disperse blob)

5) Pipet the sample to fully disperse the blob and remove an aliquot to use as a working sample.

6) 4x SDS loading buffer is added to the working sample to a concentration of 1x.

7) Samples are boiled again at 95°C for 5 minutes to denature and allow for proper dispersal of the blob.

8) Working and stock samples should be stored at -20°C.

Bradford Assay:

- Protein concentration can be obtained from the working sample immediately before addition if loading buffer (preferred) or subsequently from the stock sample. If concentration is determined from the working sample, remove 2 μl of the sample after the blob has been fully dispersed and add it to 1 ml of protein assay solution and proceed with the Bradford analysis. If the stock sample is being used, use the following procedure to determine protein concentration:

1. Preheat the stock sample at 60-80°C for 1-5 minutes.
2. Spin samples down for 1-2 minutes to cool them down and collect the sample that has condensed on the lid of the tube.

3. Pipet to fully suspend the blob

4. Remove 2 μl of the sample and add it to the 1 ml of protein assay solution and proceed with Bradford analysis.

Running the Gel:

1. Select performed gels based on the number of wells needed and set up the gel apparatus.

2. Once gel is firmly in place add running buffer to the center of the gel apparatus, between the gels, check for any leaks, then add Buffer to the outside of the gels about ½ or 2/3 the way up the apparatus.

3. Place the samples and run gel at 125 volts (constant) for ~90 minutes or until blue dye runs to the bottom of the gel.

Transferring the Membrane:

1. Fill a tray with 1x Transfer Buffer and place a transfer clip in the tray with the clear side down.

2. Prepare 3 pieces of Whatman paper cut to the size of the gel and one membrane cut a bit smaller for each gel.

3. Disassemble gel apparatus using a prying knife and trim off the stacking gel and the bottom part that has curled up.

4. Place a sponge down on the clear side of the clip, followed by two pieces of Whatman paper, then the gel.

5. Prewet the membrane in the transfer buffer for a few seconds then place the membrane on top of the gel.

6. Add another piece of Whatman paper, then the other sponge and close transfer clip.

7. Place transfer clip in transfer apparatus so that the black side of the clip is facing the red side of the apparatus. Proteins are negatively charged because of the SDS and will migrate to the positive (red) charge.

8. Pour the 1x transfer buffer in the tray into the transfer apparatus and add any additional buffer needed to fill the apparatus to the top. Place the lid on
and run at 35 volts overnight. Alternatively the transfer can run at a higher voltage for a few hours, but an ice block must be placed in the transfer apparatus to prevent overheating.

**Blocking the Membrane:**

1. Prepare 5% milk and 1x TTBS. 10x TTBS is a stock solution in the cold room. Dilute to 1x with Milli-Q H$_2$O and use for the remaining steps (usually about 500 ml is needed). 10 ml of 5% milk is needed for each blot when blocking.

2. Cut a bag that is larger than the blot as adjustments may be needed once the bag is sealed.

3. Disassemble the transfer apparatus and remove the membrane. Cut the membrane corner (bottom corner opposite side of the marker) to orient the membrane later on. Discard the gel and the Whatman paper, and rinse the rest of the transfer apparatus and let dry.

4. Immediately place the membrane in bag and seal the three sides. Add 10 ml 5% milk for each membrane/bag and steal the remaining side of the bag making sure not to seal in any bubbles.

5. Place the bag on top of the rocker with a large bag of water on top of it and block in 5% milk for 1 hour.

6. Remove the membrane from the bag and place in a tip box with enough 1x TTBS to cover and wash 3 times on rocker for 10 minutes each time.

**Primary Antibody Incubation:**

1. Prepare primary antibody by diluting in either 5% milk or 1% γ-globulins in 1x TTBS. For the anti-myc-HRP antibody, dilute 1:1000 in 1% γ-globulins in 1x TTBS (0.1 g in 10 ml).

2. Cut a bag that is larger than the membrane.

3. Remove the membrane from the tip box and trim any unneeded parts of the membrane off then place the membrane in the bag and seal three edges.

4. Add 5 ml of the primary antibody solution to the bag and seal without any bubbles. Let rock with a large bag of water in top of it for 2 hours at RT. (Can go overnight at 4°C.

5. Remove membrane from bag and wash 3 times for 10 minutes each in 1x TTBS.
Secondary Antibody Incubation

1. During final wash, prepare second antibody by diluting in BSA or non-fat dry milk.

2. Cut a bag that is larger than the membrane.

3. Remove membrane from tip box, place in bag and seal three edges.

4. Add 5 ml of the secondary antibody solution per membrane/bag and seal the final side of the bag. Incubate on the rocker with a large bag of water on top for about an hour at RT.

5. After incubation, wash membrane in a tip box with 1xTTBS 3-4 times for 10 minutes each.

Visualization:

1. During the last wash, mix ECL reagents 1:1 (for 1 membrane, mix 3 ml of each reagent and add 1/10 volume enhancer reagents (300 µl of each) in the bottom of the tip box.

2. Remove membrane from bag and expose to ECL mixture in tip box for about 2 minutes.

3. Place membrane in plastic wrap and tape plastic wrap to the inside of a film cassette.

4. Expose film to membrane in dark room and develop film.
Insulin Stimulated Glucose Uptake Assay

In the hood

1) Differentiate the 3T3L1s to >day 10
2) Wash 1x with cells with 1xKRH media
3) Serum starve the cells in 0.5% BSA/KRH for >2 hours
4) Wash the cells with KRH
5) Add 0.5 ml of 0.5% BSA/KRH (exact amount)
   --Monitor meticulous timing for the next steps.
6) **Insulin, 5μl/well: 10 minutes**

   **FAST STEPS** Use electronic pipettor
   
   - [Final]: 10nM (make 5.73μl of our stock to 1ml of KRH to give 1μM stock),
     Insulin ([stock]= 1μM; [working]= 10 nM) in KRH. 1μM stock- 5μg/ml
   
   - The insulin that we use for tissue culture is 1mg/ml, which is 174.4 μM. Add
     5.73 μl of this to 1ml of KRH to get 1 μM of insulin stock. For 0.2μM stock, dilute
     the 1 μM stock to 1:5 in KRH
   *Gently swirl and rock cells*

7) **Cytocholasin B, 1μl/well: 10 minutes**

   - [Final]: 50μM, [Stock]: 25 mM
   *Gently swirl and rock cells*

8) **DOG (2-deoxy-D[2-14C(U)], 5μl/well: 5 minutes**

   Use electronic pipettor

   [Final]: 0.1uCi/ml in 500μl buffer [Stock]: 25mM

   Cold glucose concentration= 200mM in KRH, 0.036g/1ml KRH
<table>
<thead>
<tr>
<th>Hot Reagent</th>
<th>Per Well</th>
<th>Per 70 Wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold DOG</td>
<td>0.25 μl</td>
<td>17.5 μl</td>
</tr>
<tr>
<td>Hot DOG</td>
<td>1.00 μl</td>
<td>70 μl</td>
</tr>
<tr>
<td>KRH</td>
<td>3.75 μl</td>
<td>262.5 μl</td>
</tr>
<tr>
<td>Final Volume</td>
<td>5.0 μl</td>
<td>350 μl</td>
</tr>
</tbody>
</table>

9) At exactly 30 minutes post insulin stimulation, in the hot room, aspirate the media and wash cells with ice cold 1xPBS 3 times.

10) Lyse the cells in 0.5ml/well of 0.1% SDS, rock for 30 minutes

11) Transfer to eppendorf tubes and vortex well

-- At this point they can be stored at -4°C until ready to use.

12) Take 300 μl of each in scintillation vials containing 4 ml fluid & mix well

13) Normalize to protein (BCA Protein Assay)

<table>
<thead>
<tr>
<th>Insulin (nM)</th>
<th>Cytochalasin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>
**Immunoblot Protocol**

1) Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a cell nuclear extract (Extraction buffer: 20mM HEPES, pH 7.9; 25% glycerol; 0.42 M NaCl; 1.5 mM MgCl₂; 0.2 mM EDTA; 0.5 mM PMSF; 0.5 mM DTT) and transfer the proteins to nitrocellulose. Wash the bottled nitrocellulose twice with water.

2) Block the blotted nitrocellulose in freshly prepared PBS containing 3% nonfat dairy milk (PBS-MILK) for 1 hour at 20-25°C with constant agitation.

3) Incubate the nitrocellulose in 0.5-2μg/ml of anti-Myc Tag, clone 9E10 diluted in freshly prepared PBS-MILK overnight with agitation at 4°C.

4) Wash the nitrocellulose twice with water.

5) Incubate the nitrocellulose in the appropriate secondary reagent (a goat anti-mouse HRP conjugated IgG --Cat # 12-349, 1:2000 dilution) in PBS-MILK for 1.5 hours at room temperature with agitation.

6) Wash the nitrocellulose twice with water.

7) Wash the nitrocellulose in PBS- 0.05% Tween 20 for 3-5 minutes

8) Rinse the nitrocellulose in 4-5 changes of water.

9) Use detection method of choice (enhanced chemiluminescence is preferred)

**Immunoprecipitation Protocol**

1) Dilute the cell nuclear extract before beginning the immunoprecipitation to roughly 1mg/ml total cell protein in a microcentrifuge tube with PBS.

2) Add 4μg of ant-Myc Tag, clone 9E10 to 500μg-1mg cell lysate.

3) Gently rock the reaction mixture at 4°C for 2 hours or overnight.

4) Capture the immunocomplex by adding 30μl (15μl packed beads) of washed Protein G agarose bead slurry, catalog #16-266.

5) Gently rock the reaction mixture at 4°C for 2 hours.

6) Collect the agarose beads by pulsing (5 seconds in the microcentrifuge at 14,000 x g) and drain off the supernatant. Wash the beads 3x with either ice cold cell lysis buffer or PBS.

7) Resuspend the agarose beads in 50μl 2x Laemmli sample buffer.

8) The agarose beads can either be frozen for later use or suspended in Laemmli sample buffer and boiled for 5 minutes. Collect the beads by a microcentrifuge pulse. SDS-PAGE and subsequent immunoblot analysis can be performed on a sample of the supernatant.
RNA extraction using RNeasy kit

1) Wipe down all surfaces with RNeasy inhibitor solution

2) For each sample, label:
   - one QiaShredder (in collection tube)
   - one RNeasy column (in collecting tube)
   - one 2ml collection tube
   - one 1.5 ml Eppendorf tube (sterile)

3) Allow samples (in RTL buffer) to thaw at room temperature

4) Pipette 200 μl of lysed samples into QiaShredder. Centrifuge for 2 minutes at 13,000 rpm.

5) Add 200 μl of 70% ethanol to the transferred lysate in the collection tube; mix well by pipetting.

6) Transfer RLT/EtOH mixture into the RNeasy column. Spin for 15 seconds at 13,000 rpm. Discard flow-through.

7) Add 700μl of buffer RW1 to the column. Centrifuge for 15 seconds at 10,000 rpm.

8) Transfer RNeasy column to a new 2ml collection tube. Add 500μl of buffer RPE to the column and centrifuge for 15 seconds at 10,000 rpm.

9) Discard the flow-through and repeat step 8 (use the same collection tube).

10) Discard the flow-through. Dry the column by centrifuging at 13,000 rpm for 2 minutes.

11) Elute RNA: place the column in the 1.5ml Eppendorf tube and add 35 μl of RNase free water directly onto the filter of the RNeasy column. Leave for 1.5 minutes at room temperature. Then spin the 1.5 minute at 13,200 rpm.

12) Estimate [RNA] using Nanodrop and store samples at -80°C.
Gitschier Protocol for Genomic DNA isolation from mice

10x Modified Gitschier Buffer (10x MGB)

223 mL Tris (1.5 M, pH 8.8) = 670 mM Tris
10.97 g Ammonium Sulfate = 166 mM Ammonium Sulfate
3 g Magnesium Chloride = 65 mM Magnesium Chloride
Fill to 500 ml with Milli-Q H₂O, mix well and autoclave

1x MGB (for 10 mL)
8.85 ml ddH₂O
1 ml 10x MGB
100 μl 2-mercaptoethanol
50 μl Triton X-100
Vortex vigorously to make it soluble Triton X-100

Proteinase K mastermix
10 parts 1x MGB
1 part 20 mg/ml Proteinase K

Method:

1) Clip 5mm mouse tail with a clean razor blade, place in 1.5 ml tube, and store at -20°C until further processing.
2) Add 100 μl of 1xMGB
3) Heat at 95°C for 10 minutes
4) Add 110 μl of Proteinase K mastermix (10 parts 1x MGB, 1 part 20 mg/ml Proteinase K)
5) Incubate at 55°C for 30 minutes
6) Vortex vigorously for 1 second
7) Repeat steps 5-6 two more times
8) Spin tubes at 14,000 x g for 5 seconds
9) Heat at 95°C for 10 minutes
10) Spin at 14,000 x g for 5 minutes. Store at -20°C until use. Use 1 μl per 50 μl PCR reaction.