
The Open Lab Book

Release 1.0

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The OLB is a collection of open, freely-available lab protocols collected, with permission, from across the internet. The list has been available in various formats and on various sites over that time, but finally finds a home here.

Authors are credited where known and with homepage links where available. Contributions of new protocols or amendments or corrections to existing ones are most welcome and can be made via the [github repository](#).

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You can also download the complete book [as a PDF](#).

1.1 Biochemistry

Biochemistry protocols.

1.1.1 Extracting cholesterol and lipids from cells

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

This is a general method that may not work for all cell/tissue samples - tweak the solvent volumes per quantity of cells, centrifugation time and sonication may be applied for samples that are difficult to disrupt. For chloroform - handle under a chemical safety hood.

- Homogenize 1×10^6 cells or ~10 mg tissue into either 200 μ L chloroform-methanol (v/v 2:1) or 200 μ L hexane-isopropanol (v/v 3:2).
- Centrifuge for 5-10 min at 14,000 rpm in a microcentrifuge.
- Transfer the organic phase to a clean tube and vacuum dry. Store the material in the freezer (<20°C), desiccated and protected from air, i.e., under anaerobic conditions to minimize oxidation.
- Re-dissolve the vacuum-dried lipids/cholesterol into a suitable assay buffer prior to use.

References

FOLCH J, LEES M, SLOANE STANLEY GH A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* (1957) PMID:13428781

1.1.2 In-gel Tryptic Digest for Protein ID by MS

Contributed by [Timothy Mitchison](#), Harvard Medical School, Boston, MA, United States

In-gel Tryptic Digest for Protein ID by Mass Spectrometry (David Miyamoto, 2/12/2002). This protocol is based on Shevchenko A, Wilm M, Vorm O, & Mann M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal Chem* 1996, 68:850-8. I have used it with success on both Coomassie and silver-stained gel bands.

The procedure includes reduction and acetamidation steps that may be skipped if desired. For heavily stained Coomassie bands, it is helpful to wash gel pieces for 1 hr in 100 mM NH_4HCO_3 prior to dehydrating with acetonitrile (step 2).

Method

- Excise band from Coomassie or silver stained gel. Cut gel band into 1 mm cubes using clean razor blade on a clean glass surface. Transfer to an Eppendorf tube.
- Remove excess water with pipet. Add 25-35 μL acetonitrile to tube to cover gel pieces. Incubate 10 minutes at RT to dehydrate and shrink gel pieces.
- Remove acetonitrile with pipet. Speed-vac to dryness for 10 minutes.
- Swell gel particles in 150 μL 10 mM DTT in 100 mM NH_4HCO_3 . Incubate 1 hour at 56°C .
- Cool to RT. Replace DTT solution with 150 μL 55 mM iodoacetamide in 100 mM NH_4HCO_3 . Incubate 45 minutes at RT in the dark with occasional vortexing.
- Remove solution and wash gel pieces with 150 μL 100 mM NH_4HCO_3 . Incubate 10 minutes at RT.
- Remove NH_4HCO_3 solution with pipet. Add 150 μL acetonitrile to dehydrate gel pieces. Incubate 10 minutes at RT.
- Repeat wash steps 6 through 7. Remove acetonitrile and speed-vac to dryness for 10 minutes.
- Place tubes in ice water bath and swell gel particles in 25-35 μL digestion buffer. Incubate 45 minutes in ice water bath. Digestion buffer consists of 12.5 ng/ μL trypsin (Promega sequence-grade modified porcine trypsin, Cat. #V511A) in 50 mM NH_4HCO_3 . To make the digestion buffer, dissolve 20 μg Promega trypsin in 80 μL Promega trypsin buffer solution (50 mM acetic acid), and dilute with 50 mM NH_4HCO_3 to 12.5 ng/ μL .
- Remove trypsin-containing buffer. Add 5-10 μL 50 mM NH_4HCO_3 without trypsin to keep pieces wet during cleavage. Incubate o/n at 37°C .
- Spin 1' at 14,000 rpm to spin down gel pieces. Save supernatant in a separate PCR tube.
- Add 20 μL 20 mM NH_4HCO_3 to cover gel pieces. Incubate 10 minutes at RT. Transfer supernatant to the PCR tube from step 11.
- Add 25 μL 5% formic acid, 50% acetonitrile to the gel pieces. Incubate 20 minutes at RT.
- Spin 1' at 14,000 rpm. Remove formic acid/acetonitrile solution and save in the same PCR tube from step 11.
- Repeat formic acid extraction (steps 13 through 14) twice more.
- Dry PCR tube in speed-vac to complete dryness. Store at -20°C until analysis.

References

Shevchenko A, Wilm M, Vorm O, Mann M [Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels](#). *Anal Chem* (1996) [pmid:8779443](#)

1.1.3 Purification of 6X HIS Proteins

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

Purification of 6XHIS proteins with cell extraction buffer

Requirements

Extraction buffer: 10mM imidazole, 500mM NaCl, 50mM NaH₂PO₄, pH8.0, (Optional components: 0.5mM TCEP, 1x protease inhibitor cocktail -Complete PI EDTA free tablets; Benzonase Nuclease HC,3 µl per 60ml extraction buffer).

Method

- Spin cells harboring 6XHIS proteins in large bottles in Beckman Centrifuge 6000rpm 15 minutes.
- Dump supernatant
- Resuspend in 3ml Cell extraction buffer.
- Grind protein in mortar and pestle plus liquid Nitrogen. (at least 10 minutes.) until becomes a fine powder.
- Transfer frozen powder to 15ml conical tube and bring volume up to 10 ml.
- Transfer approx 1ml to microcentrifuge tube and spin for 15 minutes at full speed 4C.
- While spinning pre-equilibrate Ni-NTA resin with extraction buffer by washing 3X in extraction buffer and resuspend in 50% slurry.
- Pool all the supernatants from step 6 into a 15ml conical (save 100ul of supernatant =Load) and add about 300 ul of Ni-NTA resin to the supernatant. Save some of the pellet (= insoluble fraction).
- Bind 6XHIS protein to resin for 20min to 1 hour room temp. Spin and save 100ul of the supernatant= unbound fraction.
- Wash resin 5 times with extraction buffer (5ml each time).
- Elute with low pH elution buffer (1ml each elution). Elute 5 X.

This method is based, with permission, on an original protocol available [here](#).

1.2 Buffers & Solutions

Buffer and solution protocols.

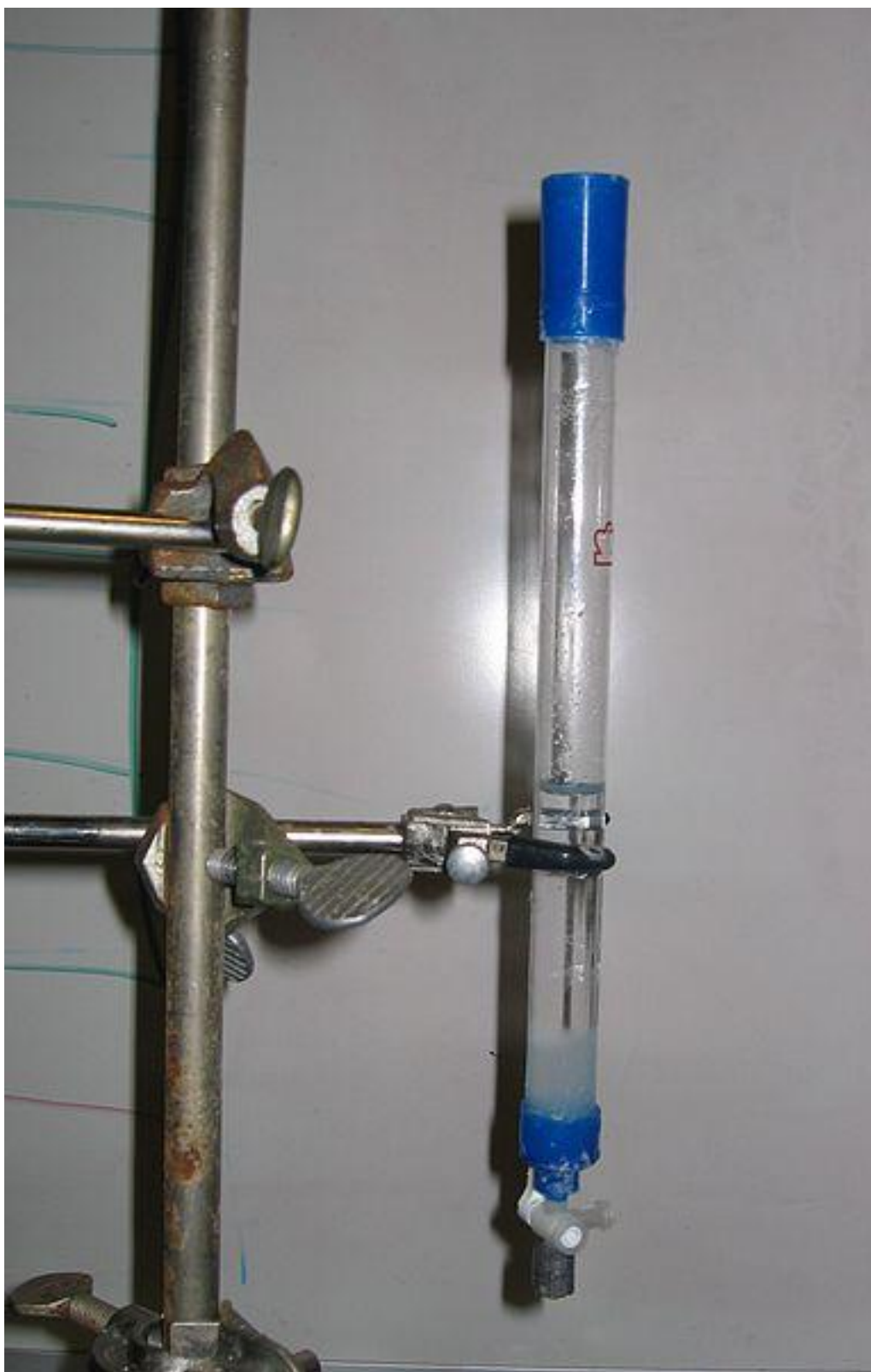
1.2.1 0.1 M Glycine-HCl, pH 2.7

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

0.1 M Glycine-HCl, pH 2.7

Requirements

11.1 g Glycine-HCl 800 ml dH₂O



Method

- Combine 11.1 g Glycine-HCl with 800 ml dH₂O
- Adjust pH to 2.7 and bring volume to 1 L with dH₂O

1.2.2 0.5 M EDTA

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

0.5 M EDTA

Requirements

16.81 g EDTA (Sodium Salt) Distilled water

Method

- Add ingredients to suitable container. Add distilled water up to 90 ml
- Adjust pH to 7.0
- Add distilled water up to a final volume of 100 ml. Store for later use.

Store at room temperature.

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays](#) (2001) 10.1002/0471143030.cb1301s00

1.2.3 0.5 M EGTA Stock

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

0.5 M EGTA Stock

Requirements

19.02 g EGTA (Sodium Salt) Distilled water

Method

- Add ingredients to suitable container. Add distilled water up to 90 ml
- Adjust pH to 7.0
- Add distilled water up to a final volume of 100 ml. Store for later use.

Store at room temperature.

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays](#) (2001) 10.1002/0471143030.cb1301s00

1.2.4 0.5 M Na₂CO₃

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

0.5 M Na₂CO₃

Requirements

531 mg Na₂CO₃ 10ml distilled water

Method

- Add 531 mg Na₂CO₃ to a suitable container.
- Make up to 10ml with distilled water. Store at room temperature until further use.

Store at room temperature.

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.5 1 M HEPES, pH = 7.0

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

1 M HEPES, pH = 7.0

Requirements

119.15 g HEPES (free acid) Distilled water Solid NaOH pellets Concentrated NaOH

Method

- Add 119.15 g HEPES (free acid) to a suitable container and make up to 400ml with distilled water.
- Add solid NaOH a few pellets at a time while mixing until the pH is ~6.8
- Add concentrated NaOH dropwise to achieve pH = 7.0
- Add distilled water to a final volume of 500 ml
- Sterile filter and store for later use.

Store at 4oC

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.6 1 M KCl Stock

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

1 M KCl Stock

Requirements

74.55 g KCl 1L distilled water

Method

- Add KCl to a suitable container. Make up to 1L with distilled water. Store for further use.

Store at room temperature

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays](#) (2001) 10.1002/0471143030.cb1301s00

1.2.7 1 M PIPES, pH = 6.9

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

1 M PIPES, pH = 6.9

Requirements

151.2 g HEPES (free acid) Distilled water Solid NaOH pellets Concentrated NaOH

Method

- Add 151.2g HEPES (free acid) to a suitable container and make up to 400ml with distilled water.
- Add solid NaOH a few pellets at a time while mixing until the pH is ~6.7
- Add concentrated NaOH dropwise to achieve pH = 6.9
- Add distilled water to a final volume of 500 ml
- Sterile filter and store for later use.

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays](#) (2001) 10.1002/0471143030.cb1301s00

1.2.8 10% Ammonium persulphate solution

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

10% solution

- Add dH2O to Falcon tube or other suitable container for the volume.
- Add 1g Ammonium persulphate per 10 ml water

e.g. 50mls add 5g APS

Mix thoroughly - APS will dissolve readily.

- Solution may be stored at 4°C up to 6 months

1.2.9 10 M NaOH

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

10 M NaOH

Requirements

10 M NaOH Stock* 40 g NaOH 100ml distilled water

Method

- Add 40 g NaOH to a suitable container. Add distilled water to make solution up to 100ml.

Store at room temperature

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays](#) (2001) 10.1002/0471143030.cb1301s00

1.2.10 10 x Column Buffer

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

10 x Column Buffer

Requirements

500 ml 1 M PIPES Stock (250 mM PIPES) 40 ml of 0.5 M EGTA Stock (10 mM EGTA) 40 ml of 1 M MgSO₄ Stock (5 mM MgSO₄) Distilled water

Method

- Combine ingredients in suitable container. Add distilled water to 1800ml.

Store at 4oC

- Adjust pH to 6.7
- Add distilled water to 2 liters

Store at 4oC.

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.11 100 mM MgATP Stock

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

100 mM MgATP Stock

Requirements

ATP 1 ml of 1M MgSO₄ Distilled water

Method

- Check the formula weight of ATP product. Calculate required quantity for 10ml of 100mM solution. Add to a suitable container.
- Add 8.5 ml distilled water
- Add 1 ml of 1M MgSO₄ Stock
- Adjust pH to 7.0
- Add distilled water to final volume of 10ml
- Aliquot in 200ml quantities and store at -20oC until further use.

Store at -20oC

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.12 100 mM MgGTP

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

100 mM MgGTP

Requirements

GTP 1 ml of 1M MgSO₄ Distilled water

Method

- Check the formula weight of GTP product. Calculate required quantity for 10ml of 100mM solution. Add to a suitable container.
- Add 8.5 ml distilled water
- Add 1 ml of 1M MgSO₄ Stock
- Adjust pH to 7.0
- Add distilled water to final volume of 10ml
- Aliquot in 200ml quantities and store at -20oC until further use.

Store at -20oC

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.13 100 mM Sodium orthovanadate

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

100 mM Sodium orthovanadate

Requirements

1.839 g Sodium orthovanadate 8ml distilled water to 8 ml

Method

- Add 1.839 g Sodium orthovanadate to a screw cap tube (falcon)
- Make up to 8ml with distilled water.
- Adjust pH to 10
- If solution is yellow, place in boiling water until clear. Re-check pH and repeat as necessary.
- Adjust to final concentration by checking A₂₆₅ nm, ext. coeff.= 2925M⁻¹cm⁻¹ and adding distilled water as needed. Store until required.

Store at -20oC

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.14 100mM Tris Buffer

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Recipe for 100mM Tris Buffer

- In a suitable container add target volume of dH₂O -10% to allow for pH adjustment.

e.g. for 1L Tris, start with 900ml

- Add 0.1211g of Tris for each 10ml dH₂O

e.g. for 900ml add 10.899g Tris

- Tris solution will be basic, therefore adjust to target pH 7.0 by addition of HCl
- Make up to final target volume with dH₂O

1.2.15 10x Tris Buffer

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Recipe for 10x Tris Buffer, dilute for use

- Fill suitable container with 1L dH₂O
- Add 30g Tris, 88g NaCl and 2g KCl to the solution, mixing thoroughly until fully dissolved.
- Adjust pH to 7.5 with HCl

1.2.16 150 mM Mg AMP-PNP

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

150 mM Mg AMP-PNP

Requirements

25 mg AMP-PNP (available from Boehringer Mannheim #102 547) 90 ul 1 M MgSO₄ Stock (150 mM MgSO₄) 225 ul distilled water

Method

- Add 25 mg AMP-PNP and 90ul 1 M MgSO₄ in a suitable container.
- Add 225 ul distilled water to give a final volume of 315 ul. Store at -20oC

Store at -20oC

References

Clare M. Waterman-Storer [Microtubule/Organellar Motility Assays](#) (2001) 10.1002/0471143030.cb1301s00

1.2.17 1M dithiothreitol (DTT)

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

1M dithiothreitol (DTT)

Requirements

1.542 g dithiothreitol 10ml Distilled water

Method

- Add dithiothreitol to a suitable container. Make up to 10ml with distilled water.
- Aliquot in 500 ml volumes and store at -20oC for future use.

Store at -20oC

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.18 1M MgSO₄

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

1M MgSO₄

Requirements

24.074 g MgSO₄

Method

- Add MgSO₄ to suitable container. Make up to 200ml with distilled water.

Store at room temperature

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.19 20 x Energy Regeneration System

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

20 x Energy Regeneration System

Requirements

150 mM creatine phosphate (Boehringer #127574) 2 ml of 100 mM MgATP Stock (20 mM ATP, 20 mM MgSO₄)
40ml 0.5 M EGTA (2 mM EGTA) Distilled water to 10 ml

Method

- Combine ingredients in suitable beaker. Make up to 10ml with distilled water.
- Aliquot in 100 ml containers and store at -20oC

Store at -20oC

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.20 2X Rapid Ligation Buffer

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

2X Rapid Ligation Buffer (ProMega)

Requirements

60mM Tris-HCl (pH 7.8) 20mM MgCl₂ 20mM DTT 2mM ATP 10% polyethylene glycol

Method

- Combine ingredients in suitable container.

This method is based, with permission, on an original protocol available [here](#).

1.2.21 3 M KI

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

3 M KI

Requirements

4.98 g KI 10ml distilled water

Method

- Add 4.98 g KI to a suitable container.
- Add distilled water to make up to final volume of 10 ml. Store at room temperature until required.

Store at room temperature

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.22 30 mM Mg GTP-g-S

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

30 mM Mg GTP-g-S

Requirements

30 mM Mg GTP-g-S* 10 mg GTP-g-S tetralithium salt (available from Boehringer Mannheim #220 467) 18 ul 1 M MgSO₄ Stock (30 mM MgSO₄) 572 ul distilled water

Method

- Add 30 mM Mg GTP-g-S, 10 mg GTP-g-S tetralithium salt and 18 ul 1 M MgSO₄ Stock (30 mM MgSO₄) to a suitable container.
- Add 572 ul distilled water to give a final volume of 592 ul. Store at -20°C until required.

Store at -20°C

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.23 30 x AlCl₃

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

30 x AlCl₃

Requirements

20 mg (1.5 mM AlCl₃) 100ml distilled water

Method

- Add 20 mg (1.5 mM AlCl₃) to a suitable container.
- Make up to 100ml with distilled water. Store until further use.

Store at room temperature.

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.24 30 x NaF

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

30 x NaF

Requirements

378 mg NaF (0.9 M NaF) 10ml distilled water

Method

- Add 378 mg NaF (0.9 M NaF) to a suitable container.
- Add distilled water up to a final volume of 10 ml

Store at -20oC

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays](#) (2001) 10.1002/0471143030.cb1301s00

1.2.25 3.6 mM Brefeldin A

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

3.6 mM Brefeldin A

Requirements

10 mg Brefeldin A (available from Epicenter Technologies Madison, WI) 12ml absolute ethanol

Method

- Add 10 mg Brefeldin A to suitable container.
- Add absolute ethanol up to a final volume of 12ml. Store at -20oC until required.

Store at -20oC

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays](#) (2001) 10.1002/0471143030.cb1301s00

1.2.26 5 M NaCl

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

5 M NaCl

Requirements

292.2 g NaCl 1L distilled water

Method

- Add 292.2 g NaCl to a suitable container. Add distilled water up to a final volume of 1litre. Store until further use.

Store at room temperature

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays](#) (2001) 10.1002/0471143030.cb1301s00

1.2.27 50 mM Tris pH 8.0 10 mM EDTA

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

50 mM Tris pH 8.0 10 mM EDTA

Requirements

50 mM 1 M Tris pH 8.0 5 ml 1 mM 0.5 M EDTA 2 ml H₂O up to 100 ml

Method

- Combine Tris & EDTA to final volume of 7ml
- Add H₂O up to 100 ml

Store at 4°C

1.2.28 5x Western blot loading buffer

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

15ml stock solution of western blot loading buffer. Dilute for use.

- To prepare base solvent add 3ml 20% SDS to add 3.75mL 1M Tris buffer at pH 6.8 in a suitable container.
- Add 9 mg bromphenol blue, 1.16 gm DTT (or 2.4ml B-mercaptoethanol) and mix well.
- Add 4.5mL glycerol to the solution, mix well.
- Make up to a final volume of 15ml with dH₂O and mix again thoroughly
- Store at 4°C. Dilute to use.

1.2.29 8% Paraformaldehyde for Immunofluorescence

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

8% Paraformaldehyde for Immunofluorescence

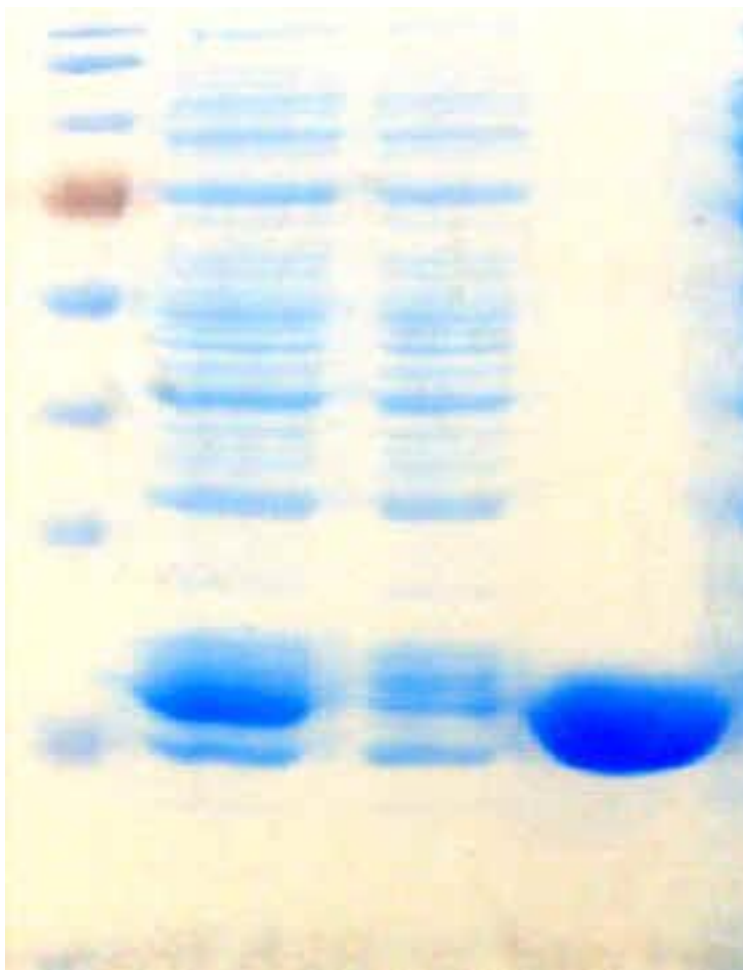
- Add 16g paraformaldehyde to approximately 160ml water in fume hood.
- Heating on setting 4.5 whilst stirring to 50°C.
- Once temperature reaches 50°C, 1M NaOH until the paraformaldehyde is just dissolved.
- Make up to final volume of 200ml with water, pH to 7.2-7.4.
- Filter with 0.22µm filter.
- Aliquot in 5ml tubes, label and freeze.

This method is based, with permission, on an original protocol available [here](#).

1.2.30 Colloidal Coomassie Blue Solution

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

Homemade Colloidal Coomassie Blue Staining



Adjust quantities as appropriate to the following %vol:

Distilled H₂O | ~650 ml Ammonium Sulfate | 10% | 100g Coomassie G-250 | 0.1% | 20 ml of % solution in H₂O Ortho-Phosphoric Acid | 3% | 30 ml Ethanol | 20% | 200 ml

Requirements

Distilled H₂O ~650 ml Ammonium Sulfate 100g Coomassie G-250 20 ml of 5% solution in H₂O Ortho-Phosphoric Acid 30 ml Ethanol 200 ml

Method

- In a large beaker add the ingredient in the order listed. Make sure the Ammonium Sulfate is dissolved before adding the Coomassie G-250 solution to a final of 0.1%.
- Bring final volume up to 1L with H₂O.
- Store at room temperature with a tightly sealed lid.

This method is based, with permission, on an original protocol available [here](#).

1.2.31 Colloidal Coomassie Blue Stain

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

Colloidal Coomassie Blue Stain for SDS-PAGE gel

- After SDS PAGE rinse Gel twice with 100 ml Distilled H₂O (3 minutes each wash).
- Add Coomassie Blue working solution to Gel and stain for at least 2 hours.
- Bands should be visible without de-staining but you can de-stain with H₂O.

References

Neumann U, Khalaf H, Rimpler M Quantitation of electrophoretically separated proteins in the submicrogram range by dye elution. *Electrophoresis* (1994) [pmid:7529171](#)

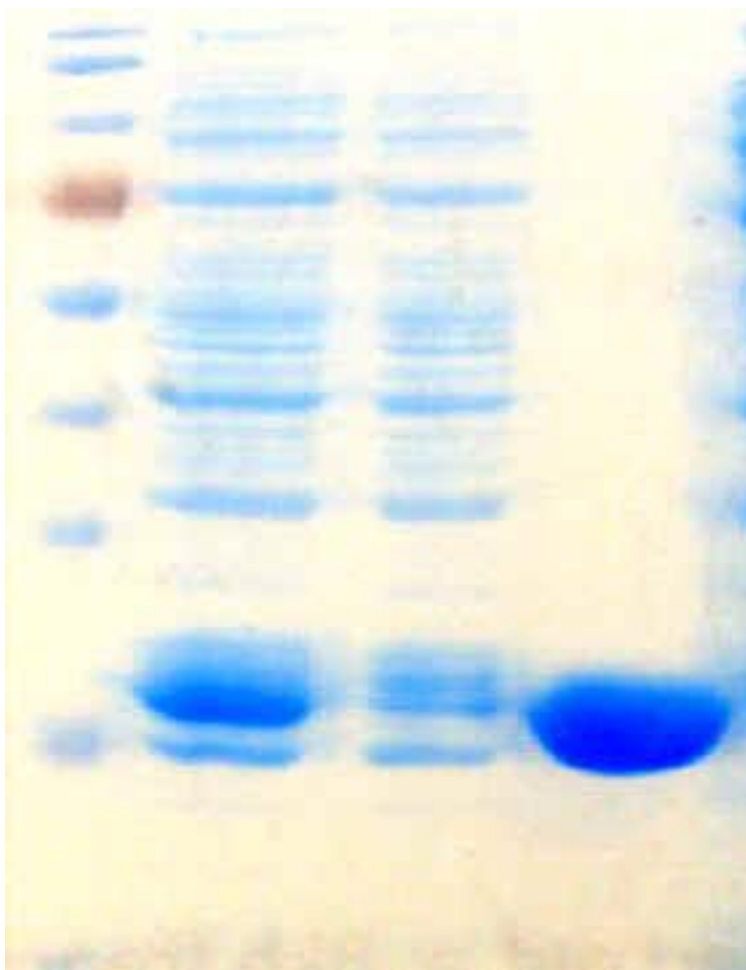
1.2.32 High Salt Buffer

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

High Salt Buffer

Requirements

120 ml of 5 M NaCl Stock (0.6 M NaCl) 4 ml of 1 M MgSO₄ Stock (4 mM MgSO₄) 2 ml of 0.5 M EDTA (1mM EDTA) 10 ml of 1 M HEPES Stock (10 mM HEPES)



Method

- Combine ingredients in suitable container. Add distilled water to 900 ml.
- Adjust pH to 7.0
- Add distilled water to final volume of 1 liter. Store until required.

Store at 4oC

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.33 Homogenization Buffer

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Homogenization Buffer

Requirements

300 ml PM buffer 52.3 mg PMSF (1mM PMSF) 3 g leupeptin (10 mg/ml leupeptin) 300 mg pepstatin A (1 ug/ml pepstatin A) 3 mg TAME (10 ug/ml TAME)

Method

- Combine ingredients in suitable container. Mix well to dissolve.

Use immediately.

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.34 Isolation Buffer

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Isolation Buffer

Requirements

20 ml 5 M NaCl Stock (0.1 M NaCl) 4 ml of 1 M MgSO₄ Stock (4 mM MgSO₄) 2 ml of 0.5 M EDTA (1mM EDTA)
10 ml of 1 M HEPES Stock (10 mM HEPES) Distilled water

Method

- Combine ingredients in suitable container. Add distilled water to 900 ml.
- Adjust pH to 7.0
- Add further distilled water to final volume of 1 litre. Store until required.

Store at 4oC

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays](#) (2001) 10.1002/0471143030.cb1301s00

1.2.35 PBS (1x in 1L)

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

PBS (1x in 1L)

Requirements

8.18 g NaCl (140 mM NaCl) 0.186 g KCl (2.5 mM KCl) 0.218 g KH₂PO₄ (1.6 mM KH₂PO₄) 2.15 g Na₂HPO₄ (15 mM Na₂HPO₄)

Distilled water to 1 liter

Method

- Combine ingredients in a suitable container. Add distilled water to 1L

Store at room temperature.

1.2.36 PBS (5x in 500 mls)

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

PBS (5x in 500 mls)

Requirements

20.45 g NaCl 0.465 g KCl 10.142 g Na₂HPO₄*7 H₂O 0.545 g KH₂PO₄

Method

- Add 500ml distilled water to a suitable container.
- Add ingredients to water and pH to 7.2

This method is based, with permission, on an original protocol available [here](#).



1.2.37 PHEM (500 mls) 2x

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

PHEM (500 mls) 2x

Requirements

18.14 g Pipes 6.5 g Hepes 3.8 g EGTA 0.99 g MgSO₄

Method

- Combine in beaker. Bring pH to pH7.0 with addition of KOH.

This method is based, with permission, on an original protocol available [here](#).

1.2.38 PLC buffer for Immunoprecipitation

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

PLC buffer for Immunoprecipitation

Requirements

50 mM Hepes 150 mM NaCl 10% glycerol 1% Triton X-100 1 mM PMSF 1.5 mM MgCl₂ 1 mM EGTA 10 mM NaPPi 10 mM NaF

Inhibitors: 1 mM sodium orthovanadate 10 mg/ml leupeptin 2 mM benzamidine

Method

- Combine the ingredients in a suitable container, and then add the inhibitors.

This method is based, with permission, on an original protocol available [here](#).

1.2.39 PM Buffer

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

PM Buffer

Requirements

100 ml 1 M PIPES (100 mM PIPES pH= 6.9) 2 ml 1 M MgSO₄, (2 mM MgSO₄) 2 ml 0.5 M EGTA (1 mM EGTA,) Distilled water

Method

- Combine ingredients in suitable container. Add distilled water to final volume of 900 ml
- Adjust pH to 6.9
- Distilled water to 1 liter. Store until required.

Store at 4oC

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.40 PMG Buffer

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

PMG Buffer

Requirements

80 ml 1 M PIPES Stock (80 mM PIPES) 2 ml 1 M MgSO₄ Stock, (2 mM MgSO₄) 2 ml 0.5 M EGTA Stock (1 mM EGTA,) 600 ml Glycerol Distilled water

Method

- Combine ingredients. Add distilled water to 900 ml, mix well
- Adjust pH to 6.9
- Add distilled water to 1 liter. Store for later use.

Store at 4oC

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays \(2001\)](#) 10.1002/0471143030.cb1301s00

1.2.41 Sodium Citrate Buffer

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

10mM Sodium Citrate, 0.05% Tween 20, pH 6.0

Requirements

Tri-sodium citrate (dihydrate) 2.94 g Distilled water 1000 ml

Method

- Mix to dissolve.
- Adjust pH to 6.0 with 1N HCl.
- Add 0.5 ml of Tween 20 and mix well.

This method is based, with permission, on an original protocol available [here](#).

1.3 Cell biology

Cell biology protocols.

1.3.1 Cell Culture Freezing Media

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Media for freezing of cells for culture.

Requirements

700ml RPMI (2mM L-Glutamine) 200ml FCS 100ml DMSO

Method

- Filter sterilize 700ml of RPMI-1640 (2mM L-Glutamine) and 200ml fetal calf serum (FCS) through a 0.22µm filter
- Add 100ml sterile dimethyl sulfoxide (DMSO)

Do not filter DMSO, it will dissolve the cellulose acetate membrane.

- Aliquot into tubes as appropriate

Store the tubes at -80oC for up to one year.

1.3.2 HL-60 differentiation to neutrophils/monocytes

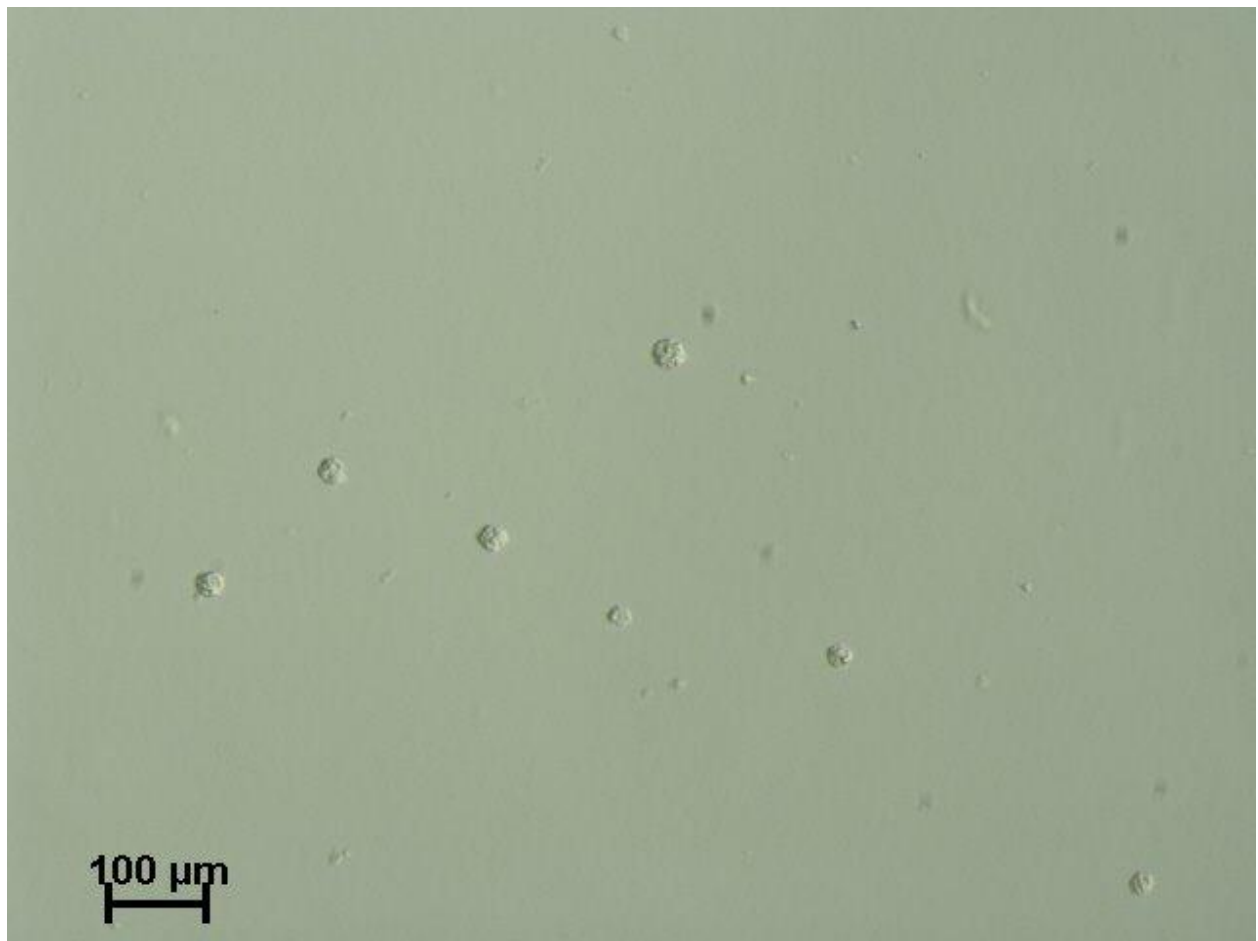
Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Differentiation of HL-60 to neutrophil or monocyte-like cells by Vitamin A (ATRA) and Vitamin D (1a,25(OH)2D3)

- Seed cells at 2.5x10⁵ cells/ml as 4ml cultures in 25cm² flasks.

If seeding to plates, seed to base-density of approximately 1x10⁶ cells/25cm² e.g. 1cm² well = 3.14cm² 25/3.14 = 8 1/8 = 0.125 x 10⁶ cells/well

- Differentiation can be induced with 100nM ATRA (neutrophils) or 100nM 1a,25(OH)2D3 (monocytes)
- Re-feed cells late on day 2 or morning day 3, ensuring stocks stay below 1x10⁶/ml to avoid spontaneous differentiation
- First signs of differentiation will appear day 3
- Complete differentiation should be apparent day 5



1.3.3 Restoration from DMSO cryopreservation

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Restoration of culture cells from storage at -80°C with 10% DMSO cryopreservation.



- For each preserved sample add 6ml media (RPMI+10% FCS) to a 15ml tube.
- Defrost frozen samples in water bath at 37°C. Rinse with EtOH to clean before transferring to tissue culture hood.
- Pasteur all cells from sample into 6ml media, transfer back and forth to rinse.
- Centrifuge 15ml tube at 1200rpm 6 minutes to pellet.
- Pour off supernatant and resuspend in 2ml media. Transfer into 25cm² flask.
- Incubate overnight.
- If cells are respiring effectively media will turn yellow. Add media up to 5ml to culture.

1.4 Histology

Histology protocols.

1.4.1 Acetic alcohol fixation

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Fixative for smears, cytopsin preparations and frozen sections

- Add 3ml glacial acetic acid to 100ml 95% methanol
- Fix section in solution for 1 minute
- Rinse section in distilled water prior to staining

This method is based, with permission, on an original protocol available [here](#).

1.4.2 Antibody diluent for IHC

Contributed by <jt03@ic.ac.uk>

Antibody diluent for IHC

Make sure you use good quality crystalline BSA e.g. fatty acid free from sigma.

Requirements

NaCl Proclin 300 RIA/IHC grade BSA 1M Tris-HCl pH 7.6

Method

- Add the following items to 90mls ultra pure water
 1. 870mg NaCl
 2. 50ul Proclin 300
 3. 1g BSA
 4. 5mls 1M Tris-HCL pH 7.6
- Make up to 100mls total volume with ultra pure water, stir until completely dissolved.
- Filter the solution through a sterile 0.2um filter, aliquot and store at -20C
- Once thawed solution is stable at 4C for at least a week.

It can also be used for long term storage (>6m) of antibody working solutions.

1.4.3 Bouin's fluid fixation

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Fixation of histological samples with Bouin's fluid

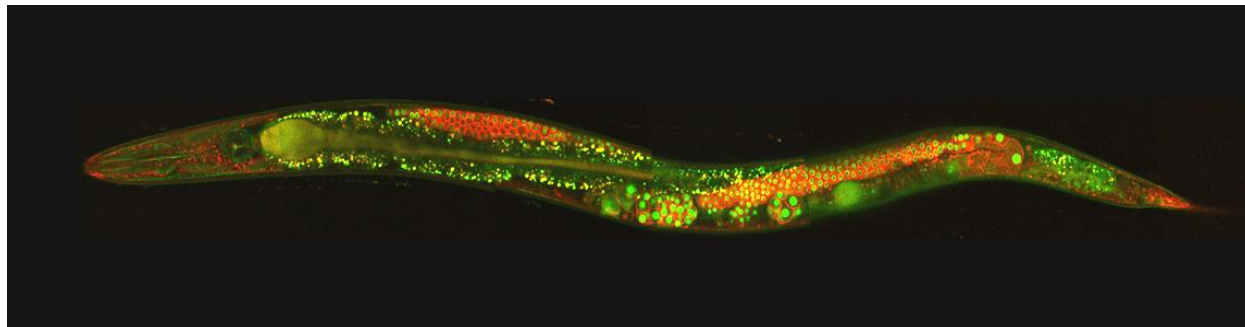
- Prepare 75ml saturated aqueous solution of picric acid
- Add 25ml formalin (40% aqueous solution of formaldehyde) to give 100ml total volume
- Add 5ml glacial acetic acid
- Fix tissue by submersion in Bouin's fluid for 6 hours
- Transfer fixed tissue to 70% alcohol

This method is based, with permission, on an original protocol available [here](#).

1.4.4 C. Elegans Embryo Staining

Contributed by Ian Chin-Sang, Queens University, ON, Canada

Staining protocol for C. Elegans embryos.



- Wash several plates of worms off with M9. Use microfuge tubes to spin worms. Wash 2X with 1ml M9 buffer. Pool worms into 1 or 2 microfuge tubes. Use the tubes that have siliconized to prevent loss of embryos.
- Add 1 ml of Bleach solution (1ml 10 N NaOH 8ml H₂O) to worm pellet. Vortex occasionally and let sit for 3min.
- Spin embryos at full speed for 30 sec. Aspirate off bleach solution and add 1ml of new bleach solution. Again vortex and let set for 3min. Try not to exceed greater than 10 min in the bleach solution as the embryos tend to get damaged.
- Wash 3X with M9 buffer (1ml each time). Be careful not to loose the embryos. They tend to stick to the tube. If they are sticking to the side of the tube try spinning at 6000 rpm for 2 min.
- On the final spin aspirate off all but 30ul of the M9 buffer.
- Add 200 ul 2X witches brew (with 2% BME). Mix.
- Add 70ul 10% paraformaldehyde solution. Mix.
- Freeze immediately in liquid Nitrogen for 1 min. At this stages embryos may be kept at -80C for several weeks until ready to use.
- Thaw on ice for for at least 20 min.
- Wash 1X with Tris Triton Buffer (2min)
- Wash 2X with Antibody Buffer A (10min each wash)
- Add primary antibody (usually 1:100- 1:500 dilutions)
- Let sit for 4 hours or overnight without rocking.
- Wash 4X with Antibody Buffer B (1ml each with 10min between each wash).
- Add 500 ul of Antibody Buffer A 0.5 to 1ul of secondary antibody (ie. FITC anti-rabbit)
- Let sit for at leas 2 hours at room temperature in the dark.
- Wash 3X with Antibody Buffer B.
- Resuspend embryos in 10% glycerol (25-50 ul) plus anti-fading reagent.
- Put 5-10ul on a slide to visualize under the microscope.

This method is based, with permission, on an original protocol available [here](#).

1.4.5 Carnoy's fluid fixation

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Fixation of samples in Carnoy's fluid

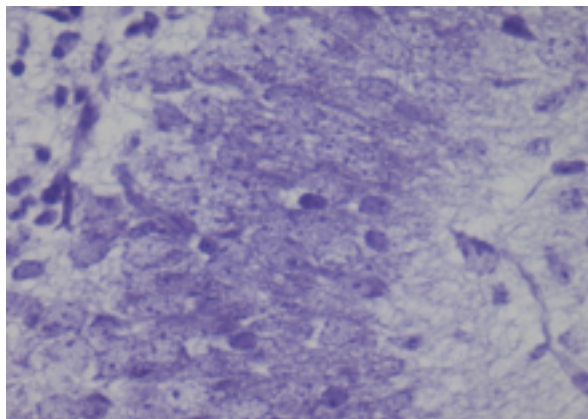
- In a fume hood pour 60ml of ethanol into a suitable container
- Add 30ml chloroform
- Add 10ml glacial acetic acid to give a total volume of 100ml
- Place tissue into fixative for 1-3 hours
- Process fixed tissues immediately or transfer to 80% alcohol for storage

This method is based, with permission, on an original protocol available [here](#).

1.4.6 Cresyl Violet Staining (Nissl Staining)

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

Cresyl Violet Staining for free floating sections, mounted and air-dried. Cresyl Violet Acetate solution is used to stain Nissl substance in the cytoplasm of neurons in paraformaldehyde or formalin-fixed tissue. The neuropil will be stained a granular purple-blue. This stain is commonly used to identify the neuronal structure in brain and spinal cord tissue.



The Cresyl Violet method uses basic aniline dye to stain RNA blue, and is used to highlight important structural features of neurons. The Nissl substance (rough endoplasmic reticulum) appears dark blue due to the staining of ribosomal RNA, giving the cytoplasm a mottled appearance. Individual granules of extra-nuclear RNA are named Nissl granules (ribosomes). DNA present in the nucleus stains a similar color.

The thicker sections require exposure to each solution for longer to allow the diffusion of reagents.

Requirements

95% Ethanol 70% Ethanol Differentiation solution: 2 drops glacial acetic acid in 95% ethanol Cresyl Violet Acetate 0.2% in Acetate Buffer (Fronine, Cat No: HH155)-Filtered

Method

- Wash slides briefly in tap water to remove any residual salts.
- Immerse slides through 2x3min changes of 100% ethanol.

- Defat the tissue: 15min in 100% xylene(2-3 changes as directed), then 10min in 100% ethanol.
- Rehydrate through alcohol (100% x2) 3min each.
- Wash in tap water.
- Stain in 0.1% Cresyl Violet 4-15min.
- Quick rinse in tap water to remove excess stain
- Wash in 70% ethanol (the stain will be removed by this method).
- If required immerse sections for 2min in Differentiation solution – check staining on microscope.
- Dehydrate through 2x3min changes of absolute ethanol.
- Clear in xylene x2 and mount in DePeX. Allow dry in the fume hood.

This method is based, with permission, on an original protocol available [here](#).

1.4.7 Dewaxing Paraffin Sections

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

Dewaxing Paraffin Sections Sections must be free of wax to allow aqueous solutions to penetrate.

Cryostat and free floating sections may need defatting prior to immunohistochemical staining. This must be optimised by the user

Method

- Xylene x2 (2-3mins)
- Absolute ethanol x2 (2-3mins)
- Water wash.

This method is based, with permission, on an original protocol available [here](#).

1.4.8 Ethanol fixation for flow cytometry

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Fixation for flow cytometry with ethanol. This method is very simple, quick and allows samples to be stored for weeks at -20°C.

- From a 35mm-10cm plate harvest cells with trypsin into a 15ml tube.

Keep media as this helps to inactivate trypsin and many mitotic and dead cells will be floating in the media

- Pre-chill 100% -20°C high grade Ethanol
- Centrifuge at low speed 1500rpm 10 minutes to pellet
- Remove supernatant media and resuspend in 1ml PBS
- Transfer to a 1.5ml eppendorf
- Centrifuge at 5000rpm for 2-5min to pellet
- IMPORTANT: Remove supernatant PBS and gently resuspend pellet in 300ul of PBS
- Add 700ul of 100% -20°C high grade Ethanol (EtOH) inverting tube a few times to ensure proper mixing and a good fixing of cells.

- Store at -20°C until required for flow cytometry/FACS

This method is based, with permission, on an original protocol available [here](#).

1.4.9 Fixation with paraformaldehyde

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Fixation with paraformaldehyde

- Add 41.5ml 2.26% sodium dihydrogen orthophosphate to a suitable heat-proof container
- Add 8.5ml sodium hydroxide 2.52%
- Heat to $60-80^{\circ}\text{C}$ in a covered container
- Add 2g 'Analar' paraformaldehyde, stirring thoroughly until dissolved.

Adding a couple of drops of 1M NaOH can help the PFA dissolve.

- Allow to cool and filter to remove particulates. Recheck pH.
- Store at RT and ideally use within 48h.

This method is based, with permission, on an original protocol available [here](#).

1.4.10 Fixing/pelleting chromatin for immunofluorescence

Contributed by [Timothy Mitchison](#), Harvard Medical School, Boston, MA, United States

Fixing and pelleting chromatin/nuclei from extracts onto coverslips for immunofluorescence.

Most protocols call for -20°C Methanol treatment of the coverslip post sedimentation. This is generally used to stick the sedimented object to the coverslip and prevent it from floating away during processing and is also thought to help antigenicity by exposing epitopes. The use of polylysine coated coverslips eliminates the first problem and as to the effect of methanol on antigenicity I don't have an opinion. Methanol sometimes drastically affects (negatively) the morphology of chromosomes and, therefore, I have stopped using it.

This protocol is optimized for mitotic chromatin but works for nuclei as well. The final structure is affected slightly by increasing magnesium concentration or adding polyamines to the fixation buffer. The buffer we use works reasonably well and therefore I haven't changed it much aside from occasionally using 4 mM Magnesium rather than 2 mM.

Some epitopes may be sensitive to formaldehyde and, therefore, lack of any signal may require more fiddling with the fixation conditions.

Frog extracts have huge pools of biotin and it is generally a good idea to avoid avidin-biotin detection systems. I highly recommend labeled donkey secondary antibodies from Jackson Immunoresearch for immunofluorescence.

Requirements

2X XBE2 (10 mM HEPES, pH7.7 (@ 10 mM), 100 mM KCl, 0.1 mM CaCl_2 , 2 mM MgCl_2 , 5 mM EGTA, 50 mM sucrose). 16 % formaldehyde (Methanol-free from Ted Pella; used for two weeks after opening a sealed vial) OR paraformaldehyde (freshly prepared) : will be used at 1% final. Poly-lysine coated coverslips. Spin down tubes (or equivalent method to pellet onto coverslips; our tubes are modified 15 ml Corex tubes). Extract fix : 1ug/ml Hoechst in 1X MMR, 50% glycerol, 10% formaldehyde (from 37% stock sold by Fisher) OR 4.5% Ted Pella MeOH-free formaldehyde.

Method

- Squash 1 ul of extract with 3 ul of Extract fix prior to beginning fixation to compare the morphology post fixation.
- To 10 ul of extract with chromatin/nuclei in extract gently add 200 ul of 1% MeOH-free formaldehyde (or 1% paraformaldehyde) in XBE4. Immediately use a cutoff pipet tip to gently mix the fix with the extract by pipeting up and down 5 times. (If this is not done promptly one often gets aggregation).

*The 1% formaldehyde in XBE2 should be made up just before use (~5'-10'). Titration of formaldehyde 1% - 4% doesn't show much difference in morphology; however, higher formaldehyde concentrations generally reduce anti-genicity. If extracts contain membranes then 0.5% Triton addition to the fix reduces particulate debris which tends to bind antibodies. *

- Incubate at RT for 12' - 15'.
- During incubation setup spindown tubes. Each tube contains a polylysine coated coverslip and is loaded with a cold 5 ml cushion of 30% glycerol (v/v) in XBE2 and stored on ice.
- After incubation in formaldehyde, gently layer the fixed sample onto the cushion using a cutoff pipet tip.
- Spin in a swinging bucket rotor (I use a Sorvall HB-4 or Beckman JS13.1) at 10,000 rpm for 10'.
- Aspirate half the cushion and then rinse the interface with a few mls of XBE2 before aspirating the rest.
- Remove the coverslip and after rinsing with TBS Tx/PBS Tx, block and process for immunofluorescence. I use 1 ug/ml Hoechst to stain the DNA just before mounting.
- Compare the fixed samples to nuclei squashed in Extract fix before fixation to determine if the fixation worked as desired.

This method is based, with permission, on an original protocol available [here](#).

1.4.11 Formaldehyde fixation for flow cytometry

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Alternative fixation procedure for FACS fixation, using formaldehyde fixative.

- Fix cells with 2% fresh formaldehyde in PBS for 5-15min, gentle rotation may help to improve fixation
- Wash cells 2-3 times with PBS to remove all of the formaldehyde
- Resuspend cells in 300ul of PBS
- Add 700ul of 100% -20°C EtOH to permeabilize the cells - they can now optionally be stored for weeks at -20°C.
- Permeabilize cells with 0.1% TritonX-100 in PBS for 15min

This method is based, with permission, on an original protocol available [here](#).

1.4.12 Formol sublimate fixation

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Fixation in formol sublimate

- In a fume hood pour 100ml formalin (40% aqueous solution of formaldehyde) into a suitable container
- Add 900ml saturated aqueous mercuric chloride

- Submerge tissue and fix for 4-6 hours
- Remove fixed tissue and transfer to 80% alcohol to store

This method is based, with permission, on an original protocol available [here](#).

1.4.13 Haematoxylin and Eosin Staining

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

Haematoxylin and eosin stain is used to stain cell nuclei blue and the cytoplasm pink/red to aid visualization of tissue structure and morphology. The staining method involves application of the basic dye haematoxylin, which stains basophilic structures, usually the ones containing nucleic acids, such as the ribosomes and the chromatin and alcohol-based acidic eosin Y, which colors eosinophilic structures, generally composed of intracellular or extracellular protein, bright pink to yellow.

Thicker sections require exposure to each solution for longer to allow the diffusion of reagents.

Requirements

Absolute alcohol Xylene 0.3% Acid Alcohol (0.3% HCl in 70% ethanol) Distilled water. Mayer's Haematoxylin (Fronine, # II007) 1% alcoholic eosin (Fronine # 11016Q) DePeX

Method

- Dewax sections in xylene (2 or 3 changes of 3minutes each)
- Rehydrate in alcohol (100%, 95%, 70%) 3minutes each.
- Stain in Mayer's haematoxylin 30sec. Watch how stain develops and check under microscope (nuclei should be blue with a pale blue tissue matrix)
- Wash and "blue" in running tap water.
- In the case of high background differentiate in acid alcohol 8-12 times briefly.
- Wash in tap water and re-blue and check under the microscope.
- Stain with the 1%eosin solution for 10seconds.
- Rinse in tap water to remove the excess stain.
- Wash in 70% ethanol and check microscopically until the tissue matrix and cellular components are differentially stained.
- Rapidly dehydrate the sections through graded ethanol clear in xylene and mount in DePeX.

This method is based, with permission, on an original protocol available [here](#).

1.4.14 Helly's fluid fixation

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Fixation using Helly's fluid

- To prepare stock solution add 1l distilled water to suitable container.
- Add 25g potassium dichromate.
- Add 50g mercuric chloride.

- Add 10g sodium sulphate and mix thoroughly. Store stock solution until required.
- Make up fixative just before use.

Add 5ml formalin (40% aqueous solution of formaldehyde) to 100ml stock solution

- Fix tissues for 12-24 hours
- Wash fixed tissue for 24hours prior to processing

This method is based, with permission, on an original protocol available [here](#).

1.4.15 Immunohistochemistry (cultured cells/monolayers)

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

Immunohistochemistry (cultured cells and monolayers)

This protocol outlines a general method for immunohistochemical staining and can be adjusted to suit the antibodies used.

Requirements

Fixative Use 4% PFA (sometimes kept as frozen 8% stock – add 2xPBS buffer to make up to 4%) Blocking Buffer 0.5% BSA/PBS (e.g. 0.5g BSA in 100ml PBS) (% = g/100ml) Permeabilisation Buffer 0.1% TX-100 in PBS (e.g. 10% Tx-100 stock then 200µl in 20ml PBS)

Method

- Fix cells: 4% paraformaldehyde in PBS, pH7.4 for 30-90 mins RT
- Wash gently x3 PBS
- Permeabilise cells with 0.1% TX-100 in PBS for 5mins
- Wash 2x in PBS
- Block for 10min in 0.5% BSA/PBS (but can be as long as you like)
- While blocking should make up primary antibody, dilute antibody in BSA/PBS, need 25-40ul/coverslip

E.g. Antibody 1:50 = 2ul in 100ul BSA/PBS

- Aliquot onto parafilm with sufficient space between coverslips
- Pick up coverslip and dry edges gently (touch corner to kimwipe) and place coverslip cells down onto antibody drop. Incubate for 60-90min RT
- Flip coverslip so cells face up and wash gently with BSA/PBS x 3
- Prepare secondary antibody (25-45ul/coverslip) and incubate for 45-60min

Many 2° Ab are used 1:400 (e.g. 1ul per 400ul) in BSA/PBS

- Wash x3 BSA/PBS
- Wash x3 PBS

This method is based, with permission, on an original protocol available [here](#).

1.4.16 Immunohistochemistry with TSA

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

Immunohistochemistry with Tyramide Signal Amplification (TSA) using Perkin Elmer kit & Protocol for Vibratome Sections

Perform all steps with slides arrayed horizontally in humidified chamber on a rotator at RT.

Requirements

Sodium citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20 pH 6) 2.94g Tri-sodium citrate // 1000ml DI water // adjust pH to 6.0 with 1N CL // add tween 20 // mix well // store at room temperature for 3months or at 4°C for longer storage 50 ml of 1xPBS pH 7.4 50ml of 1% BSA/PBS

Method

- Mount sections on SuperFrost Plus slides – dry for ~1hr to fully adhere sections to slide
- Rinse slide with PBS to rehydrate
- Fix in 4% PFA for 10min
- Wash 3x 3min in PBS
- Perform antigen retrieval in Biocare Medical decloaking chamber – heat sections to 125oC for 4min at 15 psi in sodium citrate buffer
- Wash 3x 3min in PBS
- Block sections in 0.9% H2O2/10% Normal Serum/0.2% Triton-X 100 in PBS for 2hr on rotator (serum should be same as the species from which your secondary antibody was generated)
- Incubate overnight with 1o antibody/ies at desired concentration in 2% Normal Serum/0.2% Triton-X 100 in PBS
- Wash 3x20min in PBS
- Incubate with appropriate HRP labelled IgG 2o antibody diluted 1:500 in 0.2% Triton-X 100 in PBS for 1hr
- Wash 3x10min in PBS
- Dilute TSA Amplification reagent 1:50 in 1xPlus Amplification Diluent buffer.
- Apply 150-200µL per slide and incubate for 1 – 10mins (need to optimize for individual antibodies) in humidified chamber covered in foil. For all subsequent steps, keep chamber covered in foil
- Wash 3x10min in PBS

If performing double labelling for multiple primary antibodies proceed to step 15. If only performing single labelling with TSA kit proceed to step 16 Incubate with appropriate fluorescent 2o antibody diluted in 0.2% Triton-X 100 in PBS for 3hr.

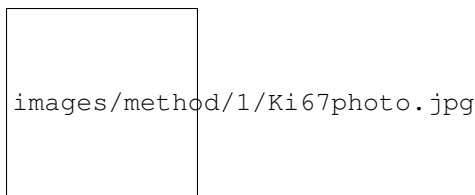
- Incubate in DAPI diluted 1:1000 in 0.2% Triton-X 100 in PBS for 20min at RT.
- Wash 3x20min PBS. NB: If using 2o antibodies at high concentrations (i.e. greater than 1:500) replace first two washes with 0.2% Triton-X 100 in PBS.
- Coverslip using ProlongGold (Invitrogen). Let mounting media set for 1hr at RT in slide book, then store at 4oC in dark. Take images within 2-3 weeks for optimum fluorescence.

This method is based, with permission, on an original protocol available [here](#).

1.4.17 Immunohistochemistry

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Basic outline protocol for immunohistochemistry on unfixed cytotast-thin tissue sections



Requirements

50 ml of 1xPBS pH 7.4 50ml of 1% BSA/PBS Primary antibody: As recommended by manufacturer Secondary antibody: As recommended by manufacturer – Alexa dyes usually work best at 1:400

Method

- Cryostat sections are picked up on subbed slides (chrome-alum gelatin coated) and air dried for at least 2 hours. Alternatively Superfrost or Poly-L-Lysine coated slides can be used.

- Draw a circle around section with hydrophobic pen.
- Wash sections with PBS 3 times
- Incubate twice in 100% Ethanol for 2 minutes each.

Incubation for 20 min in PBS + 4% formaldehyde can be used as an alternative fixative.

- Block with BSA/PBS for 20 minutes.
- Add 1o antibody for 1 hour.
- Wash with BSA/PBS 3 times.
- Add 2o Ab for 1hr
- Wash with BSA/PBS 3 times
- Wash with PBS 3 times.
- A counterstain (e.g. DAPI or PI) may be added for a few minutes, followed by PBS washing.
- Mount with aqueous based fluorescent mounting medium (mowiol / prolong gold), dry edges, seal with nail polish.

This method is based, with permission, on an original protocol available [here](#).

1.4.18 Immunostaining of Cells Adherent to Coverslips

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States

Immunostaining of Cells Adherent to Coverslips

Requirements

1L PBS-T (1000 ml) 8gm NaCl 0.2gm KH₂PO₄ 1.15gm Na₂HPO₄ 0.2gm KCl 1ml Tween 20 0.1gm Thimerosol

Method

- Immerse 18 mm² glass coverslips in EtOH. In the tissue culture hood, individually pull out and carefully flame to sterilize.
- Allow to cool then place in 35 mm dishes, or in 6 well plates. Coat overnight (4°C) with 3 ml/dish or well of 40 µg/ml BMS made up in ddH₂O.
- Remove coating solution and block with 1% BSA for 4 hr at 4°C.
- Isolate cells and plate at 6 x 10⁵ cells/ml (3 ml/well). Allow cells to adhere for 1 hr in incubator.
- Gently pull off media containing non-adherent cells and wash once with media.
- Pull off media and add 4% formaldehyde (25 ml of 16% ampule stock made up to 100 ml in PBS) to dishes or wells. Fix for 30 min at room temperature.
- Remove coverslips. Those for immunostaining are placed vertically in two ceramic coverslip holders (need to borrow from Otey lab until we purchase). The remainder can be stored at -70°C.
- Coverslips in ceramic holders are washed two times in PBS. For washes and incubations, use a 250 ml beaker and a 100 ml volume of wash or reagent. Permeabilize by immersion in PBS-Tween ('PBS-T'; PBS containing 0.1% Tween 20) for 15 min at room temperature.
- Wash four times over 5 min with PBS.
- Block by immersion in PBS containing 1% BSA for 60 min at room temperature
- Immerse one group of coverslips in preimmune sera and the other in immune sera diluted in PBS/1% BSA. Cover and incubate overnight at 4°C.
- The next day, wash five times over 40 min in PBS.
- Immerse in Pierce 'peroxidase suppressor' for 30 min at room temperature, then wash several times in PBS.
- Wash several times in PBS.
- Immerse in secondary peroxidase-labeled antibody diluted 1/1,000 in PBS containing 1% normal goat serum. Incubate for 60 min at room temp.
- The next day, wash five times over 40 min in PBS.
- Place coverslips flat on Parafilm and add Pierce 'metal enhanced DAB' diluted 1/10 in peroxide buffer. Allow reaction to go for 5 - 15 min, then replace in ceramic holders to wash two times in water.
- Dehydrate in 80%, 90% and 2x 100% EtOH, then immerse in xylene (in chemical hood; all 1 min each).
- Place cover slip cell layer down on a glass slide containing a drop of mounting medium and examine in the light microscope.

This method is based, with permission, on an original protocol available [here](#).

1.4.19 Immunostaining of Paraffin Sections

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States

Immunostaining of Paraffin Sections

- Fix tissues for 3 hr on ice in 4% formaldehyde (2.5 ml of Polysciences #18814 made up to 10 ml in 80 mM NaPO₄ [3.2 ml of 1 M NaPO₄] pH 6.8 containing 0.2 gm of sucrose).
- Wash for 2 hr in several changes of cool PBS.
- Infiltrate and embed in paraffin (use automatic processor). Embed in flat end of capped Beem capsules
- Prepare polylysine-coated slides by immersing clean slides, via a stainless steel slide rack, in 400 ml of 50 µg/ml polylysine (Sigma #P1524; make up in 10 mM Tris, pH 8) for 30 min.
- Cover with foil and let dry overnight at room temperature. Polylysine solution can be stored at -20°C and reused. Store coated slides at 4°C and use within 4 wks.
- Trim away excess paraffin so that block face is 2 - 3 mm².
- Set up on an ultramicrotome using glass knives or an old diamond knife with 'boat' filled with water. Rapidly cut away surface paraffin, then cut 1 - 2 µm sections.

*Sections cut easily and should form ribbons. *

- Pickup with the point of a 25 gauge needle and transfer to a drop of water on a polylysine-coated slide. Have two groups of sections per slide. Transfer slide to a 42°C warming plate and leave overnight.

Sections prepared in this manner can also be used for in situ hybridization

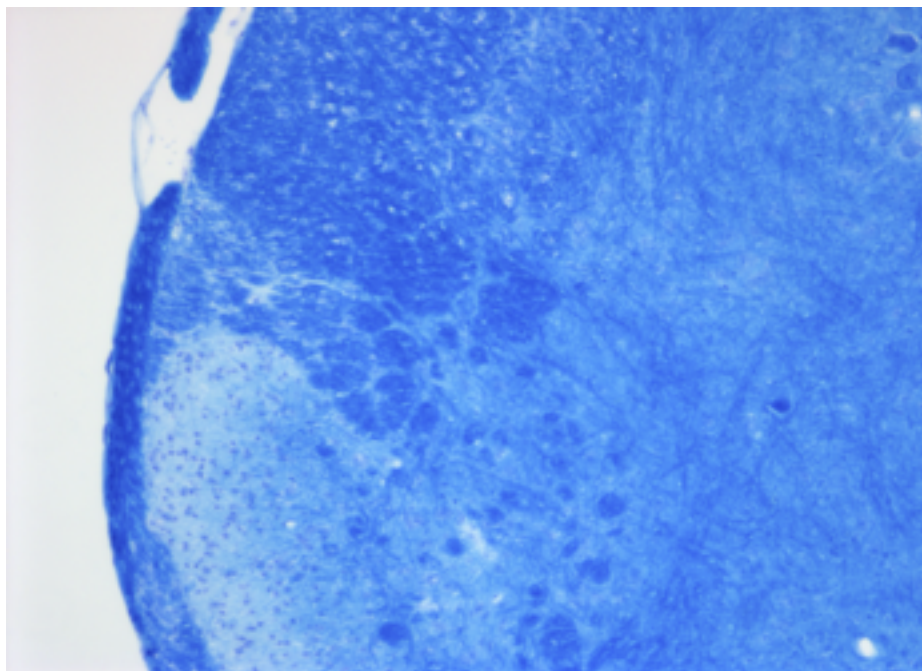
- Deparaffinize sections in xylene (2 x 10 min) using glass slide jars. Transfer to 100% EtOH (2 x 5 min), and then to PBS (2 x 5 min).
- Insert sections in Coplin jars containing 'TUF' (Target Unmasking Fluid; Signet Labs [Dedham MA] #1050) preheated to 80-90°C.
- Incubate in TUF for 10 min at 80-90°C.
- Let jar cool 5 - 10 min, then rinse two times in ddH₂O and two times in PBS.
- Place sections on a staining rack and block with PBS containing 1% BSA for 60 min at room temperature.
- Pour off, dry with kimwipe around sections. Add diluted (dilute in PBS containing 1% BSA; use 10 fold dilutions at start) antibody or preimmune control in one or two drops to sections
- Place slides in a closed 150 mm petri dish containing wet filter paper, and place overnight at 4°C.
- The next day, wash five times over 40 min in PBS containing 1% BSA.
- At this time, can immerse in Pierce 'peroxidase suppressor' (Pierce #35000) for 30 min at room temperature.
- Wash several times in PBS containing 1% BSA using squirt bottle to carefully wash around sections. Dry around sections, then add one or two drops of secondary peroxidase-labeled antibody diluted 1/1,000 in PBS containing 1% BSA.
- Incubate for 60 min at room temp on staining rack.
- Wash five times over 40 min in PBS using squirt bottle.
- Dry around sections, then add Pierce DAB metal concentrate (Pierce #1856090) diluted 1/10 in stable peroxide substrate buffer (Pierce #1855910).
- Allow reaction to go for 5 - 15 min, then wash with ddH₂O using squirt bottle.
- Dehydrate slides in 80%, 90% and 2x 100% EtOH (1 min each), then immerse in 2 changes of xylene (1 min each).
- Mount cover slips on the slides using Permount (Fisher) and examine in the light microscope.

This method is based, with permission, on an original protocol available [here](#).

1.4.20 Luxol Fast Blue staining

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

Luxol Fast Blue histological stain. This technique is a method requiring over-staining of tissues and differentiation to reveal the myelin. It is advisable to include a control showing de-myelination pathology to ensure the end point of staining is reached. Cresyl Fast Violet counter-stain is optional and results in neuronal piliform staining and a deepening of the myelin blue staining.



Cresyl Fast Violet counter-stain is optional and results in neuronal piliform staining and a deepening of the myelin blue staining.

Requirements

Mounted sections on glass slides Slide staining rack, Staining jars, Coplin jar Absolute alcohol Xylene Distilled water. DePeX Luxol Fast Blue (Fronine: FG080) Cresyl Fast Violet 0.2% (Fronine: HH155) Lithium Carbonate (Sigma Aldrich: 62470) 0.05% Lithium Carbonate(aq)

Method

- Heat the staining solution to 60°C in the incubator (this will take 30mins approximately depending on the volume to be heated).
- Dewax or defat sections prior to rehydration as necessary. Rinse in tap water.
- Immerse the slides in the LFB solution and incubate at 60°C for 4 hours.
- Rinse off excess stain in distilled water
- Differentiate slides in Lithium Carbonate for 30 seconds
- Rinse in distilled water.
- Check staining microscopically and repeat steps 4 to 5 until myelinated and unmyelinated regions are defined.

- Counterstain with Cresyl Fast Violet for 8mins
- Dehydrate through 95% ethanol and check the Cresyl Fast Violet staining at that point. (70% ethanol will continue to remove the LFB).
- Dehydrate through absolute ethanol, clear in xylene and mount in DPX

This method is based, with permission, on an original protocol available [here](#).

1.4.21 Michel's fixative for immunofluorescence

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Michel's fixative for immunofluorescence

- Pour 100mls distilled water into a suitable container
- Add 0.81g potassium citrate
- Add 0.0625g N-ethylmaleimide (Handle with care)
- Add 0.123g magnesium sulphate to prepare stock solution.
- Before use add 55g ammonium sulphate and mix thoroughly to dissolve.
- Adjust pH to 7.0-7.2 with 1M KOH.
- Place tissue in fixative for 24-48hours
- Wash tissues in buffer 3x for 10 minutes
- Freeze tissues at -70°C until ready for use

This method is based, with permission, on an original protocol available [here](#).

1.4.22 Mounting Media

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Mounting Media

Requirements

20mM Tris pH 8.0 0.5% N-propyl gallate 90% Glycerol

Method

- Combine ingredients in suitable container. Store for use.

Store at 4oC.

This method is based, with permission, on an original protocol available [here](#).

1.4.23 Neuron Recovery of Neurobiotin/biocytyn cells

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

Neuron recovery of neurobiotin/biocytyn filled neurons. The method may applied to free-floating or cryostat sectioned fixed tissue.

This technique results in a black insoluble reaction product delineating the filled neuron. Developing the chromagen is slow and should be monitored microscopically. The reaction is stopped before the background staining occurs. The labeled sections can then be counter stained and mounted permanently.

DAB is a carcinogen and all powder must be weighed in a fume hood with appropriate PPE. Disposal and deactivation of excess powder and contaminated washings with dilute bleach solution prior to disposal into the sink is mandatory. Nickel Sulfate must be disposed of appropriately.

Requirements

24 well plate Sable brush 1XPBS buffer PBS Tween Buffer: 0.05% Tween in 1XPBS Acetate buffer: 0.1M Sodium acetate trihydrate bring to pH to 6.0 with Glacial Acetic Acid NiSO₄ solution(0.13M) in Acetate buffer (40g Ni SO₄ in 2L of acetate buffer adjust to pH 6.0 with HCl) – Note: NaOH will produce a precipitate. D-glucose Ammonium Chloride Glucose Oxidase 30% H₂O₂ Fetal calf serum ABC vector elite kit PK6100 NiSO₄/DAB solution (dissolve DAB in approximately 250?l of water before adding to NiSO₄/acetate buffer)

Method

- Collect sections into multi well plate using sable-hair brush.
- Wash sections well in 3 x 5min of PBS-Tween
- Oxidise sections in PBS- H₂O₂ (750?l of 30% H₂O₂ in 25ml PBS) for 30min (this step quenches the endogenous peroxidase activity in the tissue.)
- Wash sections well in 5 x 5min of PBS.
- Incubate sections in blocking solution (25ml PBS + 0.5ml fetal calf serum) for 1 hour (this step blocks non-specific protein interactions.)
- During this time make ABC solution (6?l of part “A” and 6?l of part “B” per ml of solution required) or as stipulated by the manufacturer. Add total amount of A and B to 1/5 of the final volume of PBS and stir at room temp for at least 30min. Make up to the final volume with the remaining PBS just prior to use.
- Sections are incubated overnight in ABC solution at 4°C on a shaker.
- Wash sections well in 3 x 10min of PBS
- Wash sections 5min in acetate buffer
- Pre-incubate sections for 15min in NiSO₄/DAB solution without glucose oxidase.
- Incubate sections in fresh NiSO₄/DAB solution with glucose oxidase. Reaction can take up to 30min.
- To stop the reaction, wash quickly with acetate buffer.
- Wash again in acetate buffer for 5min to prevent background staining.
- Wash 3x5min PBS. An overnight wash can help reduce the background even further.
- Mount free floating sections onto chrome alum gelatin coated slides and dry.
- Counterstain, dehydrate, clear and mount in DPX.

References

Smith DW, Day TA Neurochemical identification of fos-positive neurons using two-colour immunoperoxidase staining. *J Neurosci Methods* (1993) pmid:8100600

1.4.24 Neutral buffered formalin fixation

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Fixation with neutral buffered formalin

- To produce 10L pour a base 1L distilled water into a suitable container.
- Add 40g sodium dihydrogen orthophosphate (monohydrate)
- Add 65g disodium hydrogen orthophosphate (anhydrous)
- Add 1L formaline (40% aqueous solution of formaldehyde)
- Add a further 8L water for use
- Immerse samples and fix for 12-24 hours
- Samples may be stored in this fixative if required

This method is based, with permission, on an original protocol available [here](#).

1.4.25 Nickel DAB (N-DAB) Labeling

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

Nickel DAB (N-DAB) Labeling for sections

Requirements

Triton-X 100 Buffer 0.2% in 2%normal serum/1x PBS buffer Primary antibody diluted as per manufacturers recommendation (titration may be necessary) Appropriate secondary antibody diluted in Triton buffer AB complex Sodium acetate 0.175M. Nickel sulphate 1.25g in 50ml Sodium acetate buffer 0.175M (Make up fresh) DAB solution 10mg DAB in 50ml Nickel Sulphate solution. Dissolve and filter before use. 1% bleach solution for DAB deactivation.

Method

- Wash section with 0.2% Triton-X 100 buffer
- Block section with Triton-X 100 0.2% normal serum -1hour
- Remove blocking solution and incubate the section in primary antibody overnight at 40C
- Wash in 1x PBS (3x 10minutes)
- Incubate sections with secondary antibody 1hour
- Wash in 1x PBS (3x 10minutes)
- Incubate in AB complex as per manufacturer's instructions
- Wash in 1x PBS (3x 10minutes)
- Incubate in DAB solution for 5minutes

- Pour off the DAB solution and add 5ul of 30% Hydrogen Peroxide to this solution, mix and re incubate the sections. Check the sections microscopically and wash in PBS to stop the reaction once the desired staining intensity is reached.
- Counterstain if necessary using a contrasting histochemical stain such as Nuclear Fast Red(note: Haematoxylin counterstain is effective only with a brown DAB reaction product)
- Prepare sections for the microscope by dehydrating clearing and mounting in DPX
- Decontaminate any DAB affected surfaces and glassware with bleach and dispose of waste as per local regulations.

This method is based, with permission, on an original protocol available [here](#).

1.4.26 Preparation for microdissection (Paraffin/Frozen)

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Basic protocol for Eosin & Haematoxylin histological preparation of microdissection slide.

- For paraffin-embedded sections, place slides into fresh xylene to remove paraffin.
- Place slide into fresh xylene
- Transfer slide to 100% ethanol, 95% ethanol, then 70% ethanol; 30 seconds/step
- Transfer slide into de-ionized water
- Transfer slide into Mayer's Haematoxylin
- Return to de-ionized water to rinse for 15 seconds x2 changing dH2O between steps
- Transfer slide to 70% ethanol
- Transfer slide into Eosin Y
- Return to de-ionized water to rinse for 30 seconds x2
- To reduce brittleness of tissue, place in 3% glycerol in de-ionized water
- Shake and air dry to remove layer of glycerol and water
- Microdissection is best performed in the following 5-10 minutes following preparation. If if tissue dries return tissue to 3% glycerol/water; step #10

1.4.27 Silane/acetone silanized slides

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Silanized (3-aminopropyltriethoxysilane) slides to prevent sections from detaching.

- Place slides in 2% silane/acetone solution for 1 minute
- Transfer slides to 100% acetone for 1 minute
- Transfer slides to double distilled water. Agitate for 1 minute
- Replace double distilled water, and wash repeat x2 for 1 minute
- Leave slides to air dry overnight at room temperature
- Slides may be kept indefinitely covered in slide box until ready for use

1.4.28 Tissue Preparation for Vibratome Sectioning

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

Tissue preparation for vibratome sectioning.

- Fix in 4% paraformaldehyde in 0.1 M PBS pH 7.2-7.4.
- Remove excess fixative by washing tissues in PBS x 3.
- Embed in warm 40°C 4% agarose in distilled water (dH2O).
- Cool tissue blocks to 25°C or until the agarose solidifies.
- Trim excess agarose away and attach blocks to metal chuck using cyanoacrylate adhesive.
- Cut sections to 30-50µm Vibratome sections.
- Collect sections in 0.1M PBS (add azide 0.05% for long term storage>1 week).

This method is based, with permission, on an original protocol available [here](#).

1.4.29 Zenker's fluid fixation

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Fixation with Zenker's fluid

- Add 950ml distilled water to a suitable container
- Add 25g potassium dichromate
- Add 50g mercuric chloride
- Add 50g glacial acetic acid
- Mix thoroughly to dissolve
- Fix samples for 4-24 hours
- Following fixation wash samples overnight in running tap water prior to processing

This method is based, with permission, on an original protocol available [here](#).

1.5 Imaging

Imaging protocols.

1.5.1 Animal Trans-cardial Perfusion Technique

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

Animal Trans-cardial Perfusion Technique

Pre requisites:

- Animal ethics approval
- Training in the safe handling of laboratory animals
- Training in animal perfusion

Requirements

Pre-perfusion buffer (for washing to clear blood vessels and open up the capillaries) Sodium Nitrite 0.01% in PBS pH 7.4 Fixative 4% Paraformaldehyde +/- 0.25% Acrolein (other fixatives can be used here) Anesthetic see “NHMRC Guidelines to promote the well-being of animals used for scientific purposes”

Method

- Set up the perfusion rig with fresh filtered solutions each time and ensure that all tubing is clean and free of blockages.
- Anesthetise the animal checking the foot pinch reflex to make sure it is fully anesthetised.
- Secure the animal on its back.
- Cut open the pericardium
- Pull the heart down to reveal the ascending aorta and insert the catheter into the ascending aorta.
- Clamp the heart at right angles to the catheter.
- Cut the right atrium.
- Make sure the pericardium does not occlude the venous outflow.

The principle of perfusion is to start at a rate equivalent to cardiac output.

- Begin the perfusion by running the wash solution through to open the capillaries and wash the erythrocytes out of the tissue.
- Once cleared switch to the fixative.
- The run through time for the fixative should be 20 to 30 mins.
- Dissect out the brain and post fix for 24 hr (this may vary depending on the sensitivity of the antigen).

This method is based, with permission, on an original protocol available [here](#).

1.5.2 Dot Blot Analysis with ImageJ

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Analyzing a dot blot in ImageJ by background subtraction and measuring the integrated density of each dot.

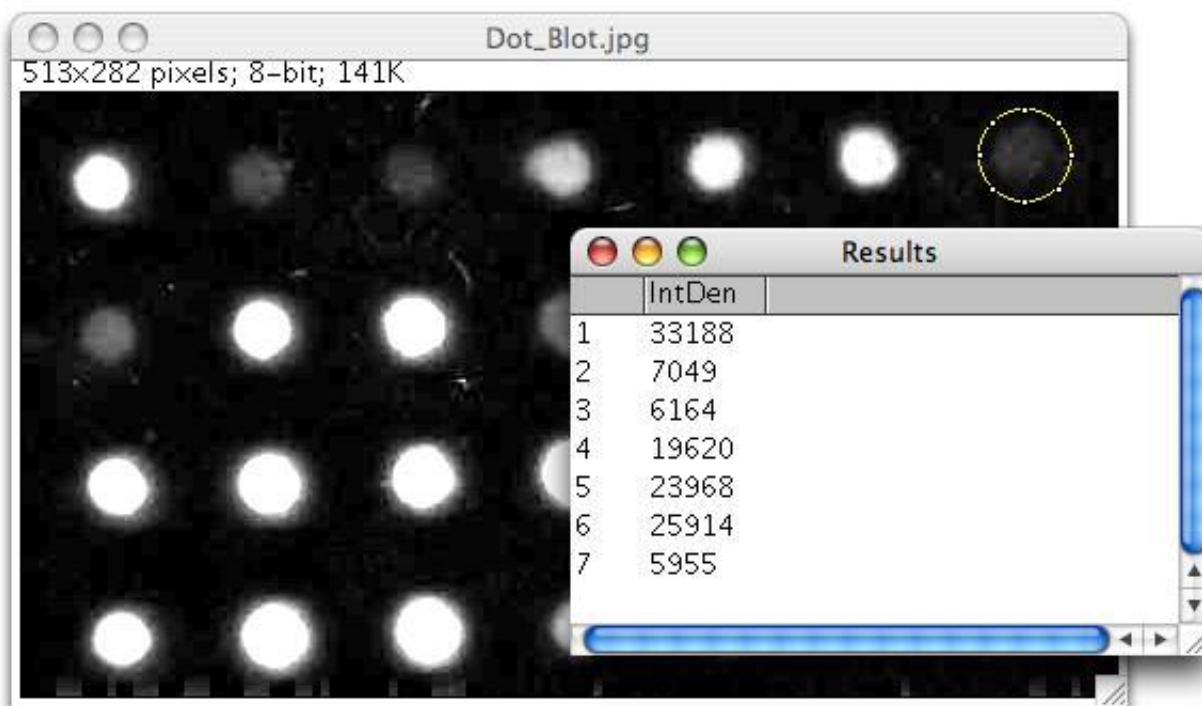
This dot blot image is available in the File/Open Samples menu in ImageJ 1.33s or later.

Method

- This method usually requires background correction of the image, which can be done using the Process/Subtract Background command.

*See image for profile plots (Analyze/Plot Profile) of the first row of dots before and after background correction was done using the Subtract Background command with the rolling ball radius set to 25 pixels. *

- After correcting the background, enable “Integrated Density” in Analyze/Set Measurements, create a circular selection, drag it over the first dot, press “m” (Analyze/Measure), then repeat for the other 27 dots.



*Notice how the image now has a black background? It was inverted (Edit/Invert) so background pixel values are near zero, which is required for correct calculation of integrated density. You can invert the lookup table (Image/Lookup Tables/Invert LUT) to restore the original appearance of the image. The “Use Inverting Lookup Table” option in Edit/Options/Image will invert the pixel data and invert the lookup table. *

This method is based, with permission, on an original protocol available [here](#).

1.5.3 Mowiol (Aqueous mounting media)

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

Aqueous mounting medium for fluorescence and Stereology. Recommended Anti-Fade for fluorescence microscopy – especially good for EGFP and other low expression reporting molecules.

Mowiol goes into solution with difficulty. It’s best to make a large batch and freeze aliquots at -20°C.

Takes about 8 hours to prepare correctly.

Note for stereology: if you have sections mounted in DPX – remove the DPX by soaking and washing in zylene, rehydrate in PBS and Azide (for 1-2 nights) then mount in Mowiol

Method

- Add 24g glycerol (analytical grade) to clean 250 ml flask or beaker
- Add 9.6g Mowiol 4-88 (Fluka, #81381 – through Sigma) and stir thoroughly to mix
- Add 24ml UHP distilled water and leave for 2hr at room temperature
- Add 48ml 0.2M Tris-HCl buffer, pH8.5

Final volume will be 200 mls

- Stir with a clean stir bar on a hot plate on warm – heat gently to 50°C stirring continually – at least 4-5 hours until the majority of the Mowiol powder goes into solution
- Aliquot into 50 ml centrifuge tubes, weigh and balance
- Centrifuge at 5000g for 15 minutes, there will be a pellet at the base, carefully remove supernatant
- Aliquot into 15 ml conical tubes – add only 10 mls for expansion
- Store at -20°C for 12 months – store at room temperature no more than one month

This method is based, with permission, on an original protocol available [here](#).

1.5.4 DAPI counterstain adherent cells/sections

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

DAPI counterstain of adherent cells or tissue sections on slides

- Perform all fixation, permeabilization and primary staining of your sample before applying the DAPI counterstain. Fixation or permeabilization is not required to stain with DAPI.
- Wash and equilibrate section in PBS for 5 minutes
- Dilute stock DAPI to 300nM concentration in PBS
- Add 300ul of diluted DAPI per spot to the coverslip. Ensure cells/section is completely covered by the droplet.
- Incubate at room temperature for 1-5minutes
- Place sample in PBS bath, washing 3x for 5 minutes then replacing the PBS.
- Add coverslip with appropriate mounting medium

1.5.5 Measuring cell fluorescence using ImageJ

Determining the level of cellular fluorescence from fluorescence microscopy images in ImageJ

- Open ImageJ. Note: ImageJ may be freely downloaded from [\[here\]\(http://rsbweb.nih.gov/ij/download.html\)](http://rsbweb.nih.gov/ij/download.html)
- Select the cell of interest using any of the drawing/selection tools (i.e. rectangle, circle, polygon or freeform)
- From the Analyze menu select “set measurements”. Make sure you have **area integrated intensity** and **mean grey value** selected (the rest can be ignored).
- Now select “Measure” from the analyze menu. You should now see a popup box with a stack of values for that first cell.
- Now go and select a region next to your cell that has no fluorescence, this will be your background.

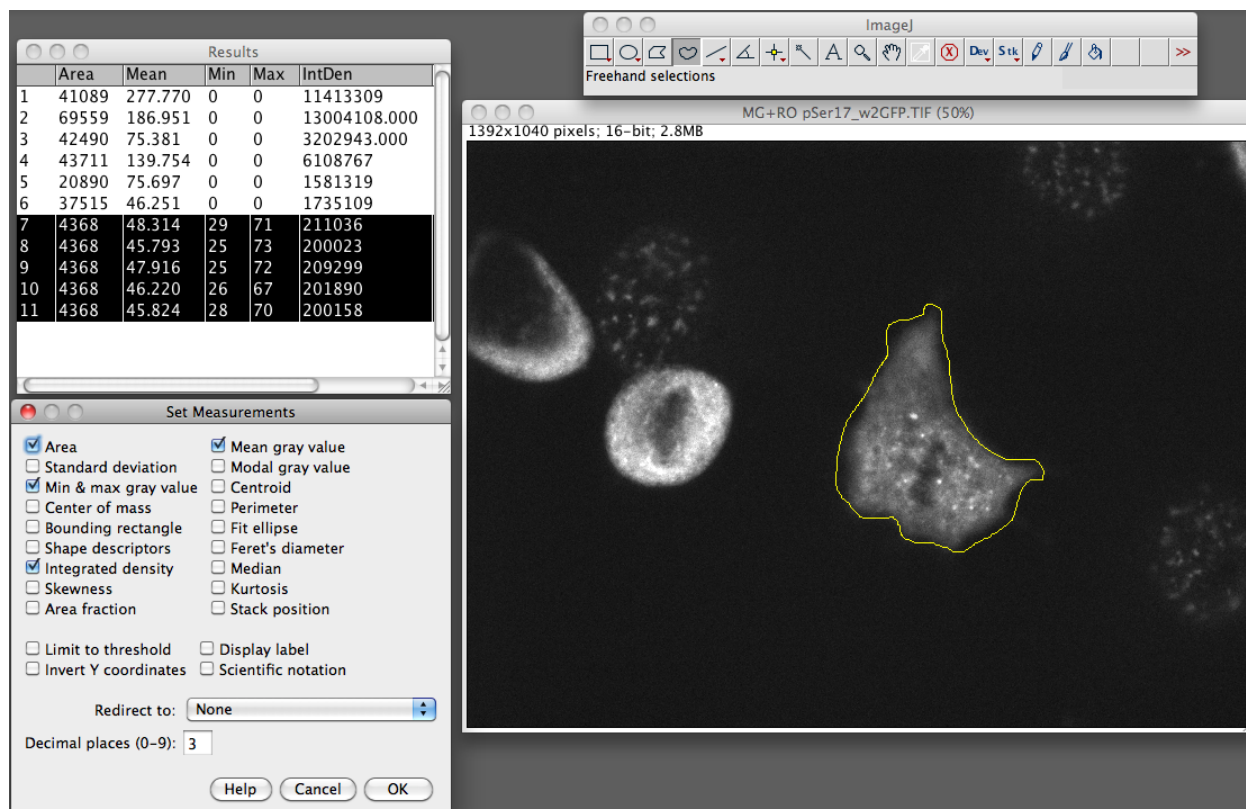
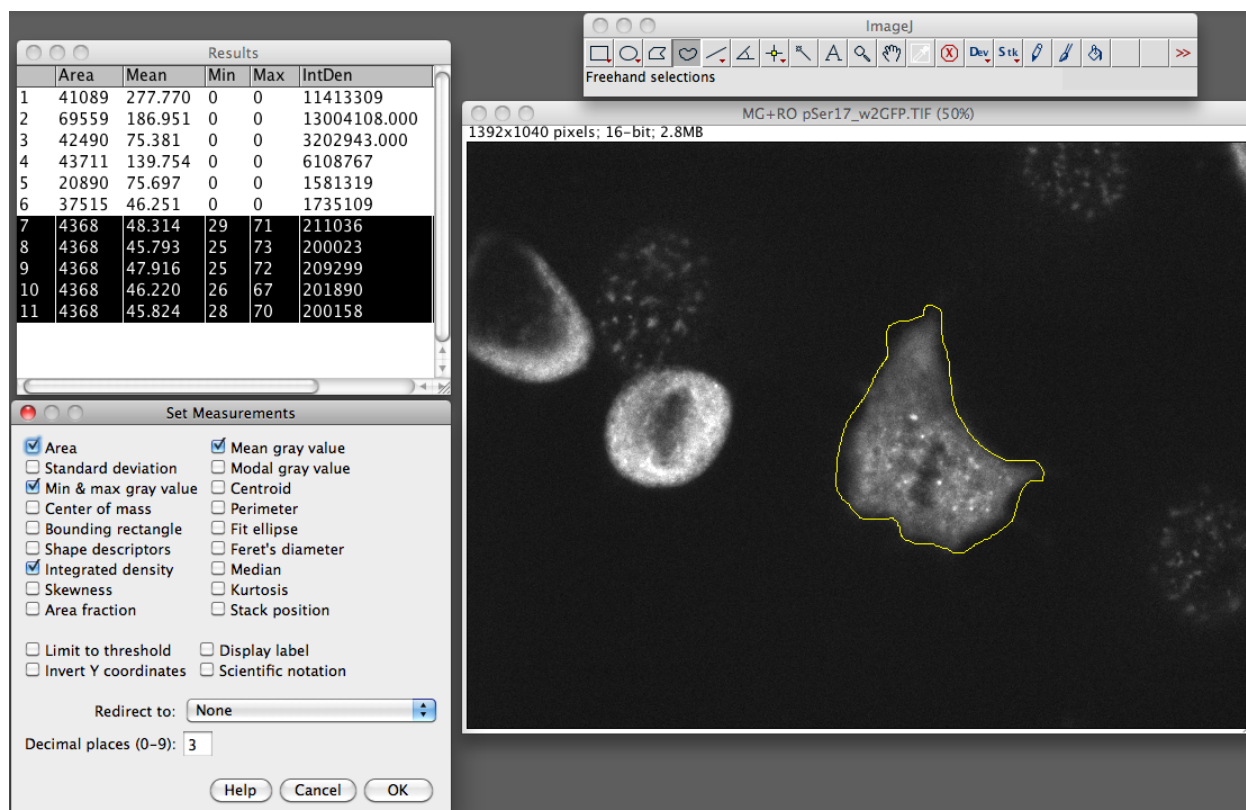
Repeat this step for the other cells in the field of view that you want to measure.

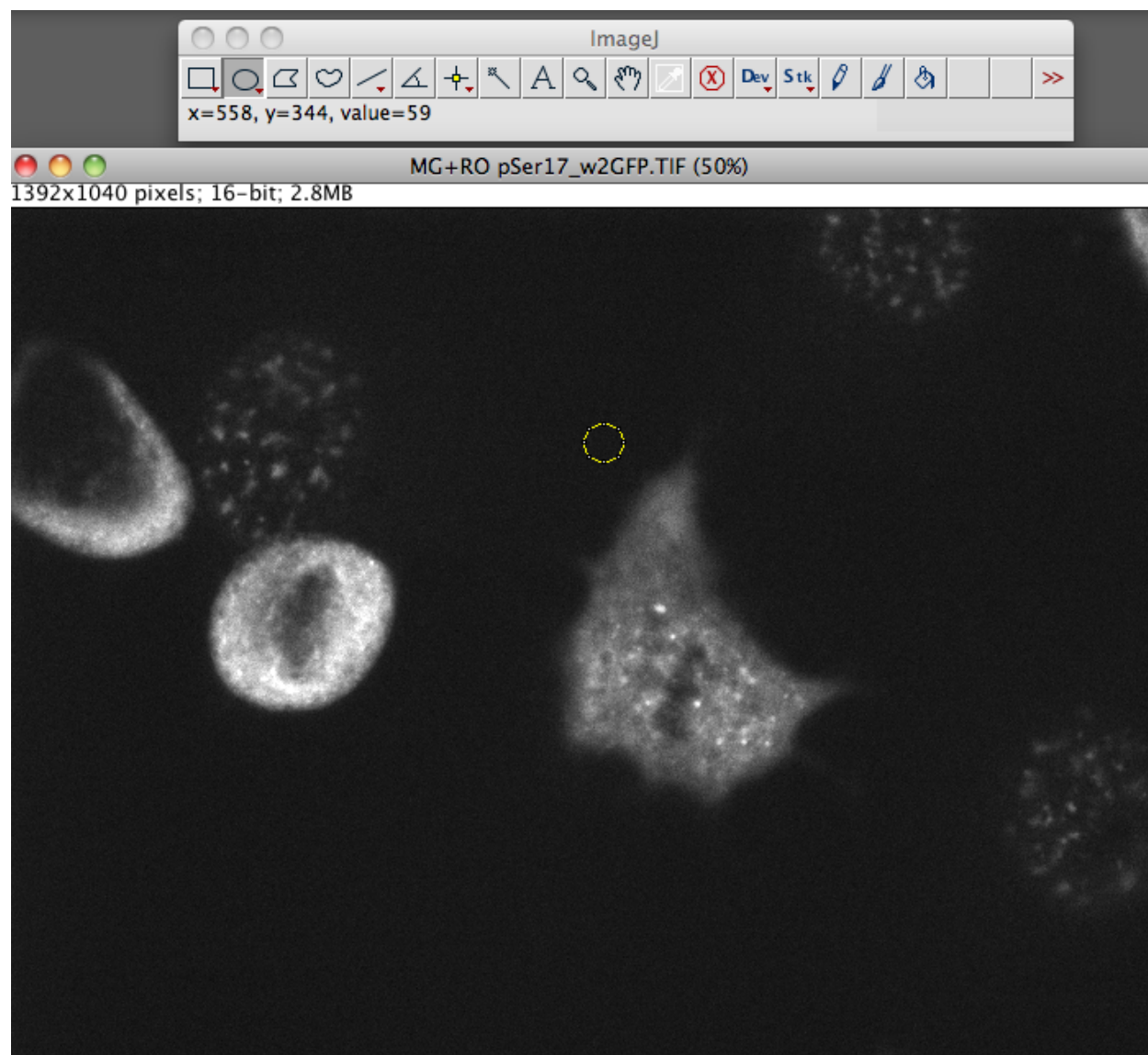
NB: Size is not important. If you want to be super accurate here take 3+ selections from around the cell.

- Once you have finished, select all the data in the Results window and copy and paste into a new spreadsheet (or similar program)
- Use this formula to calculate the corrected total cell fluorescence (CTCF).

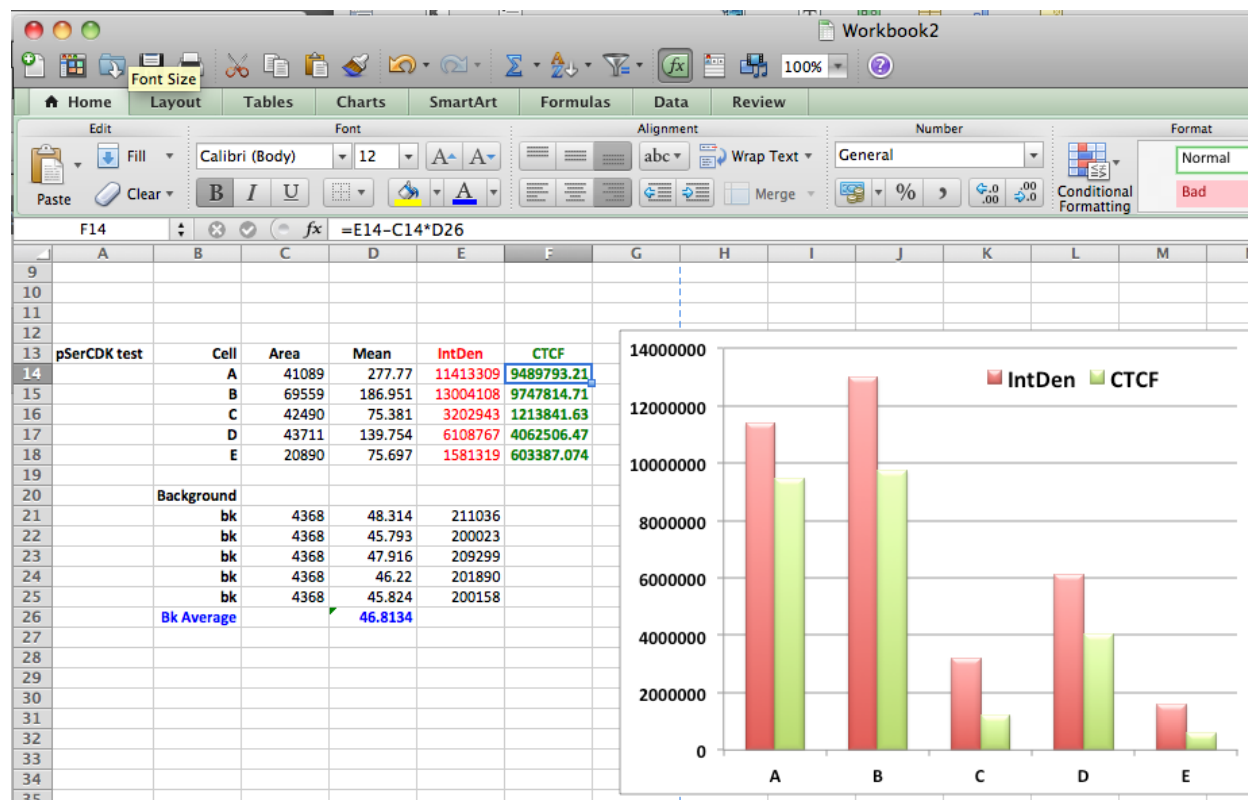
CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)

- Make a graph and your done.





Notice that rounded up mitotic cells appear to have a much higher level of staining due to its smaller size concentrating the staining in a smaller space. If you used the raw integrated density you would have data suggesting that the flattened cell has less staining then the rounded up one, when in reality they have a similar level of fluorescence.



<https://sciencetechblog.files.wordpress.com/2011/05/measuring-cell-fluorescence-using-imagej.pdf>

1.6 Immunology

Immunology protocols.

1.6.1 Antibody Purification using Blotted Antigen

Contributed by Ian Chin-Sang, Queens University, ON, Canada

Antibody Purification using Blotted Antigen

- Load antigen onto a preparative (single well) SDS PAGE gel.
- Electro-blot onto Nitrocellulose. (Transfer for 1.5 hours instead of the usual 1 hour).
- Ponceau S stain the blot for 1min. Cut out portion of Blot that contains the Antigen. Wash with copious amounts of distilled water or TBST until the Ponceau S is gone. Keep strip of Antigen in a 15ml conical tube. Block with 5% Milk in TBST for at least 20 min. Rinse 2X (10ml) TBST.
- Add crude serum with antibody. (5-10 ml.). Incubate 4hr to overnight.
- Pour off the unbound fraction and save serum.
- Wash the blot 3X with 10ml TBST.

- Elute bound antibodies with glycine buffer, pH 2.7 and mix/vortex for 5- 10 min. Collect into a new tube and add 1/10 the volume of 2 M Tris, pH 8.0 to neutralize the antibody which will prevent denaturation of antibodies, which can result from low pH. Check pH with pH paper. Measure the concentration of the protein A280. Use the neutralized glycine buffer as blank. (Concentration (mg/ml) = $(1.55 \times A_{280}) - 0.76 \times A_{260}$) Use a quartz cuvette not plastic.

For example if eluted with 10ml glycine then add 1ml 2M Tris, pH 8.0. Elute at least 3X.

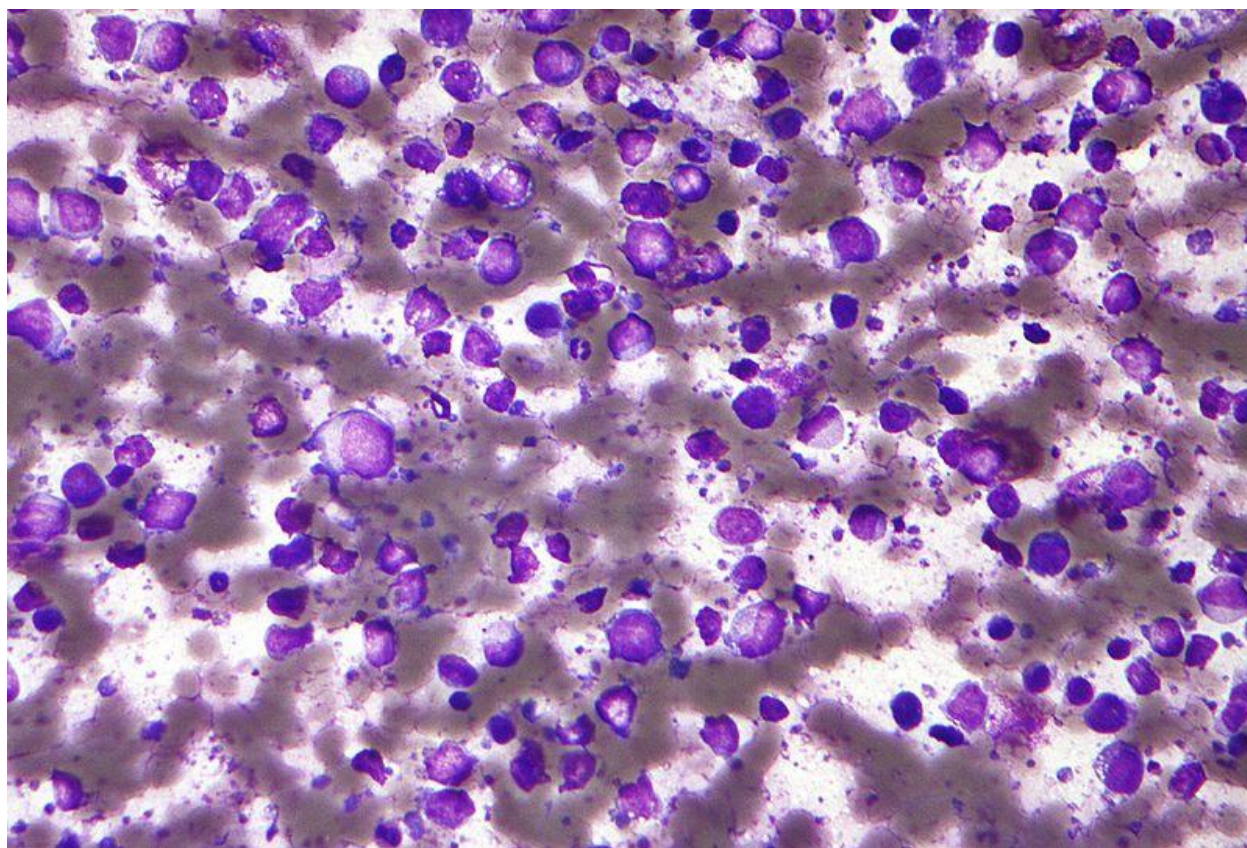
- Dialyze the antibody in 1XPBS. Lyophilize the Antibody and resuspend in 50% glycerol. Alternatively the antibody may be stored in the neutralized elution buffer.

This method is based, with permission, on an original protocol available [here](#).

1.6.2 B cell enrichment via Dynabeads

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

B cell enrichment using Invitrogen Dynabeads magnetic separation



- Count cells in PBMC sample for enrichment.

B cells constitute ~5% of total PBMC population.

- Calculate total Dynabeads for use at 4x B cell number (4 beads/cell)
- Wash x2: Centrifuge cells 1200rpm 5 minutes, resuspend cells in serum-free RPMI.

After final wash, resuspend in 100ul per 10^6 beads.

- Add B cell specific beads at number required. Stock at 4×10^8 beads/ml.

- Agitate tube at 4°C for 30 minutes; e.g. place on roller in cold-room
- Resuspend in 4mls cold RPMI + 1% FCS. Place tube into magnet for selection.

Wait for 4 minutes, then remove supernatant to fresh tube (B cell depleted PBMCs). Repeat x5

- Resuspend beads in 500ul RPMI + 1% FCS
- Add 20ul detachabeads (10ul/25ul beads used)
- Agitate at room temperature for 45 minutes; e.g. roller.
- Resuspend in 4mls RPMI + 1% FCS. Place tube into magnet for selection.

Wait for 4 minutes, then remove supernatant to fresh tube (B cells). Repeat x3

1.6.3 Generic (Quantitative) Sandwich ELISA

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

A generic ELISA protocol with optional quantitative step.

- Dilute primary antibody in coating buffer solution allowing for 50ul/well. A total of 5ml/96 well plate will provide spare for pipetting inaccuracy.
- Coat Nunc Maxisorp plate with primary antibody solution at 50ul/well. Tap gently to ensure the bottom of each well is completely covered.
- Incubate plate overnight at 4°C
- Flick off primary antibody and blot plate on tissue.
- Block plate with 200ul blocking buffer/well. A total of 20ml/96 well plate.
- Incubate the plate at room temperature for 1hr
- For quantitative ELISA prepare standards of target antigen in culture medium.

Perform serial dilutions from high of 2000pg/ml down to 31.25pg/ml, with final 1x blank culture medium alone.

- Wash the plate x6 with PBS/1% Tween. After final wash tap out plate and blot dry.
- Add supernatants (and optional standards) at 50ul/well. A 1/10 dilution plate can be produced by adding 45ul wash buffer + 5ul supernatant.
- Incubate at room temperature for 2-4 hours
- Flick off samples and standards into Vercon for disposal
- Wash the plate x6 with PBS/1% Tween. After final wash tap out plate and blot dry.
- Dilute secondary biotinylated antibody in blocking buffer to a volume for 50ul/well (5ml/96 well plate).
- Add 50ul of diluted secondary antibody/well
- Incubate at room temperature for 1-2 hours
- Flick off secondary antibody to disposal
- Wash the plate x6 with PBS/1% Tween. After final wash tap out plate and blot dry.
- Dilute Extravidin peroxidase with blocking buffer at 1/1000 to a total volume of 50ul/well (5ml/96 well plate).
- Add extravidin peroxidase to plate at 50ul/well
- Incubate at room temperature for 1/2 hour
- Wash the plate x8 with PBS/1% Tween. After final wash tap out plate and blot dry.

- Add 100ul TMB substrate per well (10ml/96 well plate)
- Leave at room temperature for ~20 minutes or until top standard has saturated.
- Stop reaction with 1M HCl at 100ul/well
- Measure absorbance on plate reader.

TMB dual read 450nm with blank at 650nm

1.6.4 MACS CD14+ Separation of monocytes

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Isolation of CD14+ subset of monocytes by MACS magnetic bead isolation.

- Centrifuge whole cell population at 1200rpm 10 minutes to pellet cells
- Remove supernatant and resuspend in 80ul MACS buffer per 10^7 cells
- Add 20ul CD14+ MACS beads per 10^7 cells
- Mix thoroughly and incubate for 15 minutes at 4°C
- Wash in 1-2ml buffer per 10^7 cells and then centrifuge at 1200rpm 10 minutes to pellet
- Resuspend in 500ul buffer per 10^8 cells (5ml/ 10^7)
- Place MACS column in magnetic field (in stand) and rinse through with 3mls MACS buffer
- Add cell suspension to column, allow to run through collecting run-off as waste
- Wash 3x 3ml with buffer, collecting run-off as waste
- Remove column to collection tube. Add 5ml buffer wash through and plunger to collect
- Count resulting cells and purity check by flow cytometry

Purification Of Anti Peptide Antibody

Contributed by [Timothy Mitchison](#), Harvard Medical School, Boston, MA, United States

Purification Of Anti Peptide Antibody

Do not pool the low pH and GuHCl eluates as they may have significantly different properties. We have found that the GuHCl may have higher affinities, but also may contain a higher fraction of partially denatured antibody that could contribute to staining background. The proportion in each pool varies with the peptide immunogen.

The quality of the anti-peptide serum seems to increase with multiple boosts - the first bleed may be feeble.

1.6.5 Method

- Pour column in TBS ($=0.15M$ NaCl, $20mM$ TrisCl pH 7.4). We use a 5 ml column for 25 mls serum. Wash extensively in TBS after prewashing as indicated in the protocol for coupling peptide to the resin.
- Thaw serum- dilute 1:1 with TBS and filter through a 0.2 um filter
- Load the serum over the column, taking at least 20 minute total.
- Run the breakthrough over the column five times. Alternatively you can use a peristaltic pump and recirculate the serum ON or just batch bind the serum ON.
- Wash with 5 col vols TBS.

- Wash with 10 col vols 0.5 M NaCl, 20mM TrisCl pH 7.4, 0.2% Triton- X-100.
- Wash 5 col vols TBS
- Elute with 0.15 M NaCl, 0.2 M Glycine-HCl pH 2.0. Collect 1 ml fraction, with each tube containing 0.1ml of 2 M TrisCl pH 8.5
- Wash with TBS until pH is reequilibrated.
- Elute with 6 M GuanidineHCl in TBS, collecting 1ml fractions.
- Wash with TBS + 0.1% NaN₃, and store at 4 deg C.
- To determine where to pool fractions, spot 1 ul of each fraction onto nitrocellulose paper and stain with ponceau S. Pool all fractions that show pink color.
- Dialyze ON into TBS or your favorite buffer.
- If necessary the antibody can be concentrated by sweating the dialysis bags, or by spin-concentrating.
- Bring the azide concentration up to 0.1% and store at 4 deg C for up to three months. For longer storage freeze in aliquots and store at -80 degC or add glycerol to 50% and store at -20 deg C.

This method is based, with permission, on an original protocol available [here](#).

1.6.6 Testing Rabbit Bleeds By ELISA

Contributed by [Timothy Mitchison](#), Harvard Medical School, Boston, MA, United States

You can test whether or not you have gotten an immune response to the peptide and how strong that immune response is by doing ELISAs against peptide conjugated to BSA. By conjugating to BSA, you will eliminate any signal for antibodies generated to KLH during immunization.

Requirements

1 ml of 2 mg/ml BSA in 0.1 M NaHCO₃. 1 ml of 0.2% glutaraldehyde in 0.1 M NaHCO₃. 0.5 ml peptide (1 mg/ml in DMSO). Peptide can be added as a solid if soluble.

Method

- Mix ingredients adding glutaraldehyde last. Peptide and BSA turn a little yellow even before adding glutaraldehyde.
 - Incubate 90 minutes at 37 deg C.
 - Add 0.1 volumes of 0.1 M NaBH₄ in 0.1 M NaHCO₃. There will be some bubbling. Add same amount of NaBH₄ after 15'.
- *Do a quick microfuge spin if there are too many bubbles. *
- Coat 10 ug/ml antigen (peptide conjugated to BSA) diluted in TBS (50 ul/well) ON at 4 deg C.
 - Remove antigen and rinse wells 2Xs with TBST.
 - Block 2 hr with 200 ul of 5% NFDM in TBST.
 - Remove blocking reagent and rinse wells 2Xs with TBST.
 - Incubate in primary antibody diluted in Blocking Buffer for 2 hr at RT. I do tripling dilutions beginning at 1/10 (50 ul/well).
 - Remove primary antibody and rinse wells 4Xs with TBST.

- Incubate in secondary antibody (1/5000 Goat anti-rabbit conjugated to AP) diluted in Blocking Buffer for 1 hr at RT (50 ul/well).
- Remove secondary antibody and rinse wells 4Xs with TBST.
- Rinse wells 2Xs with 50 mM HCO₃; 0.5 mM MgCl₂, pH 10.
- Develop in 1 mg/ml p-Nitrophenyl phosphate in 50 mM HCO₃; 0.5 mM MgCl₂, pH 10 (50 ul/well).
- Read A410 in ELISA reader.

This method is based, with permission, on an original protocol available [here](#).

1.7 Miscellaneous

Miscellaneous protocols.

1.7.1 Automatic unzip/untar using correct tool

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

An alias for bash profile to automatically unzip/untar compressed files using the correct tool, without needing to know the syntax.

- Add the following to your *.bashrc* or *.profile* file:

```
extract () {  
    if [ -f $1 ] ; then  
        case $1 in  
            *.tar.bz2) tar xjf $1 ;;  
            *.tar.gz) tar xzf $1 ;;  
            *.bz2) bunzip2 $1 ;;  
            *.rar) rar x $1 ;;  
            *.gz) gunzip $1 ;;  
            *.tar) tar xf $1 ;;  
            *.tbz2) tar xjf $1 ;;  
            *.tgz) tar xzf $1 ;;  
            *.zip) unzip $1 ;;  
            *.Z) uncompress $1 ;;  
            *) echo "$1" cannot be extracted via extract() ;;  
        esac  
    else  
        echo "$1" is not a valid file  
    fi  
}
```

- From the prompt compress any file with *extract <filename>*

This method is based, with permission, on an original protocol available [here](#).

1.7.2 BrdU incorporation for raft cultures

Contributed by Matt Lewis <hop2kin@yahoo.co.uk>

BrdU incorporation for raft cultures

- Prepare stocks of BrdU at 10mg/ml in H₂O, filter sterilise and store in working aliquots (eg. 200 microlitres) at -20C.
- Incubate rafts with BrdU (100 microgram per ml) for 12 hours prior to harvesting.
- Detect incorporated BrdU via antibody staining (Zymed 18-0103) using standard immunohistochemical techniques

High temperature antigen retrieval is required to open up the DNA

This method is based, with permission, on an original protocol available [here](#).

1.7.3 Cell Adhesion

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States

Cell Adhesion assay protocol from the Laurie Lab

- Dissolve/dilute coating substrate in ddH₂O at 4°C. A common working dilution for the laminin-1 positive control and BSA (Sigma A8412) negative control is 40 µg/ml. For SN-peptide, use 20 µM (MW of SN-peptide is 2412) for plateau or 2.5 - 5 µM for half-maximal adhesion. For fragment E8 or laminin-1 plateau and half-maximal adhesion are usually achieved at 0.5 and 0.05 µM, respectively.

*SN-peptide should be used right after dissolution in ddH₂O. Activity decreases with storage, even frozen. A reliable approach is to weigh out 100 µg aliquots with a microbalance for use with individual experiments. *

- Add coating solution (100 µl/well) with multipipetter to wells of a 96 well tissue culture plate (Costar #3595), cover, and place at 4°C overnight. Coat in triplicate or quadruplicate.
- Invert plate and shake out coating solution. Pull off remaining coating solution from each well with a yellow tip pipetter.
- Dilute 7.5% BSA (Sigma A8412) to 1% in ddH₂O. Add 100 µl/well with multipipetter, cover and place at 4°C for 4 hrs. Thaw 10 x trypsin/EDTA (then dilute to 1 x in PBS), warm PBS and serum-free medium.
- In last 45 min of block, pull off medium from cells in T75 flask, and add serum-free medium. Replace in incubator for 30 min.
- Subsequently, pull off medium, wash with PBS, add 1 x trypsin/EDTA for 1 - 3 min, pull off released cells, wash flask with 20 ml of serum-free medium, pellet cells in 40 ml of serum-free medium, make up in 6 ml of serum-free medium and count (15 µl of suspended cells plus 15 µl of trypan blue; add 15 µl to each side of hemocytometer; cell#/ml = combined count from both sides x 104). Dilute cells to 2.0 x 10⁵/ml in serum-free medium.
- Invert plate and shake out BSA blocking solution. Pull off remaining blocking solution from each well with a yellow tip pipetter.
- Pour cells in Reagent Reservoir (Costar # 4870), rock to suspend, remove 100 µl/well with multipipetter and add to wells. Repeat rock/resuspension prior to removing cell suspension for each row. Place in incubator for 30 - 60 min (37°C).
- Examine plate in invert microscope. Photograph selected wells if desired. Invert plate gently onto an absorbent diaper pad. Pull off remaining cell solution from each well with pipetter.
- With multipipetter, slowly add 100 µl/well of serum-free medium down the side of each well (tilt plate; PBS is not recommended for this wash). Invert plate gently onto an absorbent diaper pad. Pull off remaining wash solution from each well with pipetter.
- Slowly add 100 µl/well of serum-free medium down the side of each well. Examine plate in invert microscope.

*Cells in BSA negative control wells should be rare (if not, repeat wash). Adherent cells in SN-peptide wells remain mainly rounded or slightly spread. An exception is M2 melanoma, which spreads rapidly on SN-peptide. Adherent cells in laminin-1 wells should be all spread. *

- Invert plate gently onto an absorbent diaper pad. Pull off remaining wash solution from each well with pipetter.
- With multipipetter, slowly add 100 µl/well of freshly diluted 1% glutaraldehyde in PBS. Fix for 10 min at room temp.
- Invert plate gently onto an absorbent diaper pad. Pull off remaining fix solution from each well with pipetter.
- With multipipetter, add 100 µl/well of freshly filtered (use 0.2 µm syringe filter) crystal violet (0.1% in ddH₂O; Serva # 27335). Stain for 25 min at room temp. Invert plate onto an absorbent diaper pad, then wash plate gently by immersion in a plastic tray containing tap water. Invert plate onto an absorbent diaper pad. Pull off remaining

wash from each well with pipetter. Reimmerse in fresh tap water. Invert plate onto an absorbent diaper pad. Pull off remaining wash from each well with pipetter. Repeat an additional time if required.

- Allow to dry for 5-10 min at room temp.
- With multipipetter, add 50 µl/well of 0.5% Triton X-100 (diluted in ddH₂O). Allow to solubilize overnight at room temp. in a drawer. Read at OD 595.

*BSA background should be less than 0.1 OD. Laminin value should be about 1.0 OD. Plateau SN-peptide value is usually 70-80% of laminin. *

This method is based, with permission, on an original protocol available [here](#).

1.7.4 Cells in suspension

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

DAPI counterstain of cells in suspension, e.g. for FACS analysis

- Dilute stock DAPI mix to a concentration of 3µM in staining buffer, allowing a total of 1ml per sample.
- Centrifuge cell suspension at 1200rpm for 5 minutes to pellet. Pour off supernatant.
- Tap/agitate to dislodge and re-suspend cells in remaining buffer.
- Add 1ml of diluted DAPI to each sample
- Incubate for 15 minutes at room temperature
- Analyze cells as required:

For *flow cytometry* leave cells in DAPI solution.

For *fluorescence microscopy* pellet cells by centrifugation, remove supernatant and resuspend in PBS. Add a drop of cells to a slide and cover with coverslip.

1.7.5 Chemiluminescent Detection

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

Homemade chemiluminescent detection recipe

Requirements

p-coumaric acid (Sigma #9008) 3-aminophthalhydrazide (Luminol, Sigma #09253) 1 M Tris pH 8.5 30 % H₂O₂ 90mM p-coumaric acid in DMSO (store at -20°C) 250 mM Luminol in DMSO (store at -20°C)

Method

- Solution A (50ml):
- 5 ml 1M Tris pH 8.5
- 45 ml water
- 110 µl 90 mM coumaric acid (0.2mM coumaric acid)
- 250 µl 250 mM Luminol (final 1.25mM Luminol)

Keep 2-3 weeks at 4°C

- Solution B (1ml):
- 100 ul 30% H₂O₂
- 900 ul water

Keep 2-3 weeks at 4°C

- Combine 1 ml Solution A + 3 ul Solution B (5ml Solution A +15ul Solution B for each blot)

This method is based, with permission, on an original protocol available [here](#).

1.7.6 Chrome Alum Gelatin Slide Coating

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

Chrome Alum Gelatin Slide Coating

Clean grease-free slides are needed for the slide coating to be effective.

Requirements

200ml Distilled water 0.5g Gelatin 0.05g Chromic potassium Sulphate

Method

- Microwave briefly to dissolve the gelatin
- Cool
- Dip clean slides and allow to drain vertically.
- Once completely dry pack into Slide boxes in a dust free environment.

This method is based, with permission, on an original protocol available [here](#).

1.7.7 Chromosome FISH

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

DAPI counterstain for chromosome FISH analysis

- After FISH sample preparation briefly rinse sample in distilled water to remove residual salts
- Dilute stock DAPI to a concentration of 30nM in PBS
- Add 300ul of diluted DAPI solution directly onto the sample, ensuring sample is covered equally
- Incubate at room temperature for 30 minutes in the dark
- Rinse specimen with distilled water or PBS to remove DAPI solution
- Blot edge of slide with tissue to remove excess water/solution from the side
- Mount slide with appropriate slide mountant, nail polish or wax.

1.7.8 Collagen gel containing 3T3 fibroblasts

Contributed by Matt Lewis <hop2kin@yahoo.co.uk>

Collagen gel containing 3T3 fibroblasts (dermal equivalent for raft culture)

- Pre-chill pipettes and place 12mL rat tail type 1 collagen (>3.8mg/mL) on ice

Everything is kept cold to avoid the collagen solidifying

- Mix 1.5mL of 10x DMEM with 1.5mL of 10x reconstitution buffer, keep on ice.
- Count J2-3T3s and pellet required number in a universal.
- Add the 3mL of [1:1, 10x DMEM and 10x reconstitution buffer] and swirl to resuspend the cells, keep on ice.
- Using chilled pipette, add the 12mL of collagen gently to the cells and tilt to mix, avoiding bubbles as far as possible.
- Add 10N NaOH to bring the pH up to 7

You may judge the pH visually by the phenol red in the DMEM. Approximately 30–60mL will be required. Use the glacial AcCOOH if necessary but more mixing means more bubbles.

- Pipette 2–2.5mL into each well and incubate O/N
- Add 2mL raft media on top of each matrix.
- Use within 1 week, change media every 2 days.

This method is based, with permission, on an original protocol available [here](#).

1.7.9 Cosmid / Low Copy Plasmid Prep

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

Prep for cosmids or low copy number plasmids.

Requirements

LB: 10 g tryptone, 5 g yeast extract, 10 g NaCl per liter (pH to 7.0) Resuspension buffer: 50 mM Tris, 10 mM EDTA (pH 8.0) Lysis buffer: 200 mM NaOH, 1% SDS 4 M KOAc: pH to 5.3 with glacial acetic acid Qiagen tip-100 QBT buffer: 0.75 M NaCl, 50 mM MOPS, 15% isopropanol, 0.15% TritonX-100 (pH 7.0) QC buffer: 1.00 M NaCl, 50 mM MOPS, 15% isopropanol (pH 7.0) QF buffer: 1.25 M NaCl, 50 mM Tris, 15% isopropanol (pH 8.5) Isopropanol 70% ethanol

Method

- Grow up cells from which to purify DNA
- Inoculate 100 mls LB + appropriate antibiotic with 1 ml saturated culture
- Grow overnight or to saturation
- Spin down cells 7' @ 5000K (SLA-1500 rotor)
- Resuspend cells in 5 mls resuspension buffer - make sure cells are thoroughly resuspended
- Transfer cell suspension to 50 ml conical tube
- Lyse cells to release cosmid DNA

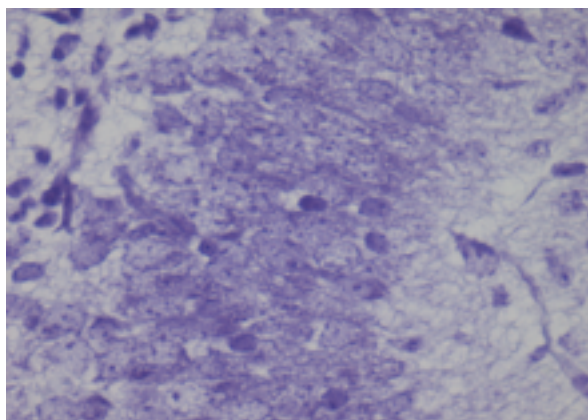
- Add 5 mls fresh lysis solution and invert gently twice to mix
- Incubate on ice for 5 min
- Add 5 mls 4 M KOAc and invert gently 2-3 times to mix
- Incubate on ice for 10 min
- Prepare an empty 50 ml syringe barrel by plugging a KimWipe into the bottom
- Place syringe over clean 50 ml conical tube
- Dump cell lysate into syringe and allow clarified solution to drain into 50 ml conical tube while the precipitate will be captured atop the KimWipe
- Add RNase to 50 ug/ml and let sit at room temperature while equilibrating Qiagen column

This method is based, with permission, on an original protocol available [here](#).

1.7.10 Cresyl Violet Staining (Nissl Staining)

Contributed by [Luke Hammond](#), QBI, The University of Queensland, Australia

Cresyl Violet Staining for paraffin embedded sections. Cresyl Violet Acetate solution is used to stain Nissl substance in the cytoplasm of neurons in paraformaldehyde or formalin-fixed tissue. The neuropil will be stained a granular purple-blue. This stain is commonly used to identify the neuronal structure in brain and spinal cord tissue.



The Cresyl Violet method uses basic aniline dye to stain RNA blue, and is used to highlight important structural features of neurons. The Nissl substance (rough endoplasmic reticulum) appears dark blue due to the staining of ribosomal RNA, giving the cytoplasm a mottled appearance. Individual granules of extra-nuclear RNA are named Nissl granules (ribosomes). DNA present in the nucleus stains a similar color.

Requirements

95% Ethanol 70% Ethanol Differentiation solution: 2 drops glacial acetic acid in 95% ethanol Cresyl Violet Acetate 0.2% in Acetate Buffer (Fronine, Cat No: HH155)-Filtered

Method

- De-wax sections in xylene (2 or 3 changes of 3min each)
- Rehydrate in alcohol (100% x2), 3min each.

- Stain in 0.1% Cresyl Violet 4-15min.
- Quick rinse in tap water to remove excess stain
- Wash in 70% ethanol (the stain will be removed by this method).
- If required immerse sections for 2min in Differentiation solution – check staining on microscope.
- Dehydrate through 2x3min changes of absolute ethanol.
- Clear in xylene x2 and mount in DePeX. Allow dry in the fume hood.

This method is based, with permission, on an original protocol available [here](#).

1.7.11 Crude Depletion Conditions for XKCM1

Contributed by [Timothy Mitchison](#), Harvard Medical School, Boston, MA, United States

Crude Depletion Conditions for XKCM1. The following is for depleting a protein present at ~ 10-20 ug/ml in the extract with high affinity polyclonal antibodies, using anti-XKCM1 as an example. (Arshad Desai 3/17/95)

The main problem with immunodepletion of crude CSF extracts is that they activate during or soon after immunodepletion. En

- a) During extract prep, after washing out cysteine with XB, wash eggs 2-3x with CSF-XB (no PIs) before washing with CSF-XB + PIs and then process as usual.
- b) Crush the eggs at 10K, full brake in SW55 at 16 deg.C. Even 12.5K doesn't work - you can't get decent spindles after immunodepletion !
- c) Use BioRad AffiPrep beads - these beads are really heavy and easily pellet; (agarose beads will not pellet very well in crude).

However, we have never been able to cycle a depleted crude - all assays were performed in straight CSF. Attempts to cycle have always failed so we gave up on cycling.

Method

- Put 25 ul of bead slurry into two 0.5 ml tubes labeled IgG and XKCM1.
 - Wash beads 3X with 0.5 ml TBST each wash.
 - Add Rb IgG (4 ug) or anti-XKCM1 Gly (4 ug) and bring volume to 400 ul total.
 - Bind antibody to beads at 4 deg.C for 1 hr on rotator. Make sure beads are rolling around.
 - Pellet in ufuge in coldroom and wash 1X TBST, 3X CSFXB + PIs.
 - Add 150 - 200 ul of crude extract to each tube, resuspend beads in extract gently with a cutoff tip and then immediately place on rotator. Avoid tapping or vigorous agitation.
 - Rotate for 1 hr at 4 deg.C ensuring that beads are mixing well.
 - Pellet and transfer supe to a different tube. Save a small supe aliquot for western blots and use supe for assays
 - Processing beads for gel:
1. Wash beads 2x with CSFXB +PIs.
 2. Wash beads 2x with TBST
 3. Wash beads 1x with TBS
 4. Add 50 ul SB w/ DTT.

5. Also add 3 ul of each supe in 60 ul of SB
6. Boil for 5', pellet out the beads and transfer supe and freeze gel samples at -20 deg.C.
 - Assay depletions by blots; also run rest of pellet on coomassie gel to estimate cleanliness of IP.

This method is based, with permission, on an original protocol available [here](#).

1.7.12 CSF Extract Prep for Spindle Assembly

Contributed by [Timothy Mitchison](#), Harvard Medical School, Boston, MA, United States

This protocol is essentially as described by Murray (1991), Cell Cycle Extracts. In Methods in Cell Biology, B.K. Kay and B. Peng, eds. (San Diego: Academic Press), pp. 581-605. I've included a protocol which emphasizes the points that we find are most important for obtaining good CSF extracts that are competent for CSF spindle assembly and for cycled spindle assembly. The indicated buffer amounts are sufficient for a 4 frog prep. For sperm nucleus preparation, see above protocol. For spindle assembly, see Sawin and Mitchison (1991) J. Cell Biol. 112: 925-940. (Claire Walczak 11/1995)

The quality of the eggs is essential for good CSF extracts. Always sacrifice quantity for quality when trying to make functional extracts. Discard any batches of eggs that have 'puff balls' or activated eggs as more than 10% of the eggs. We routinely use laid eggs and collect at about 16-17 hours. If you are trying to make extracts that will form spindles competent of anaphase chromosome segregation, we find it necessary to use only freshly squeezed eggs. Keep the eggs cool (16 deg C incubator) and only bring them to RT right before you are ready to prepare the extract.

Requirements

20X XB Salts: 2 M KCl, 20 mM MgCl₂, 2 mM CaCl₂ (store at 4 deg C) 2 M Sucrose: Sterile filter and store in aliquots at - 20 deg C. 1 M HEPES, pH 7.7: Sterile filter and store at 4 deg C. 0.5 M K-EGTA, pH 7.7: Sterile filter and store at RT.

MMR: 5 mM HEPES, pH 7.8, 0.1 mM EDTA, 100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂ (make 2 l- store at RT). XB: 10 mM HEPES, pH 7.7, 1 mM MgCl₂, 0.1 mM CaCl₂, 100 mM KCl, 50 mM sucrose (make 250 ml- make fresh). CSF-XB: 10 mM HEPES, pH 7.7, 2 mM MgCl₂, 0.1 mM CaCl₂, 100 mM KCl, 5 mM EGTA, 50 mM sucrose (make 250 ml- make fresh).

Dejelling solution: 2 % cysteine; 1X XB salts, pH 7.8 (water to 200 ml- make within 1 hour of use).

Energy Mix: 150 mM creatine phosphate, 20 mM ATP, 2 mM EGTA, 20 mM MgCl₂, 100 ul aliquots- store at -20 deg C.

1 600 ml beaker 1 150 X 75 mm petri dishes 5% gelatin in ddH₂O (at 37 !C) Flame polished cut-off pasteur pipettes (diameter of opening approx. 2-3 mm) LPC (10 mg/ml each of leupeptin, pepstatin, chymostatin in DMSO) Cytochalasin D (10 mg/ml in DMSO) 13 X 51 mm ultraclear tubes SW55.1 @ 16 deg C in ultra

Method

- Get all solutions ready and tubes in the rack. Have gelatin @ 37 deg C. Coat petri dish with 100 ul/dish of gelatin, swirl and replace with XB. Bring frogs to room temp at the last minute.
- Collect laid eggs: keep eggs in separate batches if distinguishable difference in quality
- Wash eggs in MMR till all the crap and dirt is removed in 600 ml beaker.
- Garden away the bad eggs (pick out individually with pasteur)
- Remove as much MMR as possible.
- Dejelly in 2% cysteine till packed (~ 5 min)- remove all cysteine

- Wash dejellied eggs 2-3 X with XB in gelatin-coated petri dish-remove all XB. For each wash swirl the eggs around the dish and then let the eggs settle back down. They should pack tightly after the jelly coat is removed.
- Wash 2-3 X in CSF-XB (150 ml total volume)- remove as much buffer as possible.
- Wash 2X in CSF-XB + 10 5g/ml PIs (100 ml total volume)
- Transfer into 1 ml of CSF-XB + PIs + 100 ug/ml cytochalasin D in 13 X 51 ultraclear tubes (let eggs drop in)
- Suck off all buffer from top (pretty dry)
- Put into falcon tube and spin for 10 sec @ #4 in a clinical centrifuge.
- Remove all buffer (pretty dry) and put in 1 ml versilube
- Spin at #5 for 30 sec and full speed for 15 sec in a clinical centrifuge.
- Remove all buffer and versilube (as dry as possible)
- Crush @ 16 deg C: 15 min @ 10,000 rpm (full brake) in an SW55 rotor. We find that using the ultracentrifuge at this step gives much more reproducible extracts.
- Collect extract with 18 gauge needle by puncturing the side of the tube and gently sucking out the cloudy cytoplasmic layer. You should be able to obtain about 0.5-0.75 ml of extract/tube.
- Add 1/1000 volume of LPC and cyto D; 1/20 vol of 20X energy mix; 1/40 vol 2M sucrose. Extract is Ready to go!

This method is based, with permission, on an original protocol available [here](#).

1.7.13 EMS Mutagenesis

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

EMS Mutagenesis protocol

EMS is a potent carcinogen. Use caution when dispensing. Double your gloves and if you get your gloves contaminated, remove by turning inside out and dispose of in the solid EMS waste. Any paper towel or kimwipes that are contaminated can be held in a gloved hand and the glove turned inside out to dispose of. All tips that come in contact with the EMS should be soaked in 1M NaOH solution prior to throwing away.

Method

- Wash plates of worms (mixed staged or synchronized) with M9 buffer (1ml/plate).
- Put worms in a microfuge tubes. Spin for 1 min at high speed. Aspirate M9 and pool all worms into one microfuge tube. Resuspend (wash) in 1ml M9 and spin (use 1ml H₂O as balance). Wash 2 more times.
- Resuspend worms in a volume of about 1ml M9. Add 5 microliters of EMS (concentrated from bottle) to the 1ml of worms and mix immediately. This makes a final concentration of 50mM EMS (Note: 20 microliters of EMS in 4 ml M9 \approx 47mM).

50mM EMS is standard, but others have found this to be too high. Vary concentrations from 10 to 50mM EMS. If large volumes of worms are going to be used then carry out the EMS mutagenesis in 4 ml of M9 and use a 15ml polypropylene tube.

- Incubate worms at 22C for 4-5hours with mild rocking. Use the “labquake” rocker
- Spin 30 seconds. Aspirate off supernatant making sure not to suck up the worms. Note, you must use the vacuum with the side arm flask trap. Put a solution of 2M NaOH in the trap so the EMS will be inactivated. Wash worms at least 4 times with 1ml M9 buffer.

- This method is based, with permission, on an original protocol available [here](#).

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States



- Use endotoxin-free ('LAL Reagent') water for all dilutions. Charles River provides endotoxin at 12 EU/ml (after reconstitution with 1.5 ml 'LAL Reagent' water. Vortex to reconstitute and vortex immediately before use. Store at 4°C for up to 2 wks after reconstitution (should test to see if we can freeze aliquots).*

- ## 1.7. Miscellaneous

Sample (µl)|5|25|50 -|-|- LAL Reagent (µl)|45|25|0

- Incubate in 37°C incubator for 5 min.
- Add 50 µl of 'LAL Solution' and mix on rotating platform in 37°C incubator for 18 minutes.
- Add 100 µl of 'Substrate-Buffer Solution' mix on rotating platform in 37°C incubator for 8 minutes.
- Read on plate reader at 405nm. Look at analyzed graph to get EU values for test samples and see standard curve. Divide by volume applied.

This method is based, with permission, on an original protocol available [here](#).

1.7.15 Frozen Yeast Competent Cells

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

Frozen Yeast Competent Cells

Requirements

1M Lithium Acetate (LioAC) 10.2 g in 100ml dH₂O and autoclave. Dilute to 100mM with dH₂O for working solution. Glycerol or DMSO

Method

- Grow up intended stain (i.e. Y190) in appropriate media (YPAD) overnight. (Approximately 1ml for every 100ml intended to inoculate the next day (i.e. 2.5 ml overnight to inoculate 250ml). If you are growing a yeast strain with a selectable plasmid. Grow in appropriate selection media about 20 ml for every 100ml intended to inoculate the next day. Some yeast strains in selection media may take 2-3 days to reach saturation. You should grow just to the point of saturation.
- Inoculate 250ml YPAD- this will be enough for about every seventy-five 100ul aliquots of frozen competent cells. Scale up as desired. Let them grow to log phase (~0.7 OD). Doubling time for Y190 is about 3.5 hours. If you are inoculating a strain with a selectable marker inoculate to ~OD 0.3 so it goes through one doubling time. You don't want too many doubling times as they may be lost due to non selection.
- Spin down cells (6,000 for 10 minutes) and wash in 0.4 volumes of starting volume (i.e 40 ml if you grew up 100ml culture) of a 100mM solution of LioAC.

We do not wash in water as in other protocols and this allows us to skip the 30 min incubation step

- Spin down cells again and wash in a 0.2 volumes starting volume with 100 mM LioAC.
- Spin down a final time and resuspend cells in 100mM LioAC with 15% DMSO or glycerol to a final volume of 0.03 of your starting volume.

Glycerol seems to work better for us.

- Aliquot 100ul shots in microfuge tubes and put cells into a cardboard box and allow to freeze slowly in -80C.

Unlike E. coli competent cells, flash freezing in liquid nitrogen will severely reduce their competency.

This method is based, with permission, on an original protocol available [here](#).

1.7.16 Heparan Sulfate Chain Analysis

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States

Analysis of SDC1 HS Chains

- Generate 50% confluent cell cultures in 150-mm culture dishes.
- Metabolically label with 50 mCi/ml Na²³⁵SO₄ (1494 Ci/mmol; PerkinElmer, Boston MA) in DMEM for 48 hours as described by Zako et al. (2003). Use both normal and heparanase-1 depleted cells.
- After washing three times with PBS, generate cell lysates (use 'Lacritin Affinity Binding of SDC1' lysis buffer).
- Preclear lysates by centrifugation (20,000'g) at 4°C, then by passage of lysate supernatant over beads.
- Determine protein concentration of supernatant by the BCA assay (Pierce, Rockford IL).
- Affinity precipitate with FGF2-GST or lacritin-intein beads overnight at 4°C.
- Digest SDC1 bound to beads using chondroitin ABC lyase (MP Biochemicals, Aurora Ohio) for 3 hours at 37°C.
- Elute SDC1 from beads with 2 M NaCl and then subject to eliminative cleavage and reduction of HS by adjusting to 100 mM NaOH/1 M NaBH₄ for 24 hours at 37°C.
- Neutralize released HS by drop wise addition of 1M HCl.
- Subject to Sepharose CL-6B column (1'57cm) gel filtration chromatography in PBS at a flow rate 16 ml/h.
- Measure radioactivity by liquid scintillation counting.
- Prior to running the columns, determine the void volume (V₀, fraction 26) and total column volume (V_t, fraction 62) using dextran blue and sodium dichromate as markers.

References

Zako M, Dong J, Goldberger O, Bernfield M, Gallagher JT, Deakin JA *Syndecan-1 and -4 synthesized simultaneously by mouse mammary gland epithelial cells bear heparan sulfate chains that are apparently structurally indistinguishable.* *J Biol Chem* (2003) 10.1074/jbc.M209658200

1.7.17 Heparanase Western Blotting

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States

Heparanase Detection method for Western Blotting

- Generate cell lysates (use 'Lacritin Affinity Binding of SDC1' lysis buffer).
- Dialyze lysates overnight against binding buffer (10 mM sodium phosphate, pH 7) and apply to a HiTrap heparin affinity purification (Amersham Biosciences, Piscataway NJ).
- Wash with 10 column volumes of binding buffer, then elute using 5 column volumes of elution buffer (10 mM sodium phosphate, 2 M NaCl, pH 7).
- Determine protein concentration by BCA
- Analyze by 10% SDS-PAGE. Detect HPSE with rabbit polyclonal antibodies directed against human heparanase (kindly provided by Israel Vlodavsky, Rappaport Faculty of Medicine, Haifa, Israel)
- Add HRP-conjugated secondary antibody and ECL.

This method is based, with permission, on an original protocol available [here](#).

1.7.18 HSS Depletion Conditions for XKCM1

Contributed by [Timothy Mitchison](#), Harvard Medical School, Boston, MA, United States

HSS Depletion Conditions for XKCM1 (Arshad Desai 3/17/95)

HSS is less sensitive to activation - I find it to be very stably CSF. Other types of protein A beads can also be used for this purpose - it is not necessary to use Affiprep beads. I have successfully scaled up this depletion to 500 ul of extract increasing amount of beads and antibody proportionally.

Method

- Put 25 ul of Bio-Rad Affi Prep bead slurry into two 0.5 ml tubes labeled IgG and XKCM1.
- Wash beads 3X with 0.5 ml TBST each wash.
- Add Rb IgG (4 ug) or anti-XKCM1 Gly (4 ug) and bring volume to 100 ul total.
- Bind antibody to beads at 4 deg.C for 1 hr 15' on rotator. Make sure beads are rolling around.
- Pellet in uforce in coldroom and wash 1X TBST, 3X CSFXB + PIs.
- Add 150 ul of clarified extract to each tube.
- Rotate for 1 hr at 4 deg.C ensuring that beads are mixing well.
- Pellet and transfer supe to a different tube. Aliquot and freeze 20 ul aliquots in green tubes (XKCM1 deplete) and in yellow tubes (IgG deplete).
- Processing beads for gel:
 1. Wash beads 2x with CSFXB +PIs.
 2. Wash beads 2x with TBST
 3. Wash beads 1x with TBS
 4. Add 50 ul SB w/ DTT.
 5. Also add 3 ul of each supe in 60 ul of SB
 6. Boil for 5', pellet out the beads and transfer supe and freeze gel samples at -20 deg.C.

This method is based, with permission, on an original protocol available [here](#).

1.7.19 Isolation of LDL From Human Plasma

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

This procedure involves use of human plasma, a potentially dangerous source of blood-borne disease. Wear long-cuffed gloves, and eye-protection.

Requirements

Human Plasma (purchased from the Red-Cross. It should be 3-4 days old, from testing).

Method

- The plasma bag is emptied by cutting the long tube with a pair of sharp scissors (be careful not to splash).
- To the plasma (300mL) is added 3mL of 100mM EDTA (sterile filtered) to a final concentration of 1mM. Protease Inhibitors may also be added as preservative.
- Plasma (~300mL) is spun at 12C@20,000 RPM for 20min in a TI-70 Rotor. The tubes are removed and the chylomicrons are discarded. If the plasma donor fasted (as instructed) there should be little chylomicrons in the upper fraction. Discard the upper white, chylomicrons and transfer the samples to new tubes.
- Plasma (~250mL) is spun at 12C@52,000 RPM for 24h in TI-70-Rotor. ~ 25mL per tube. Tubes using an inverted plug are used, with a red, aluminum screw-cap. Tubes are sealed and placed in Ultra Centrifuge.
- Mix the lower layer, leaving the greenish-pellet intact. The pellet contains glycogen and fibrinogen, and some LDL, but not enough to worry about. Tubes may be inverted and squeezed to remove the contents. Discard pellet, and tubes.
- Adjust the density of the LDL-Plasma to 1.06 using Potassium Bromide (KBr). If density is not adjusted LDL will not spin down. Calibrate pipette to ensure accuracy, using dH₂O.
- Add .075gKBr/mL Plasma.
- Check density weighing 1mL of solution. If not high enough, add KBr.
- Spin at 12C@40,000 RPM for 48h in TI-70 Rotor.
- Stop the rotor, without using the brake.
- Collect the LDL (uppermost fraction) taking care not to mix the layers or fractions.
- The LDL should be kept under nitrogen, dark and at 4C until use.

This preparation according to other sources is good for up to 2 weeks.

1.7.20 L B Broth

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

L B Broth

Requirements

1 liter of ddH₂O 10 grams Bacto-tryptone 5 grams Bacto-yeast Extract 5 grams NaCl

Method

- In 1 liter of ddH₂O combine 10 grams Bacto-tryptone, 5 grams Bacto-yeast Extract and 5 grams NaCl
- Stir until dissolved.
- Autoclave for at least 15min @120C
- Once cooled below 55C add antibiotic of choice and store at 4C. Use within 2 weeks

This method is based, with permission, on an original protocol available [here](#).

1.7.21 Lacritin Calcium Signaling Assay

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States

Lacritin Calcium Signaling Assay - Ratiometric method as performed by the Putney Lab (see references).

- Read the Bird, Putney chapter very carefully. Plan positive and negative controls. Prepare for the possibility that experimental optimization may be necessary. This may include optimization of fura-5F concentration*, loading time and temperature may be required for lacritin signaling studies.
- HEK293 cells (ATCC) are cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine and maintained in a humidified 95% air, 5% CO₂ incubator at 37 °C.
- In preparation for cDNA or siRNA transfection, cells are transferred to 6-well plates.
- Allowed to grow to 90% confluence overnight (Day 1).
- The next day (Day 2) cells are transfected with Lipofectamine 2000 (2 µl/well; Invitrogen) and cDNA (0.5 - 3 µg/well). Alternatively on Day 2, cells are transfected with siRNA (10 -100 nM) including siGLO (Dharmacon) as a marker.
- Incubate for 6 hours.
- After a 6-h incubation period, the medium bathing the cells is replaced with complete DMEM and maintained in culture overnight.
- On Day 3, siRNA treated cells can be transfected with cDNA.
- In preparation for Ca²⁺ measurements (Day 3 or 4), cells are transferred onto 30-mm round glass coverslips (no. 1 thickness) as a 0.5 ml cell suspension (400,000 cells/ml).
- Allow cells to attach for a period of 12 hours
- Additional DMEM is then added to the coverslip.
- Maintain cells in culture for an additional 12 - 36 hours before use in Ca²⁺ measurements.
- For intracellular Ca²⁺ measurements (Day 4 or 5), cells are loaded with 1 µM Fura-5F/AM (Invitrogen).
- Cells plated on 30-mm round coverslips and mounted in a Teflon chamber were incubated in DMEM with 1 µM acetoxymethyl ester of fura-5F* (fura-5F/AM, Molecular Probes) at 37 °C in the dark for 25 minutes.
- While cells are loading, turn on the 120 watt mercury-xenon lamp.

The lever near the objective should be in the position of 0% eye piece to 100% camera or vice versa. Fluorescence studies should be done utilizing the 20X objective (10X for phase contrast). Insight IM1 software is for single wave-length excitation. Insight IM2 software is for ratiometric analysis of Ca²⁺.

- Turn on all other components by switching on the isobar surge protector. Start computer and click on InCyt Im2 icon for ratiometric imaging. Initial Settings: use default, collect 150 images. Setup/Calibration: set <frame size> to quarter size and develop a new graph from standard solutions (0, 38, 65, 100, 150, 225, 351, 602 nM). To do this, place 100 nM calcium standard on the stage and adjust the focus until the spot is as sharp as possible. Select calibrate from the setup option on the tool bar, select new graph from solutions, adjust the exposure times at 340 nm and 380 nm. If exposure time ratio is 1/1- faster, 2/1-slow, 3/1- more slow. Adjust the focus for the calcium standard. Capture background by defocusing the calcium stand slide. Refocus the calcium standard again. Capture image pair for each concentration of the standard.

Alternatively, changes in intracellular Ca²⁺ are represented by and expressed as the ratio of fura-5F fluorescence because of excitation at 340 nm and 380 nm (F₃₄₀/F₃₈₀) (as per recent Bird, Putney articles).

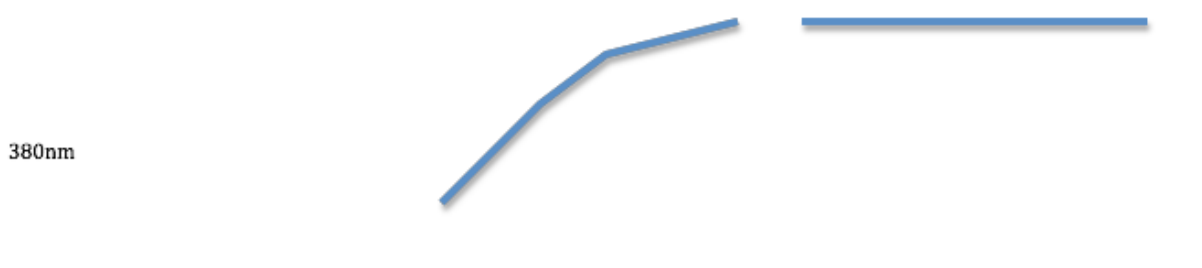
- Cells are then bathed in HEPES-buffered salt solution (HBSS: NaCl 120; KCl 5.4; Mg₂SO₄ 0.8; HEPES 20; CaCl₂ 1.8; and glucose 10 mM, with pH 7.4 adjusted by NaOH) at room temperature. Nominally Ca²⁺- free solutions were HBSS with no added CaCl₂.

- Before starting an experiment, regions of interest identifying cells to be measured or transfected cells expressing the EYFP fluorescence tag are created by observing cells at _ or at a 530-nm emission wavelength and illuminated with 477-nm excitation light. In all cases, ratio values are corrected for contributions by autofluorescence, which is measured after treating cells with 10 μ M ionomycin and 20 mM MnCl₂.

*Typically, 20 to 30 cells are monitored per experiment. *

- Select experiment from the tool bar. Set threshold levels for the Ca²⁺ (this is used to remove false calcium signal from the experiment). Adjust the lower limit and higher limit to adjust threshold. Adjust the exposure time ratio for the data capture (be sure to adjust it same as the exposure time ratio of the standards. Adjust the appropriate rate of data capture (it depends on the number of images you wanted to capture per second. Start the experiment, wait for the base line. Then add lacritin or C-25 by marking the time of addition. Save the experiment.
- Select the measure option from the tool bar. Look for the graph or values (depends on your convenience). Data (Ca²⁺) can be averaged or the time point and amount of maximum Ca²⁺ can be viewed.

Typical standard graph of Ca²⁺ measurement



For editing, images of your choice can be selected. Graph of particular time point can be selected. A mosaic of different images at different time points, with scale can be presented. Change in the calcium levels with time can be viewed as an animated video.

References

DeHaven WI, Smyth JT, Boyles RR, Bird GS, Putney JW Jr [Complex actions of 2-aminoethyldiphenyl borate on store-operated calcium entry.](#) *J Biol Chem* (2008) 10.1074/jbc.M801535200

1.7.22 Lacritin IP3 Assay

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States

Lacritin IP3 Assay based on Webb et al. (Eur. J. Biochem. 234:714-722,1995)

- Grow cells to confluence in serum-containing medium. Wash twice with inositol-free medium.
- Incubate in serum-free/inositol-free medium containing 8 μ Ci/ml of 2-[³H]myo-inositol for 24 h at 37°C.

- Wash five times with HBSS supplemented with 10 mM HEPES, 3.5 mM NaHCO₃, 10 mM LiCl, 1 mM MgCl₂ and 1 mM CaCl₂ pH 7.4.
- Incubate in the same wash solution at 37°C for 15 min.
- Replace with the same wash solution containing 10 nM LACRT or C-25 or 100 µM CCH for 0, 15, 30 and 60 sec.
- Add the samples to 2ml Dowex AG1-X8 columns. Wash off free inositol with water, then elute InsP with 0.2 M ammonium formate/0.1 M formic acid; InsP₂ with 0.4 M ammonium formate/0.1 M formic acid; and InsP₃ with 1.0 M ammonium formate/0.1 M formic acid.
- Measure radioactivity in scintillation counter.

Processing was as described by Webb et al. (Eur. J. Biochem. 234:714-722,1995)

References

Donna J. Webb, Isa M. Hussaini, Alissa M. Weaver, Tara L. Atkins, Charleen T. Chu, Salvatore V. Pizzo, Gary K. Owens, Steven L. Gonias *Activated alpha2-Macroglobulin Promotes Mitogenesis in Rat Vascular Smooth Muscle Cells by a Mechanism that is Independent of Growth-Factor-Carrier Activity* *European Journal of Biochemistry* (1995) 10.1111/j.1432-1033.1995.714_a.x

1.7.23 Lacritin PKCa Signaling Assay

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States

Lacritin PKCa Signaling Assay

- Seed Cells in serum-containing media overnight in 24-well plates at a density of 0.5 x 10⁵ cells/mm² corresponding to 20 – 30% confluency after cell attachment.
- Incubate cells in serum-free medium without supplements overnight to 24 hours.
- Reconstitute lacritin or lacritin fragment in water or serum-free medium to 10 µM stock. Wash cells with serum-free media without supplements three times and then replace media with serum-free medium containing lacritin (1 - 1000 nM), PMA (100 nM), EGF (1.6 nM) or 10% fetal bovine serum. In negative controls, incubate cells with equal molar amounts of C-25 or bovine serum albumin or without additive in serum-free media without supplements.
- Incubate for 1 – 15 min in the hood at room temperature.
- Stop incubation by adding ice-cold PBS containing sodium orthovanadate (2 mg/ml).
- Aspirate PBS and extract cells in 1% NP40 containing sodium orthovanadate (2 mg/ml), DTT (5 mg/ml), protease inhibitors (1 µg/ml leupeptin, 2 µg/ml aprotinin, 0.4 mg/ml sodium fluoride), 50 mM HEPES, 100 mM NaCl and 2 mM EDTA.
- Separate by 10% SDS PAGE, transfer and blot.
- Detect with anti-phospho-PKCa/bII (Cat. no. 9375) from Cell Signaling Technology (Beverly MA). or for loading control with anti-PKCa (Cat. no. 05-154) from Millipore-Upstate (Temecula CA). For other phospho signaling, detect with anti-PLCg2 (Cat. no. 3874) or antiphosphotyrosine antibody (P-Tyr-100; Cat. no. 9411) also from Cell Signaling Technology.
- After washing, bound antibodies were detected with peroxidase-labeled secondary antibody and ECL.

This method is based, with permission, on an original protocol available [here](#).

1.7.24 LB Agar Plates

Contributed by [Paul Barber](#)

LB agar plates for cloning

Batch makes about 40 plates.

Requirements

Special Additives (to be added to LB Agar right before pouring plates) Ampicillin (VWR 80055-786) 50 mg dissolved in a small amount of dH₂O (concentration 100 ug/mL) X-gal (VWR IB02260)

Stock solutions 50 mg dissolved in a small amount of DMSO 200mg in 10mL dH₂O (store at 4 in 1mL aliquots) use 100mM 238 mg IPTG in 10mL dH₂O (store at -20 Ampicillin 20mg/mL 50uL on each plate IPTG (VWR EM-5800) in 1mL aliquots) use 40uL on each plate X-gal 40 mg/mL 400 mg X-gal in 10mL DMSO (store at -20 in 1mL aliquots foil wrapped tubes) use 40uL on each plate

Method

- Add 250 mL of dH₂O to a graduated cylinder.
- Weigh out 20g of premix LB Agar powder (VWR DF0445-17) or:
 - 5.0 g tryptone
 - 2.5 g yeast extract
 - 5.0 g NaCl
 - 7.5 g agar
- Mix powder well to bring into solution
- Add dH₂O to total volume of 500 mL and transfer to 1 L flask
- Put on stirring hot plate and heat to boil for 1 min while stirring.
- Transfer to 1 L pyrex jar and label with autoclave tape.
- Autoclave at liquid setting for 20 minutes in a basin making sure to loosen top
- Let agar cool to ~55C (you should be able to pick up the jar without a glove)
- Add your antibiotic at this point.
- Make sure bench top has wiped down with bleach/EtOH.
- Remove sterile Petri dishes (VWR 25384-208) from plastic bag.

Save the bag for storage

• Pour a thin layer (5mm) of LB Agar (~10mL) into each plate being careful to not lift the cover off excessively.

You should be able to just open up enough to pour.

- Swirl plate in a circular motion to distribute agar on bottom completely.
- Let each plate cool until its solid (~20 minutes) then flip so as to avoid condensation on the agar.

- Store plates in plastic bags in fridge with: name, date and contents

Note any additive

This method is based, with permission, on an original protocol available [here](#).

1.7.25 M9 Plates -Leu

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

The following recipe is for one litre of plates.

The salts need to be autoclaved separately from the agar, for this reason prepare the following two mixtures in flasks (make sure one is a 2 litre flask since you are going to combine the mixes after autoclaving):

Method

- Mix 1: Salt mix in 500ml of water:
 - 6g Na₂HPO₄
 - 3g KH₂PO₄
 - 0.5g NH₄Cl
- Mix 2: Agar:
 - 15g Agar
 - 500ml Water
- Autoclave mixes 1 and 2—after autoclaving combine the two mixes and add the following sterile solutions to the mixture:
 - 1ml 1M MgSO₄
 - 0.1ml 1M CaCl₂
 - 4.0ml 50% Glucose (filter sterilized)
 - 0.69g -Leu Drop Out
- Pour plates and label appropriately. 1L = ~40 plates

This method is based, with permission, on an original protocol available [here](#).

1.7.26 Microtubule Spindowns from Extracts

Contributed by [Timothy Mitchison](#), Harvard Medical School, Boston, MA, United States

MAP pelleting protocol.

The key variable in MT spindown experiments is ATP. Under high ATP conditions, conventional MAPs are selectively co-sedimented with microtubules. In the absence of ATP (or in presence of AMPPNP which induces rigor binding of motor to MTs), both motors and MAPs will bind to MTs and pellet with themicrotubules. For MAP and motor analysis, I often supplement the extract with exogenous taxol-stabilized MTs to ensure that binding sites are not limiting and I am not seeing competition effects. For a unknown protein, it is best to try a variety of conditions. Below is a protocol for MAP pelleting under high ATP conditions. I have listed the modifications for MAP/motor protocols afterwards.

Method

- Prespin extract in TLA100.3 at 70K for 20' at 4 deg.C. Transfer supe to tube on ice.

*Extracts must have a source of GTP. For *Xenopus* egg extracts, we simply add an energy regeneration mix to extracts and the extract maintains physiological GTP levels. For dilute tissue culture extracts, it is best to supplement the extract with 0.5 mM MgGTP. This can be done before or after the prespin. *

- Add 2 mM MgATP to extracts, warm to RT and add taxol to 5 uM. Mix well and incubate for 2'-3' before adding an additional 15 uM taxol (final is 20 uM taxol). Incubate for 20'-30' at RT - 37 deg.C.

*We use *Xenopus* extracts for which physiological temperature is RT; for tissue culture cells, tubulin will polymerize better at higher temperatures. 30 - 33 deg.C is a good compromise range to balance polymerization and proteolysis. Extracts must be supplemented with a peptide protease inhibitor cocktail just before warming up to prevent excessive proteolysis. *

- Layer the polymerized mixture onto a 1M sucrose cushion in BRB80 containing 0.5 mM ATP, 10 uM taxol and protease inhibitors and pellet in a TLS55 at 40K for 20' at 22 deg.C.

*You can also use a fixed angle rotor such as a TLA100.3 or TLA100.4. I like to pellet MTs at 100-150,000 g for 20 - 30' in TLA100 rotors over 30-40% glycerol/sucrose cushions. For larger ultra rotors, increase the speed and/or time to reach an equivalent clearing factor. *

- Save supe for gel/blot, aspirate cushion while washing 2-3x with BRB80 and remove as much of the cushion as possible (MTs form a clear, gelatinous pellet).
- Boil pellet in sample buffer and analyze.

This method is based, with permission, on an original protocol available [here](#).

1.7.27 Organotypic raft culture of PHKs

Contributed by Matt Lewis <hop2kin@yahoo.co.uk>

Organotypic raft culture of primary human keratinocytes.

- Trypsinize the PHKs off the flask and resuspend in (say) 2ml raft medium.
- Count cells via haemocytometry and isolate 10⁶ keratinocytes per raft from cell source to fresh medium.
- Remove the medium from the collagen plugs and overlay with the new medium containing PHKs.
- Incubate for 1-2 days to allow the PHKs to settle and become confluent.
- Separate the edge of the collagen plug from the wall of the well by going round it with a sterile scalpel.
- Allow the collagen plug to contract away from the walls for a few hours.

*This is like cutting round a cake in a cake tin. The PHK 'membrane' may contract from the edge also and detach a little from the collagen *

- Remove media, scoop out plug and optionally rinse in PBS. Deposit onto the mesh in the petri-dish, keeping everything sterile.
- This transfer is the trickiest part of the whole procedure. If you get it intact, right side up (ie. PHKs up), and roundish on the mesh, without having scraped off all the PHKs, then you've done well. Usually it ends up on the floor. Sometimes the PHKs all come away like a membrane; try to spread it back onto the collagen.*
- Feed the raft by adding 12-15ml of raft medium to the petri-dish.

*The idea is to wet the underside of the mesh but not to let the liquid come onto the upper surface. If the top of the raft becomes wet it will destroy the gradient and spoil the experiment. Watch out for air bubbles loitering under the raft. *

- Re-feed with raft media; wait 2 days
- Re-feed with raft media; wait 2 days
- Re-feed with raft media; wait 2 days
- After 8-14 days of growth/differentiation add in BrdU 8-12 hours before harvesting; see [BrdU protocol](<http://lab.methodmint.com/tasks/38/>)

This method is based, with permission, on an original protocol available [here](#).

1.7.28 Preparation for microdissection (Polyester)

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Basic protocol for Eosin & Haematoxylin histological preparation of microdissection slide.

- Place slides in 100% ethanol for 5 minutes
- Transfer slide to 100% ethanol, 95% ethanol, then 70% ethanol; 30 seconds/step
- Transfer slide into de-ionized water
- Transfer slide into Mayer's Haematoxylin
- Return to de-ionized water to rinse for 15 seconds x2 changing dH2O between steps
- Transfer slide to 70% ethanol
- Transfer slide into Eosin Y
- Return to de-ionized water to rinse for 30 seconds x2
- To reduce brittleness of tissue, place in 3% glycerol in de-ionized water
- Shake and air dry to remove layer of glycerol and water
- Microdissection is best performed in the following 5-10 minutes following preparation. If if tissue dries return tissue to 3% glycerol/water; step #10

1.7.29 Raft culture media

Contributed by Matt Lewis <hop2kin@yahoo.co.uk>

Raft culture medium, including standard supplements used.

Dilution factors are given here for any quantity but absolute quantities for a target of 100ml raft culture medium are shown in brackets.

- Add 3 parts (70ml) DMEM (including glutamine or glutamax) to a suitable container
- Add 1 part Ham's F12 (25ml)
- Add 5% FCS (5ml) and mix thoroughly
- Adenine (400ul)

(Sigma A8626), MW=135, final concentration=180mM, working stock=45mM, Dilution factor=250.

Dissolve stock in 0.05N HCl to 45mM and store working aliquots at -20C.

- Insulin (100ul)

(Sigma I6634), MW=5733, final concentration=5mg/ml, working stock=5mg/ml, Dilution factor=1000.

Dissolve stock in acidified H2O (0.1ml glacial AcCOOH + 9.9ml H2O) and store working aliquots at 4C.

- Hydrocortisone (40ul)

(Sigma H0135), MW=362, final concentration=400ng/ml, working stock=1mg/ml, Dilution factor=2500.

Follow instructions with vial (dissolve in EtOH then add equal volume of H₂O) and store working aliquots at -20C.

- Transferrin (1ml)

(Sigma T2036), final concentration=5mg/ml, working stock=500mg/ml, Dilution factor=100.

Dissolve stock in media (DMEM or KGM) and store working aliquots at -20C.

- Penicillin and streptomycin (1ml); and fungizone (200ul) may optionally be added to help prevent accidental infection of cultures.

- T3 (13.5ul)

(aka 3,3',5-triiodo-L-thyronine) (Sigma T6397), MW=673, final concentration=0.2nM, working stock=1mM, Dilution factor=7500.

Dissolve stock in 1N NaOH to 1mg/ml. Dilute 1000-fold in 0.1N NaOH and store working aliquots at -20C.

- Cholera toxin (18ul)

(Sigma), MW=87kD, final concentration=0.1nM, working stock=50mg/ml, Dilution factor=5750.

- EGF (5ul)

(Sigma E1264), final concentration=5ng/ml, working stock=100mg/ml, Dilution factor=20000.

Dissolve in media (DMEM or KGM) and store working aliquots at -20C.

- Mix thoroughly before use

This method is based, with permission, on an original protocol available [here](#).

1.7.30 Rotary Shadowing

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States

In EM rotary shadowing allows resolution of portions of the specimen that might otherwise have been obscured by a static shadow.

- Make 20µg of protein (per macromolecule of interest to be shadowed) up to a final volume of 500µl with PBS (or 0.155M ammonium acetate, pH7.4) then dialyze overnight against 500ml of 0.155M ammonium acetate (pH7.4)
- Turn on Edward's 306A coater (power, water, rotary pump, and diffusion pump).
- **Prepare the coater for shadowing:**

With the main valve closed allow air to enter the bell jar.

- Make sure that the twin electron beam gun power supply is off then wearing sterile gloves detach electrode wires from gun and remove gun from mounting pillar.
- Place gun face up on table, unscrew and remove front shields, then use air gun to clear away loose flaking metal. Remove old tungsten filament and discard. Remove and retain old tungsten rod.
- Reinstall a new acetone-cleaned filament and an acetone-cleaned tungsten rod containing 5cm of 100% platinum (better than platinum-palladium) wrapped about one end. In reinstallation, platinum-wire should be positioned immediately behind filament. Replace front shields.
- Remount gun in chamber at height indicated to give a 9deg angle from gun to stage. Use a metal rod to make sure gun is correctly aimed (angle may be checked with a protractor).

- Use air gun to clean carbon source then reload.
- Check the rotating stage is set at 0deg (graded at back surface of stage).
- Place a piece of filter paper on inside of bell jar to catch evaporated material.
- Replace bell jar; put on implosion protector then evacuate.
- **Prepare for spraying on of mica:**

Cleave in half previously cut 3mm x 3mm mica pieces and place cleaved surface up inside a 9cm petri dish. Have 6 cleaved pieces/petri dish; keep covered. Optionally cut a cross on cleaved surface with fine forceps.

- Take a microscope slide from ETOH wash and dry. Place 1mm x 1mm pieces of double adhesive tape in line at midpoint of slide, then place slide into another petri dish.
- Place covering paper in fume hood and attach airbrush to air source.
- Add 500µl of glycerol to the 500µl of protein in 0.155M ammonium acetate (pH7.4). Mix gently by moving contents in and out of a plastic pipette, making sure that the glycerol goes into the solution.
- Add mixture to airbrush cup. Before attaching cup to airbrush loosen conical nozzle as much as possible without detachment.
- Attach cup to airbrush, then turn on air supply. place petri dish with cleaved mica in a box in fume hood and remove top of dish exposing mica.
- Aim airbrush at mica standing 2-3 feet away and press trigger to initiate spraying.
- At end of spraying, place top on petri dish; wash out cup and airgun with dH₂O
- Sprayed mica can be stored at 4deg C for one week. For rotary shadowing place two of sprayed mica pieces onto microscope slide containing double adhesive tape.
- Aerate bell jar and attach microscope slide to stage with tape. Replace bell jar and implosion protector; evacuate and turn on film thickness monitor.
- When vacuum reaches 2×10^{-5} mbar turn on rotating stage (120rpm).
- Turn on twin electron gun power supply setting voltage at 2.5kV and setting amperage dial to 4.
- Allow vacuum to return to 2×10^{-5} mbar then set amperage to 0.
- Turn voltage to 4.0kV and turn amperage up slowly to 50mamp.

If arcing occurs turn amperage down quickly then slowly increase again.

- Once 50mamp is reached open shutter on film thickness monitor and shadow for 30 seconds.
- At end of shadowing close shutter, turn down amperage to 0 and voltage to 0.

Filter paper colour indicates if platinum-palladium evaporated (grey colour). When mamp is over 50 tungsten evaporates giving a light brown colour.

- Put on welders goggles.
- Turn on carbon source and increase to setting 7 watching evaporation. Evaporate for 10 to 15 seconds or almost half of carbon point then turn off. Turn off rotation stage.
- Aerate bell jar and remove glass slide.
- Float replica off of mica by insertion of gH₂O. Come up from below with copper electron microscope grid. Place grid on filter paper in petri dish and allow to dry.
- Examine in electron microscope.

References

Klaus Kühn, Hanna Wiedemann, Rupert Timpl, Juha Risteli, Hans Dieringer, Tilman Voss, Robert W. Glanville
Macromolecular structure of basement membrane collagens *FEBS Letters* (1981) 10.1016/0014-5793(81)81012-5

1.7.31 Silica-based Plasmid Miniprep (vacuum manifold)

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

Silica-based Plasmid Miniprep (vacuum manifold version)

Requirements

GTE: 50 mM Glucose, 25 mM Tris 8.0, 10 mM EDTA RNase A (0.1mg/ml) Lysis Buffer: 200 mM NaOH, 1% SDS (prepare fresh) 4.0 M KOAc: (pH 5.5 with acetic acid) Silica Suspension: resuspend immediately before use by shaking vigorously Wash Solution: 50 mM NaCl, 10 mM Tris 7.5, 2.5 mM EDTA, 50% Ethanol

Method

- Prepare Silica Suspension
- Suspend 2-10 grams of Silica (Sigma S-5631) in 20 mls H₂O and allow to settle for 2 hours
- Remove milky supernatant and suspend settled silica in 20 mls H₂O and allow to settle
- Repeat two more times
- Estimate volume and resuspend silica in 2 vols 6M Guanidine Hydrochloride-1M KOAc buffer, pH 5.5.
- Lyse bacteria
- Pellet 1.5 mls culture in microcentrifuge tube 10 sec @ 14,000 rpm
- Resuspend cells in 200 ul GTE
- Lyse cells with 200 ul lysis solution for 1 minute
- Add 200 ul KOAc and mix by inversion
- Remove debris by centrifugation 10 minutes @ 14,000 rpm
- Put a cleaned Qiagen mini prep1 column on manifold (i.e. QIAvac 24 Cat#19403) and load 200ul of silica suspension. Do not have the vacuum on at this stage

We use the used Qiagen columns from the mini prep and gel extraction kits. To clean these columns pass 1-2ml H₂O (65°C) followed by 0.5ml EtOH wash.

- Isolate plasmid DNA
- Transfer the supernatant to the silica slurry and mix by pipetting.
- Turn on vacuum and Wash 2X 1ml Wash solution.
- Spin the column 1min high speed to dry (this is very important)
- Elute the plasmid DNA with 50 to 100ul H₂O or EB. Yields approx 100-200 ng/ul plasmid DNA

References

J BROWN A rapid, non-toxic protocol for sequence-ready plasmid DNA *Technical Tips Online* (1997) 10.1016/S1366-2120(08)70079-5

1.7.32 Silica Suspension for plasmid recovery

Contributed by Ian Chin-Sang, Queens University, ON, Canada

Silica Suspension for plasmid recovery

- Suspend 2-10 grams of Silica (Sigma S-5631) in 20 mls H₂O
- Allow to settle for 2 hours
- Remove milky supernatant and suspend settled silica in 20 mls H₂O
- Allow to settle for 2 hours (repeat 3 times)
- Estimate volume and resuspend silica in 2 vols 6M Guanidine Hydrochloride-1M KOAc buffer, pH 5.5.

This method is based, with permission, on an original protocol available [here](#).

1.7.33 Tear Collection Method

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States

Tear Collection Method; Scot Bower and Denise Sediq 4.13.09 developed from Jones 1997

- After informed consent, the tear sample should be collected from one eye (left eye) of each subject. Subject's wearing contact lenses must remove their lenses and wait 5 minutes before proceeding with the tear collection procedure.
- For basal tear collection instil one (1) drop of 0.5% proparacaine on the cornea and conjunctiva.
- Instruct the patient to sit with eyes gently closed for 2 minutes before tear collection.
- Tear collection will be accomplished by using a 2.0 mm x 10.0 mm polyester fiber rod (Transorb Wick, Filtrona, Richmond, VA) as previously described [Jones 1997]
- Place the wick in contact intermittently with the tear fluid at the lower cul-de-sac for a minimum time to obtain fluid without irritating the subject's eye (30 to 60 seconds)
- Place the collection wick gently into a 1.5-ml Eppendorf tube, and freeze at -70 C for storage.

References

Jones DT, Monroy D, Pflugfelder SC A novel method of tear collection: comparison of glass capillary micropipettes with porous polyester rods. *Cornea* (1997) PMID:9220244

1.7.34 TMP UV Integration

Contributed by Ian Chin-Sang, Queens University, ON, Canada

TMP UV Integration (from Scott Clark)

- Recover L4 array-containing animals in 200ml of M9.

- Add 20ml of 1mg/ml TMP (Trimethyl Psoralen in DMSO) to 380ml M9, resulting in final concentration 50mg/ml.

Note, Although freshly made TMP solution is probably best, I have used TMP sol'n that has been stored wrapped in foil at -20C as well. It appears OK for a few weeks or more

- Add worms to TMP sol'n.
- Let worms sit for 15 minutes, covered at RT.
- Transfer to unseeded large plate in dark.
- Expose worms to 350mJ(x100) long wave UV in Stratalinker 1800 (without lid).
- Add some concentrated OP50, and maintain worms covered for 5 hours at RT or O/N at 15C.
- Pick 2-3 L4 animals/plate for 8 plates, as P0s. (way extra, but convenient)
- Transfer P0s to new plates every 24 hours for 2-3 days.
- Pick 100-120 F1, one per plate.
- Pick 2 F2 from each F1 plate. one/plate.

As some F1s will be sterile, I will pick extra F2s from some plates to get 200-250 total F2 animals

- Score for 100% non-Muv, Rol, or gfp or whatever.
- Typically recover 1-3 integrants/100 F1. (compared with 1-3 integrants/3-400 F1s by X-ray mutagenesis.)

This method is based, with permission, on an original protocol available [here](#).

1.7.35 Transformation of Frozen *Saccharomyces cerevisiae*

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

Transformation of (Frozen) Competent *Saccharomyces cerevisiae*

1M Lithium Acetate: 10.2 g in 100 ml ddH₂O – autoclave

50% PEG: Dissolve 50g in 30ml ddH₂O and stir, bring total volume up to 100ml and heat and stir to combine. Filter sterilize (this might take about 10- 20 min). Bubbles will disappear once you filter sterilize.

ssDNA: Single-stranded salmon sperm carrier DNA is found in the -20C in its own box at a concentration of 1.5mg/ml in water (we do not sonicate). It must be boiled before use, but you can refreeze 3 times after the initial boil before you need to boil it again. Keep a few tubes in your own box for you personal use.

Requirements

1M Lithium Acetate 50% PEG ssDNA

Method

- Spin down 100ul frozen competent cells from -80C(or made fresh) 1 minute at ~14,000 rpm. Use one tube per single or double transformation.
- Aspirate supernatant and add the following in order.
- 240ul 50% PEG
- 36ul 1M LiAc
- 79ul ssDNA

- 5ul yourDNA

To give you a total solution of 360ul

A master mix may be made, by leaving out your DNA and mixing the other three ingredients, vortex lightly to mix before aliquotting. If doing a double transformation use 3ul of each of the DNA and reduce the amount of ssDNA to 78ul per transformation to keep the DNA/PEG-LiAc ratio the same.

- Resuspend the yeast cells in the mixture by vortexing well to remove any clumps.
- Incubate on rocker in 30oC room for 30 minutes.
- Heat shock in 42oC water bath for 15 minutes.
- Spin down at 14,000 rpm for 1 min., aspirate off the supernatant.
- For double transformation resuspend cells in 100 ul ddH2O and plate out all cells. For single transformation resuspend cells in 200ul ddH2O and plate out half (100ul). Use appropriate media plates. (-trp, -trp -leu, etc. . .)
- Incubate plates in 30oCvroom for 2-4 days until colonies appear.

This method is based, with permission, on an original protocol available [here](#).

1.7.36 Using a Gel Doc system

Contributed by [Paul Barber](#)

Using a Gel Doc system for imaging gels via UV

- Turn camera on. To access camera lift the top cover of the hood.
- Open Kodak Imaging program by clicking on desktop icon.
- Click on Capture Image button on top left of screen.
- Place stained gel on UV box. Position gel on left edge and center.
- Close hood door. Turn on UV.
- Click on Take Picture button.
- After photo appears on screen turn off UV
- Crop gel by selecting crop tool and select area to be cropped. Click on Edit menu then select crop.
- Click on File menu and select page set up. Change paper size to gel. Select roll paper. Click on banner and save paper.
- Click on File menu and select print. Then click on options. Select suppress header and footer. Click on scale to paper size. Then print.
- Cut photo from roll paper. Hit the roll paper button on the printer for paper to be sucked back up.

This method is based, with permission, on an original protocol available [here](#).

1.7.37 Valap

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Valap

Requirements

50 g vaseline 50 g lanolin 50 g paraffin

Method

- Combine 50 g Vaseline, 50 g lanolin, and 50 g paraffin into a 1L Pyrex beaker
- Heat gently on hotplate, stirring occasionally, until components have melted and are well-mixed.
- Aliquot into small screw-cap jars (~50 ml capacity) for storage.

Store at room temperature

References

Clare M. Waterman-Storer [Microtubule/Organelle Motility Assays](#) (2001) 10.1002/0471143030.cb1301s00

1.7.38 X-gal overlay assay for Yeast plates

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

X-gal overlay assay for Yeast plates

We find that this method is less sensitive than the “standard” lacZ filter lifts and can be useful if your bait in the yeast two hybrid assay has weak self activation.

Use half of stock solution if you are only overlaying up to eight large plates. Use 3.5 ml per small plates (60mmX15mm), or 7ml for large plates (100mmX15mm).

Requirements

.5 M Potassium Phosphate Buffer pH 7.0: (mix 61ml of 1M K₂HPO₄ and 39ml of 1M KH₂PO₄ and add 100ml dH₂O)
 Dimethyl Formamide (DMF) 10%SDS: 10g in 100ml H₂O beta-mercaptoethanol X-gal: 100mg/ml in DMF Low melt Agarose 6% DMF 0.1% SDS in 0.5M KPO₄: for 100ml mix 93ml of phosphate buffer, 6ml of DMF and 1 ml of 10% SDS

Method

- Preparation of Low-Melt-Agarose:

To 100ml Stock Solution add 0.5 g low melt agar and microwave in ten or fifteen-second intervals until solution becomes clear and over 65C.

- Let solution cool for a minute or two then add:

10ul of Xgal (100mg/ml) for every ml of solution you are using. (you can use the unused portions of the gel for up to a week) 0.5ul of beta mercaptoethanol (BME) for every ml of solution.

From the time you add the BME until gel solidifies it is best to work in a fume hood.

- Apply directly onto yeast plates (3.5ml small or 7ml large plates) w/ plastic pipette or simply pour from a measured falcon tube.
- Cover immediately with foil (to keep dark) and let sit for 10 minutes.

- Wrap in parafilm and put at 30C. Strong inducers should turn blue within 1-2 hours, weaker ones over the next 12-24 hours.
- Colonies may be picked through the top agar for a few days later and grown if appropriate selection media.

This method is based, with permission, on an original protocol available [here](#).

1.7.39 Zymolyase plasmid recovery from Yeast

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

Yeast plasmid Extraction Protocol Using Zymolyase and Manifold Miniprep Method. This is a simple and efficient way to recover plasmids from yeast. There are no glass beads and no phenol:chloroform steps.

The Lysis (aka Sol'n II) and Neutralizing (aka Sol'n III) solutions are the same as the ones used in our bacteria miniprep solutions.

Requirements

67 mM KH₂PO₄ pH 7.5 (This is the resuspension solution used in Clontech Yeastmaker™)

25 ug/ul Zymolyase/Lyticase (~0.5 units/ul) (Sigma #L2524 or ICN Zymolyase 20T cat#320921 20,000units/g) in 0.01M Na₂HPO₄ pH=7.5 in 50% glycerol (store at -20C). You can also use 10mM Tris pH=7.5 as the buffer.

Lysis sol'n (1ml 10%SDS, 200ul 10N NaOH in 10 ml of H₂O)

Neutralizing sol'n (4M KoAc pH 5.5)

Method

- Obtain 1ml of saturated yeast culture grown in selection media (i.e. -Leu) and spin down at full speed for 1 min., then aspirate the supernatant.
- Resuspend pellet in 50ul- 100ul of 67mM KH₂PO₄ pH 7.5 and vortex.
- Add 10ul Zymolyase (25 ug/ul (0.5 units/ul) in 0.01M Na₂HPO₄ and 50% glycerol) Incubate at 37°C for 1 hour. This step degrades the yeast cell wall creating spheroplasts and from this step onward we treat just like a plasmid mini prep for E. coli. Note: other protocols use 10ul of 5 uint/ul zymolyase or up to 200 units. You may have to use a higher concentration if spheroplasts are not formed.
- Add 200ul Lysis Sol'n (1ml 10%SDS, 200ul 10N NaOH in 10 ml of H₂O) and mix by inversion.
- Add 200ul Neutralizing Sol'n 4M KoAc pH 5.5) and mix by inversion.
- Spin at full speed for 10 mins.
- Add supernatant to manifold columns containing 200ul celite (silica) slurry and isolate DNA using our miniprep method (wash 2X with Wash Sol'n, spin to dry, and elute columns in new microfuge tube). Alternatively you can pass the supernatant over a Qiagen mini prep column. Elute in 50 to 100ul EB or TE.

*Use 5 to 10 ul of DNA to transform E. coli (i.e. MH6 -LEU) competent cells and grow colonies on Amp plates. (note: If pGBKT7 (Kan R) was used for your bait vector then you can just transform into XL1 Blue competent cells and select for Amp resistance.)

Patch single colonies onto M9 -LEU plates (if your library is -LEU). If colony grows overnight at 37°C then grow up colony in 2XTY + AMP overnight, and purify by usual bacteria Miniprep Method.*

This method is based, with permission, on an original protocol available [here](#).

1.8 Model organisms

Model organism protocols.

1.8.1 *C. elegans* Immunoprecipitation

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

Immunoprecipitation protocol for use with *C. elegans* worm lysate

- Preparation of worm lysate:
- Add 2 ml of PLC buffer to 0.25 ml of worm pellet.
- Break up the worms by sonicating.
- Spin 10 minutes @ 13000 g in microfuge, save supernatant.
- Quantitate protein in lysate using Pierce BCA kit.
- Wash 100 ml (actually 10 ml of beads since it comes as a 10% suspension) of Protein A Sepharose in PLC buffer. Spin 3 minutes @ 13000 g in microfuge.
- Immunoprecipitate using 1 mg of lysate protein (may have to use more depending on the protein you're looking at) mixed with 5 mg of antibody and 10 ml of the washed Protein A Sepharose. Total volume of the mixture should be 1 ml.
- Incubate on a rocker platform at 4°C for 1 hour.
- Wash 3x with 1 ml of PLC buffer.
- Add 50 ml 1x sample buffer.
- Boil 5 min.
- Load 20 ml on an SDS PAGE gel.

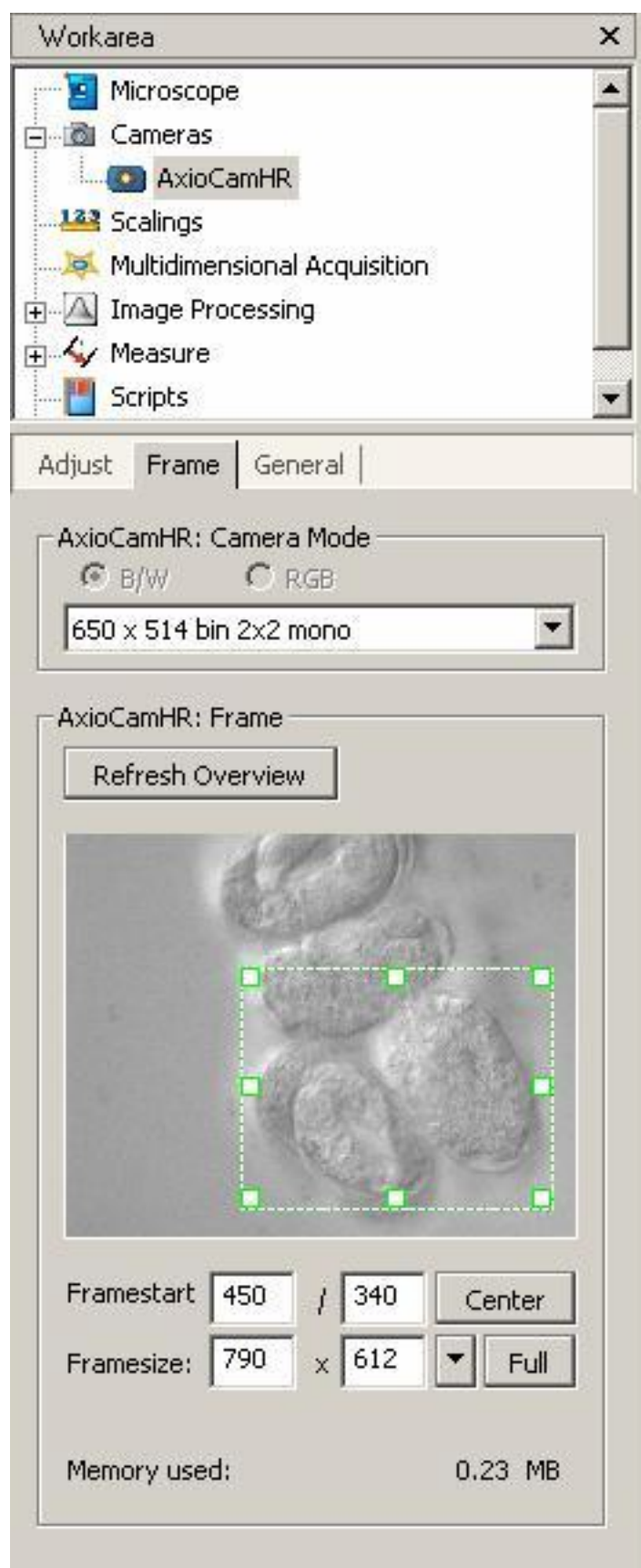
This method is based, with permission, on an original protocol available [here](#).

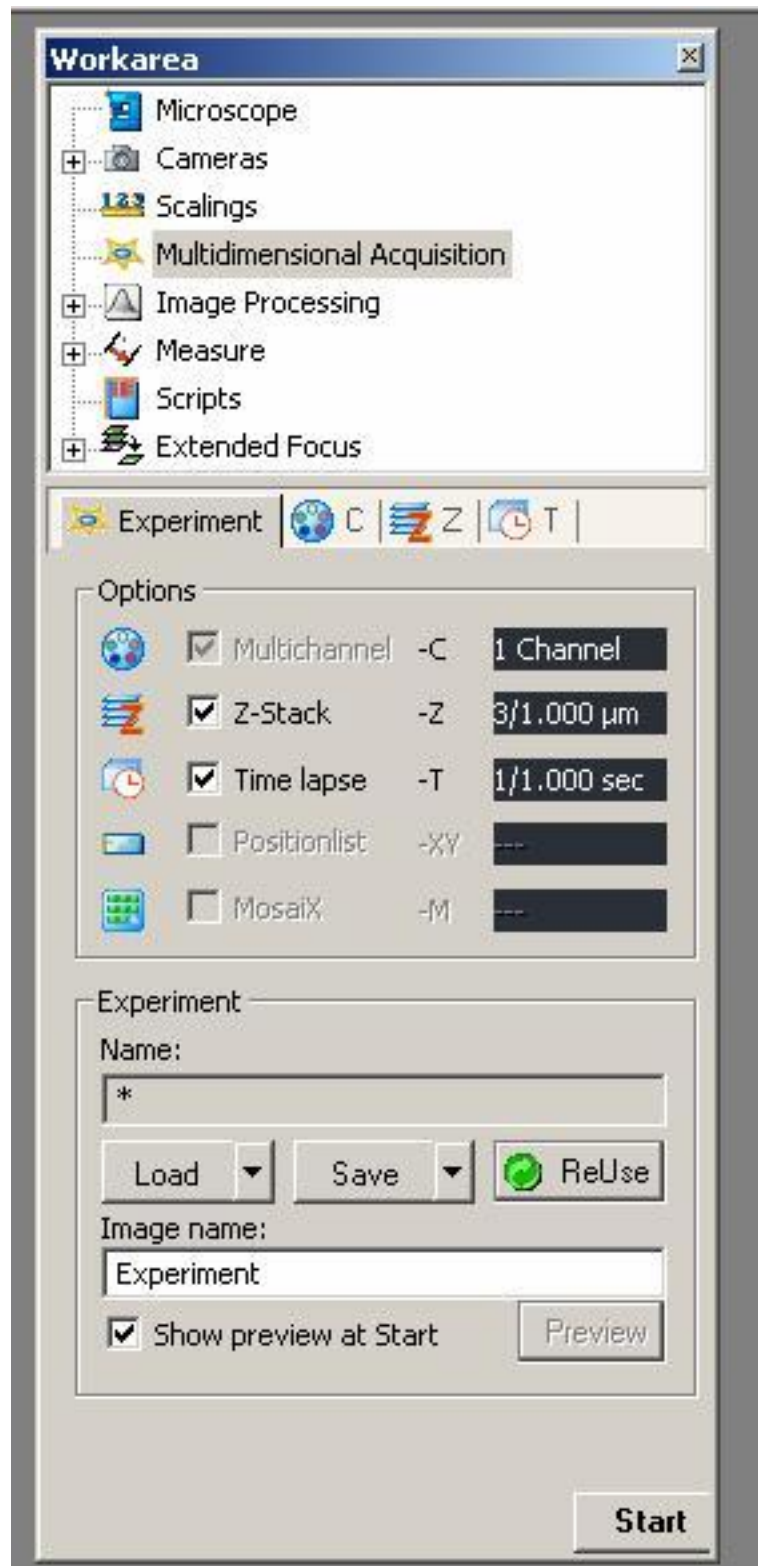
1.8.2 Embryo 4D Micro (Zeiss Axioplan 2 Axiovision 4.5)

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

Embryo 4D Microscopy Using the Zeiss Axioplan 2 and Axiovision 4.5 Software

- Start Axiovision Rev 4.5 (make sure the camera and microscope are turned on). You want to setup the Axiovision parameters before putting the embryo on the slide
- Microscope settings: Objective 10X or 20X (before oil). Transmitted light filters should be set at 4 and 2 for low neutral setting (not much light comes through). If you choose low neutral for 4D in channels settings for during acquisition these filter setting will be set for you automatically.
- Camera Settings: You want to frame your image as 1 embryo will not take up all the camera capture area. This will also reduce the size of your movie file. See boxed area below. You also want to choose 650 X 514 bin 2X2 mono in the camera mode. If you use standard mode 1300X1030 your file will be greater than 2GB after 8 hours of time lapsed capture.
- Multidimensional Acquisition: You can load a saved experiment or setup a new one with the following properties. Experiment: make sure that the Z stack and Time lapse boxes are checked.





- You can give the experiment a name (and save the parameters for future use) and you can also give the Image a name.*
- Channel Assignments (C):

During Acquisition choose DIC (low neutral setting for 4D) You can take a measurement but it will change at 40X or 63X oil. You want to make sure that the exposure time is less than 100ms at 63X objective lens. If the embryo looks too dark do not worry about this as we can increase brightness and contrast after we captured the recordings. You don't want too much light and too much exposure as the embryo could heat up and die.

After Acquisition setting choose: nothing- i.e. leave this blank (This is very important!) we used to have the shutter closed here but this results in the shutter being close between each Z-section. Instead close the transmitted light shutter after the Z-stack. See below. 10X to 40X oil -> 63X oil

Make sure you have the DIC prism matched with the objective lens i.e DIC prism III with 40X and 63X and DIC II with 20X

- Z stack: While your are in 40X or 63X oil objectives focus in the middle of the embryo.

The Z stack box should be checked and you want the Start/Stop mode (not the Center Mode). You want to choose a Z stack of about 20mm (10 slices at 2 mm each). To do this first focus to the bottom of the embryo by turning the focusing knob on the right counter clockwise (i.e. your right thumb moves downward). Focus until you just reach out of focus. Press the "Start" button . Now move up in the focus (right thumb move up) so you get about 10 slices. If you need to move up more to get out of focus then put in more slices. If you want to add more slices add to the top of the embryo (i.e focus with right thumb moving up and then press "stop" The start and stop buttons sets the interval.

If you are making short movies (less than 2 hours) then you may take sections at 1mm and take more sections to get greater details. Important! Make sure to choose after Z-stack... "transmitted shutter close" This way the light will be off the embryo between time points.

- Time points (T): Choose interval as 1 minute and the duration should be about 8 hours.
- Once all parameters are set you can hit "start" at the bottom right hand of the work area. Also when you start the capture, make sure to pull the right knob out all the way and the left one pushed in to allow all the light to go to the camera port (See green arrows below).
- After the recording is done save your file. Make sure to return the objective lens to 10X with the stage up and clean off any oil from the objectives. Close Axiovision and turn off the microscope and the camera Don't forget to cover the microscope.

This method is based, with permission, on an original protocol available [here](#).

1.8.3 Embryo 4D microscopy mounting

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

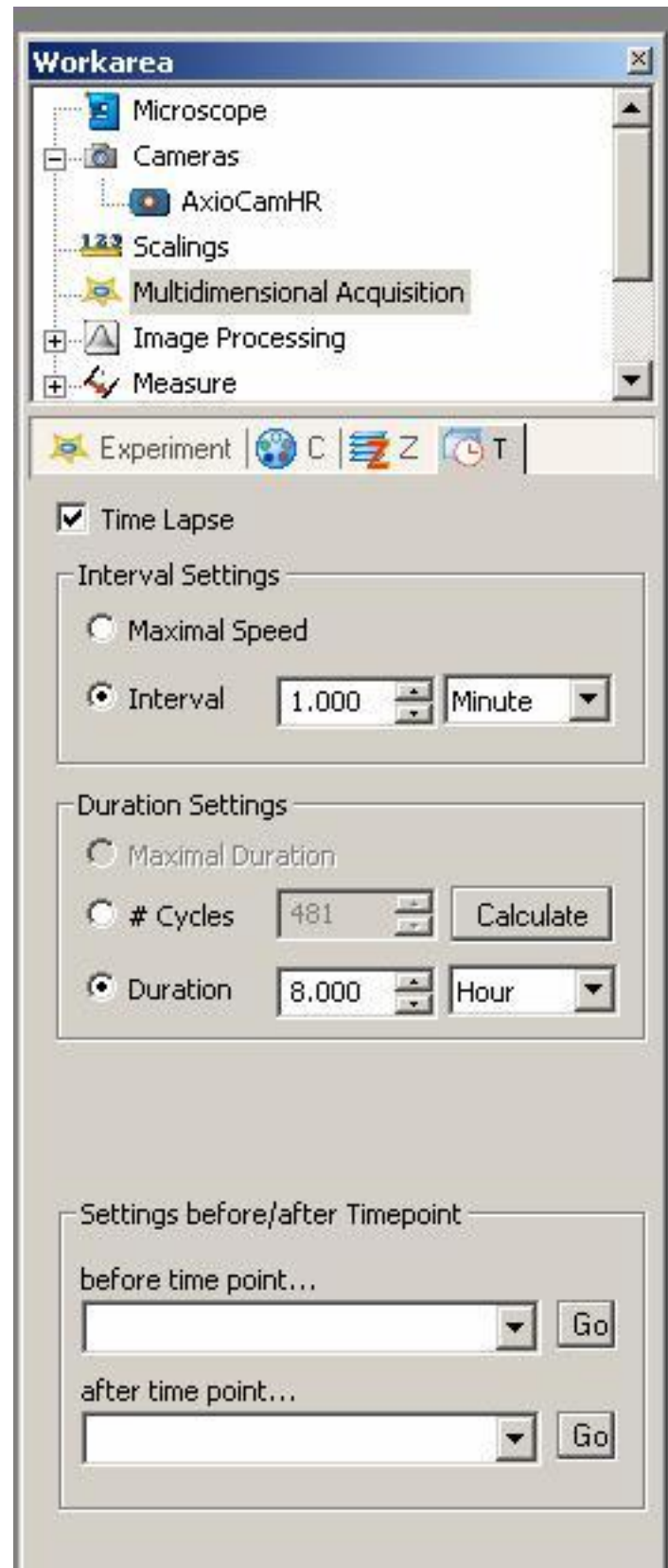
Embryo 4D video Microscopy preparation of slides and embryos.

Perform the following steps in order. Have all the materials ready and on hand and try to be quick as possible.

Before you start dissecting out the embryos make sure that you log on to the microscope computer and that the microscope and camera are on.

Method

- Prepare 50-100ml of 3.5% agarose (the one used for DNA electrophoresis) in distilled water. This concentration works the best for me. Other labs use agar. Dissolve the agarose by heating 2-3 times for at most 30 sec in the



microwave. Don't allow it to overflow. Distribute about 2-5 ml in 10 ml test tubes. Seal the tubes with parafilm and keep them in the fridge until use.

- Melt the agarose by heating it directly in the flame, and try not to burn it. You can keep the agarose fluid in the heating block set up at about 50-60°C for 24h.
- Fill a well of a the spot plate (made of glass) with about 100 ul M9 and place about 10 gravid hermaphrodites in it.
- Prepare the pad: Stick a 0.1mm thick tape (paper tape) along a slide. Make one more and use them as spacers. Place a third slide in parallel between the spacer slides. With a Pasteur pipette spot a drop of agarose in the middle of the third plate (the one without the tape). Flatten the agarose by dropping immediately a fourth slide perpendicularly over the other 3 slides. Do not press. Wait 15-30 seconds to cast and gently slid out the underneath slide with the pad. The pad should be smooth. From this step you should be relatively quick. Don't allow the pad to dry out.

This step is also described in Ledwich D et al., Methods in Enzymology, vol. 322, 76-88.

- With a thin needle (27G1/2) cut the worms in the middle of the body under dissecting scope trying to avoid the zones of the gonad with 1-2 cell embryos. The embryos will pop out from the mother. Using a mouth pipette (Sima-Aldrich Cat# A5177 Aspirator tube assemblies for calibrated microcapillary pipettes) and a drawn out microcapillary suck the embryos together with a little M9 and place them on the agarose pad. Remove most of the excess M9 by sucking with the mouth pipette, and cover the pad with a 22x22 coverslip. However, the M9 should cover the whole pad. Remove the remaining excess M9 with a paper towel inserted between the coverslip and the slide up to the pad. Try to remove all excess M9 or the recording embryo will become out of focus in time.
- Seal with immersion oil. Put 1-2 drops of immersion oil at the edge of the coverslip. It will diffuse under the coverslip and completely surround the pad. If it is necessary add one more drop of oil. Use of immersion oil instead of vaseline for sealing has the advantage that it does not mess up your optics. I use cheaper immersion oil for sealing and keep the Zeiss oil only for immersion optics.
- Mount the slide under the microscope. With 10x optics find a 1-2 cell stage embryo, and then switch to 60x. Take 10-12 focal planes at 2-2.2mm distance, for 8h (see below)

References

Duncan Ledwich, Yi-Chun Wu, Monica Driscoll, Ding Xue [Analysis of programmed cell death in the nematode *Caenorhabditis elegans* \(2000\) 10.1016/S0076-6879\(00\)22009-0](#)

This method is based, with permission, on an original protocol available [here](#).

1.9 Molecular biology

Molecular biology protocols.

1.9.1 5X Long PCR Buffer

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

5X Long PCR Buffer (store in 1ml aliquots in -20°C).

This buffer seems to work well with Worm Genomic DNA using Taq/Pfu (3:1) mixture.

Requirements

425 mM KOAc 125 mM Tricine pH 8.7 (adjust pH of Tricine stock solution with KOH) 40% glycerol 5% DMSO 1.2 mM Mg(OAc)₂

Method

- Combine ingredients in a suitable container. For 10ml 5X long PCR buffer use:
- 4.25 ml 1M KOAc
- 1.25 ml 1M Tricine, pH8.7 @ 25 degrees C (with KOH)
- 4.00 ml glycerol
- 0.50 ml DMSO
- 120 ul 500 mM Mg(OAc)₂
- Store in 1ml aliquots at -20°C until required.

This method is based, with permission, on an original protocol available [here](#).

1.9.2 6X DNA Loading Buffer for Agarose

Contributed by <jt03@ic.ac.uk>

Easy to use, add 2ul per 10ul of DNA solution.

Ficoll 400 performs better than glycerol or sucrose based loading buffers.

EDTA and SDS are included as enzyme inhibitor and protein denaturant respectively, they help sharpen up bands a bit.

Orange G is the dye of choice as it won't obscure smaller DNA bands like other dyes.

Requirements

1. Ficoll 400
2. 0.5 M EDTA solution
3. 10% SDS solution
4. Ultrapure H₂O
5. Orange G crystals

Method

- Combine the following in a 50ml falcon tube (you'll need the extra space for mixing)
- 1. 2.5 g Ficoll 400
- 2. 400 ul 0.5 M EDTA
- 3. 18 ul 10% SDS
- 4. Ultrapure H₂O to 10 mls
- Add a small pinch of Orange G (Don't use your fingers). You're aiming for a deep orange colour.

- Leave on a shaker/shaking incubator for an hour or so (it takes a while to dissolve)
- Once dissolved it's ready to use. Store at RT, lasts forever.

1.9.3 Agarose gel electrophoresis

Contributed by Ivan Delgado <ivanjdo@gmail.com>

The preparation, and running, of an agarose gel for nucleic acid separation.

Requirements

10X DNA loading buffer (25% Ficoll (Type-400) in dH₂O (note: 25% Ficoll is dense and will not go into solution readily, just stir for about 1-2 hours). The buffer is ready for use. But it is a very good idea to add a dye to not only be able to see your sample as you load it on a gel, but to track what size fragments during the run. Any combination of these dyes can be used: 0.25% Bromo-Phenol-blue (300 bp); 0.25% Xylene Cyanol (1-2 kb); 0.25% Orange-G (50 bp). Also, EtBr can be added to the buffer or the gel at a concentration of ~1 mL/10 mL of EtBr. I find it to be better to add it directly to the gel to prevent degradation during storage of the buffer) 5X TBE (Mix 54 g of Tris base, 27.5 g of boric acid, 20 mL of 0.5M EDTA, pH 8.0, and dH₂O to 1 L) 1X TBE (Mix 100 mL of 5X TBE with 900 mL of dH₂O)

Method

- The concentration of agarose needed to resolve the following fragment sizes:

1.2% = 100 bp - 5 kb 2% = 100 bp - 2 kb 4% = 20 bp - 500 bp

- Mix the desired amount of agarose with 1X TBE in a flask. For a 1% gel, add 1 g of agarose to 100 mL of 1X TBE
- Microwave into solution (while microwaving, take flask out of microwave swirl a few times). It is important the agarose is completely into solution
- Add EtBr if desired

RECOMMENDED: add EtBr to the gel, this way it is quicker to visualize the DNA fragments right after the gel run

- Pour gel into agarose gel set up
- Wait until the gel solidified (~1 hr)
- Mix DNA samples with loading buffer (1 mL of 10X loading buffer for each 9 mL of DNA sample)
- Carefully load DNA samples into the wells of the agarose gel
- Using 1X TBE as running buffer, run the agarose gel (100 V is typically more than enough)
- Visualize the DNA bands on a UV box or Imaging system

This method is based, with permission, on an original protocol available [here](#).

1.9.4 APES treatment of slides

Contributed by Matt Lewis <hop2kin@yahoo.co.uk>

This is an alternative to poly-L-lysine treatment. The APES coating makes the slides more adhesive for fixed tissue and living cells.

- Prepare 1% acid alcohol with [1% (v/v) concentrated HCl, 70% Ethanol, 29% H₂O]

- Immerse slide in 1% acid/alcohol for 30 mins to clean the slides.
- Rinse in running water, then immerse in distilled water, then remove and allow to dry.
- Immerse slide in acetone for 10 minutes
- Immerse in [2%(v/v) APES in acetone] for 5 minutes. APES is aminopropyltriethoxysilane (Sigma A3648)
- Immerse briefly in 2 sequential rinses of distilled H₂O
- Allow to dry and store in a dust-free container. Slides will keep well for months but not years.

This method is based, with permission, on an original protocol available [here](#).

1.9.5 Bacterial colony screening by PCR

Contributed by Matt Lewis <hop2kin@yahoo.co.uk>

One of the fastest way to screen bacterial colonies.

With a PCR machine that takes 24 tubes you can routinely screen 22 colonies + 1 negative + 1 positive control.

- Plan your primers.

*Ideally you want a primer pair that can only work if the correct construct is present eg. a vector flanking primer and a gene specific primer. However, this may not allow you a positive control (essential) so you might have to use both vector flanking primers instead. If you have enough space in your PCR machine you could do both. *

- Set up 24 PCR tubes each containing 5ml H₂O.
- Touch a fresh toothpick (or yellow tip) onto a colony, dip it into a PCR tube, then streak it onto a fresh replicate agar plate using a numbered template (that is, all 24 colonies on a single agar plate).
- Repeat this for all colonies.

For negative control use a colony that is negative (or use nothing).

For positive control use a colony that will yield a product with your primers. If you don't have a +ve colony then use a tiny amount of plasmid DNA

- Incubate the replicate agar plate at 37C overnight.
- Set up an appropriate number of PCR pre-mix as follows (e.g. 25x):

1 x pre-mix|25 x pre-mix -|-| 11.5ul|H₂O|287.5ul 2ul|10 x PCR buffer|50ul 0.6ul|MgCl₂ (50mM)|15ul 0.5ul|10mM dNTPs (i.e. a mixture of all four at 10mM each)|12.5ul 0.1ul|primer 1 (100mM)|2.5ul 0.1ul|primer 2 (100mM)|2.5ul 5ul|Templatel- ul 0.2ul|cheap Taq|5ul

- Add 15ml of pre-mix to each PCR tube.
- Set up PCR programme as follows:

Cycles|Temperature|Duration -|-| 1|94C|60 seconds 25|94C|30 seconds 55C|30 seconds 72C|1 minute

- While the PCR is running prepare the agarose gel ready to analyze the PCR products.
- When you have the result you can go to the replica agar plate on the same day and set up miniprep cultures of the likely candidate colonies.

This method is based, with permission, on an original protocol available [here](#).

1.9.6 Bacterial mediated RNAi

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

Protocol for bacterial mediated RNAi

- Transform 0.5 ul of RNAi plasmid vector (i.e. L4440, LT61 etc.) into HT115 competent cells.
- Plate on Amp (50ug/ml) Tet (15 ug/ml) LB plates and grow O/N 37oC.
- Pick colonies and inoculate 10ml 2XTY Amp (50ug/ml) Tet (15ug/ml) and grow overnight at 37oC (saturated).
- Once saturated store cultures at 4oC. You may also want to freeze down a sample in 15% glycerol at -80oC. RNAi bacterial cultures should only be kept for no more than 1 month at 4oC. New plasmid transformations or inoculations from frozen stock should be made every month or when needed.
- Prepare RNAi plates by spreading 65ul (flame hockey stick) to each MYOB worm plate:
- 5ul Amp (100mg/ml)
- 5ul IPTG (0.8M)
- 55ul H2O

If we assume there is about 10ml of MYOB on each plate the final concentrations are roughly 50ug/ml Amp, and 0.4mMIPTG (note we leave out Tet on the plates see below). Make a master mix and scale up for how many plates required. Note the IPTG induction is done on the plate.

- Seed 100ul (flame hockey stick) of saturated bacteria culture on each plate. Don't forget to include an empty vector (L4440) control with every RNAi experiment. Also a positive control i.e. fem-1 (LT63 or D9) unc-22 (LT61 or D7) or GFP (L4417 or D11) vectors from the Fire Lab kit. Let seeded plates grow overnight at room temperature and then store at 15oC.
- Pick 5-6 L 4 animals to each plate. Observe progeny for RNAi phenotypes. It may be necessary to transfer some of the progeny to a new RNAi plate.

This method is based, with permission, on an original protocol available [here](#).

1.9.7 C. elegans microinjection

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

One way to make transgenic animals in C. elegans we use a microinjection technique. Briefly, a DNA construct (plasmid, cosmid or YAC) or PCR product with your gene(s) of interest is mixed with a co-injection marker and injected into the distal gonad (syncytium). The injected DNA is taken up into the mature oocyte's nucleus. The DNA exists as an extrachromosomal array (i.e. not integrated in the chromosome) which segregates randomly and can be lost, that is why we need a marker to follow which animals have the array. F1 progeny that show the co-injection marker phenotype are picked and transgenic lines are established by keeping those animals that segregate the array in their F2 generation. Usually 1 in 10 F1 progeny with the array will give you a transgenic line.

Typical injection concentration:

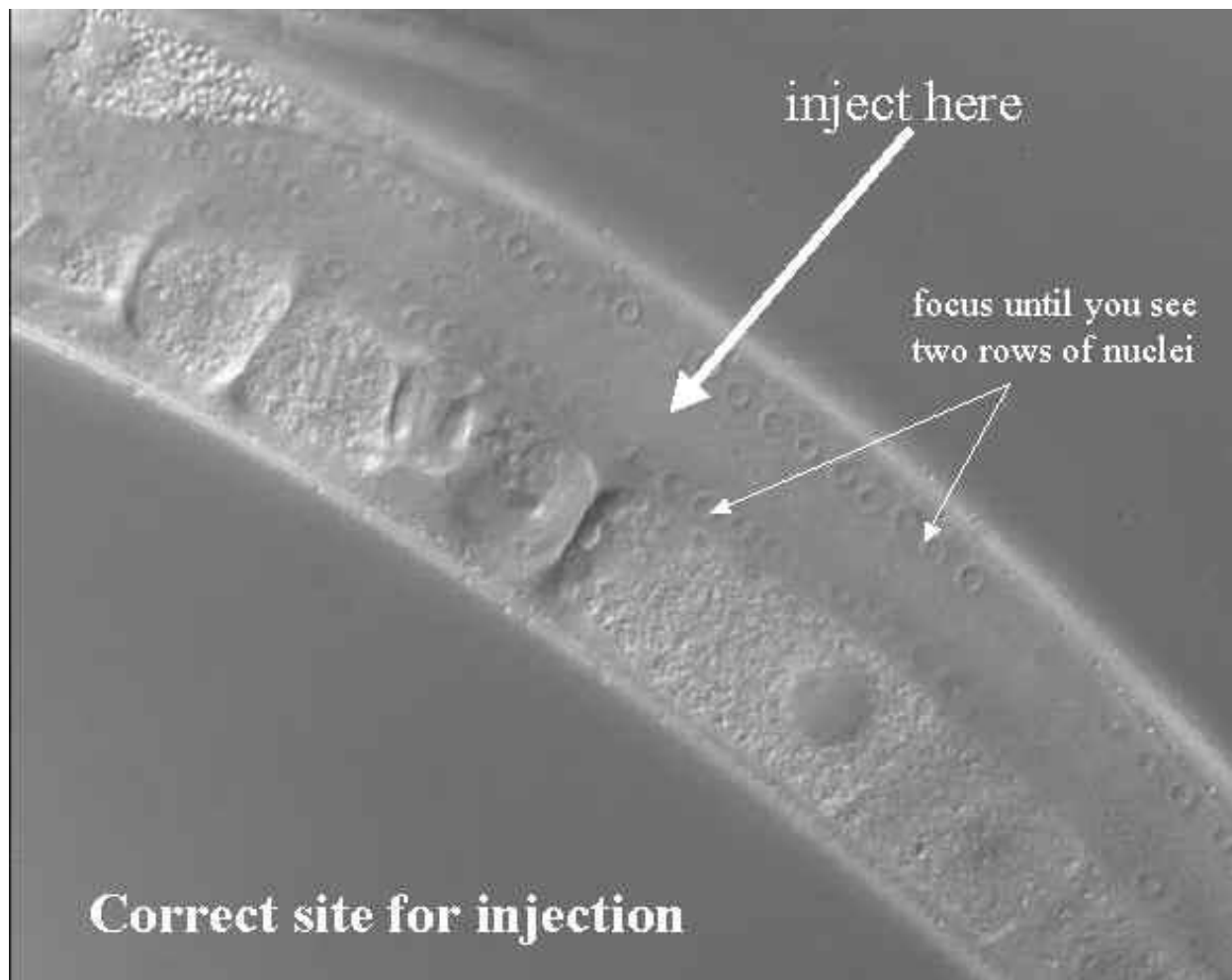
- 1-30 ng/ul Test plasmid
- 30 ng/ul co-injection marker (common markers pRF4 (roller) or p

Use pBluescript (or other plasmid) to bring total concentration of DNA to 100 ng/ul.

We do not use injection buffer T.E./E.B. or water seems to work fine.

Method

- Finding the correct spot to inject: A common problem for novice injectors is not aiming for the center of the gonad. To find the middle of the gonad arm you need to focus so that there are two rows of nuclei on each side of the gonad (see figure below) this ensures that you are at the core of the gonad.



- If you see plenty of nuclei you are at the surface of the gonad and when you try and inject you will certainly miss.

This method is based, with permission, on an original protocol available [here](#).

1.9.8 C. elegans Single Worm PCR

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

Single Worm PCR for use with *C. elegans*

Requirements

Lysis Buffer* 1 X PCR buffer (see below for 10X PCR buffer) Proteinase K: 20 mg/ml 10X PCR Buffer: 100 mM Tris, 500 mM KCl, 15 mM MgCl₂ pH 8.3 dNTP mix: 25 mM/each Primers: 5-10 uM Taq Polymerase: approx 5U/ul



Method

- Add proteinase K to 1 X PCR buffer (95 ul 1 XPCR buffer + 5 ul 20mg/ml proteinase K)

*We do not use the “Lysis buffer” recipe anymore because 1 X PCR buffer seems to work fine. *

- Place 5-10 ul of 1 X PCR buffer + proteinase K in top of 200 ul PCR tube (if you use more than 1 worm use 10ul instead)
- Pick single worm (or multiple worms) into lysis buffer.
- Immediately (don't let sit too long in lysis buffer) spin down to bottom of tube by spinning in microfuge 15 seconds @ 14,000 rpm or just flick down.
- Freeze tube in Liquid Nitrogen at least 10min.
- Lysis of worm and release of genomic DNA (You can use the PCR machine –“worm lysis” program)
- Heat tube to 65 degrees for 60-90 minutes
- Inactivate proteinase K by heating to 95 degrees for 15 minutes. If you do not use the worm DNA right away store at -80C.
- Perform PCR (50ul reaction)
- Add 45-40 ul of PCR “master mix” to each tube
- Master mix: 1X PCR Buffer, 0.5 uM primers, 0.2 mM/each dNTPs
- Add 0.5ul of Taq to tubes at 95C (hot start) If you have many samples the master mix can include the Taq so you can skip this step.
- Run PCR reaction for 30-35 cycles

References

Williams BD, Schrank B, Huynh C, Shownkeen R, Waterston RH A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. *Genetics* (1992) pmid:1321065

1.9.9 C. elegans total genomic DNA preparation

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

C. elegans total genomic DNA preparation

Avoid multiple freeze thaws of ProteinaseK! Either store in 50% glycerol or aliquot in small volumes and throw out after 2-5 thaws.

Requirements

Worm lysis buffer: 0.1M Tris-Cl pH 8.5, 0.1M NaCl, 50 mM EDTA pH 8.0, 1% SDS. 95% EtOH and 70% EtOH (ice cold) 3M NaOAc Phenol:Chloroform (TE saturated) 20mg/ml Proteinase K (in 50mM Tris pH=8, 5mM CaCl₂ and 50% glycerol (optional))

Method

- Add 4.5 ml of worm lysis buffer to a frozen 500 ul aliquot of worms (about 5 frozen worm pellets) in a 15 ml conical tube.
- Add 200 ul of 20 mg/ml Protease K to worms and vortex. Add 100ul of 10mg/ml RNaseA (boiled to inactivate DNases)
- Incubate at 65C for 60 minutes. Vortex 4-5 times during the incubation. The solution should clear as the worms disintegrate.
- Add 5ml of TE saturated Phenol:Chloroform (take the bottom layer) and vortex hard for 1 minute. Spin at high speed on the bench top centrifuge.

*Make sure the lid is sealed properly and wear gloves. Phenol burns can be very nasty and can even kill you. *

- Extract the aqueous (top) layer and transfer to a new 15ml tube. Do not be too greedy and leave a bit behind ensuring you do not get the phenol layer.
- Add 500ul 3M NaOAc mix. Aliquot into 300ul volumes in 1.5ml microfuge tubes.
- Add 2.5 volumes (750ul) of ice cold 95% EtOH. Invert to mix. The stringy white DNA should be obvious. Spin high speed at 4C for 5 minutes.
- Wash with 70 % EtOH. Be very careful here as this step is where most people lose the pellet.
- Dry (speed vac or air dry) and resuspend DNA in 50 ul TE for each tube and store at -80C.

Use 1-5ul in a PCR.

This method is based, with permission, on an original protocol available [here](#).

1.9.10 Determining DNA concentrations for ligations

Contributed by Ivan Delgado <ivanjdo@gmail.com>

Calculate DNA concentration for efficient DNA ligations.

- As an example, for any given ligation, use the following formula:

amount of insert = insert size / vector size x 3 (molar ratio of insert / vector) x amount of vector to be used

In the case of a 4.0 kb sized vector (after proper digestion) and a 0.4 kb sized fragment:

In order to use 1 mg of vector (cohesive end ligation), the following calculations are needed:

insert size / vector size = $0.4 / 4 = 0.1$ (1/10) molar ratio of insert / vector (since this is a cohesive end ligation with two different ends, there is no problem with multiple inserts, so a high ratio of insert to vector is possible -for blunt end ligations this ratio should be much lower, even 1:1) = 3/1 Therefore, for a ligation between a 4.0 kb vector and a 0.4 kb fragment, starting with 1 ug of vector, we get:

amount of insert = $0.4/4.0 \times 3 (3/1) \times 1$ ug amount of insert for 1 mg of vector = 0.9 ug

If this had been a blunt end ligation, a 1:1 ratio would have been used and the amount of insert necessary would have been 0.3 ug

These calculations were made for the equivalent of 10 units of ligase

This method is based, with permission, on an original protocol available [here](#).

1.9.11 DNA Extraction: Chelex

Contributed by [Paul Barber](#)

DNA Extraction via Chelex

Chelex is notorious for being as fickle as it is cheap and easy. Here are some tips for good amplifications: 1. Sometimes samples work best if used immediately, sometimes it is better to wait overnight before using them. Experiment and find what works for your species. Results can vary by taxa. 2. When doing initial PCRs, do a serial dilution of template. The amount of a Chelex DNA extraction used in a PCR can be as high as half of the volume of the PCR or as low as 1 microliter of a 1:10,000 dilution. I find that 1 microliter of a 1:1 is good for most applications. 3. If you don't get amplifications from your PCR the first time with a Chelex extract, repeat the vortex, spin, incubate, vortex, spin, sit overnight procedure described above. Often this will make a negative PCR work.

Method

- With Bleach, sterilize a dry reagent spatula, and a small magnetic stir-bar.
- Prepare a 5-10% by weight slurry of Chelex100 Resin (Biorad part 143- 3832, 100-200 mesh Chelex, sodium form) and UV sterilized HPLC water. The most effective way to do this is to take a 50 ml sterile falcon tube, place in on a scale inside a small beaker and zero the scale. Then add 5 grams of Chelex and fill to 50ml mark with water.

Precision is not critical. Sterile technique is.

- Place sterile stirbar in tube and place on magnetic stirrer. Chelex settles quickly so if the slurry is not well mixed, your concentrations and results will be variable. Keeping the slurry well mixed, aliquot 300-500 microliters into 0.6 or 1.6ml eppendorf tubes (again, sterile) and cap immediately. If you have access to a laminar flow hood, that is a good place to do all of this. You may want to wipe down the scale and stirrer with a 10% bleach solution and/or UV sterilize prior to use.
- Turn on heating block. Set to 95°C. Fill holes with water.
- Using sterile forceps (Flame over alcohol burner several times to sterilize), remove a small piece of tissue from your sample. This piece of tissue should be big enough to be visible, but not so big as to be easily visible. Imagine cutting a 0.2mm section of a standard staple. This is plenty big. Too much tissue may inhibit your reactions.

Sterilize forceps between samples.

- When finished, make a negative Chelex control by dipping your sterilized forceps into a tube of Chelex slurry.
- Vortex sample and chelex slurry for 10-15 seconds.

Be sure lids are snapped on tightly before beginning.

- Spin samples briefly at high speed in a microcentrifuge
- Incubate samples for 20 minutes at 95°C

*The block temperature may drop slightly when doing this step. This drop is normal. Check tubes while incubating to ensure that lids have not popped off. *

- Vortex samples again for 10-15 seconds.

Be careful as steam may pop lid off of centrifuge tube. Hold lids down.

- Spin tubes again at high speed in microcentrifuge.
- Samples are ready to use.













Only use supernate for PCR reactions. Chlex bead will inactivate Taq!

This method is based, with permission, on an original protocol available [here](#).

1.9.12 DNA extraction from agarose gels (Dialysis-tubing)

Contributed by Matt Lewis <hop2kin@yahoo.co.uk>

Dialysis tubing (semi-permeable membrane, Visking tubing)

- Freeze the gel slice at -20°C for 30 minutes.

This is to make it easier to handle the gel slice

- Cut a 5cm length of dialysis tubing and rinse it inside and out with distilled water. Then rinse it with the same buffer used for the gel (eg. 0.5 x TBE) and leave it submerged in a small beaker of this buffer. Seal one end with a dialysis clip

Dialysis tubing (Sigma D9777) is purchased in rolls (dried), prepared by boiling and stored submerged in buffer at 4°C . Dialysis clips are Sigma Z37,096-7.

- Insert the frozen gel-slice into the tubing and add 200–400 μL of buffer (eg. 0.5 x TBE). Seal the other end of the tubing with a second dialysis clip.

The buffer around the gel-slice must be the same as the buffer inside the gel

- Immerse the sealed tubing in an electrophoresis tank so that the DNA band is parallel to the electrodes and apply 5V/cm electric field.

The DNA will migrate out if the gel towards the positive electrode. It will be retained by the dialysis tubing. You can see this happening under long-wavelength UV if you like. It takes about 10–15 minutes.

- Remove the buffer from the tubing and place into a 1.5mL microfuge tube.
- Phenol/chloroform extract and ethanol precipitate the DNA. Re-dissolve the DNA pellet in an appropriate volume of water or TE buffer (e.g. 10 μL).

**The pellet is often so small that it is invisible **

This method is based, with permission, on an original protocol available [here](#).

1.9.13 DNA extraction from agarose gels (Paper-strip)

Contributed by Matt Lewis <hop2kin@yahoo.co.uk>

Paper strip method for DNA extraction from agarose gels

- Using a scalpel blade, cut a slit immediately in front of the band to be extracted.

Do not remove the band from the gel

- Cut a piece of filter paper (e.g. 3MM paper) to size to fit inside the slit.

For example, 3mm x 10mm

- Place the paper strip in the slit, return the gel to the electrophoresis tank (submerged in buffer) and switch on the current for 2–5 minutes.

The DNA runs onward into the paper and is delayed in the smaller mesh size of the paper. Eventually the DNA will pass through so you have to keep checking it under long-wavelength UV so as not to leave it too long.

- Remove the strip of paper (carrying at least some of the DNA) and place into a 0.5ml microfuge tube, DNA side down.

- Make a tiny hole in the bottom of the tube using a needle (CAREFUL!)
- Place the 0.5mL tube inside a 1.5mL tube and spin for 30 seconds.

You may have to remove the lid of the 1.5mL tube. The buffer and DNA are retained in the larger tube. You can add 100µL of TE to the paper and re-spin to get a little more DNA out.

- Phenol/chloroform extract and ethanol precipitate the DNA. Re-dissolve the DNA pellet in an appropriate volume of water or TE buffer (e.g. 10µL).

*The pellet is often so small that it is invisible *

This method is based, with permission, on an original protocol available [here](#).

1.9.14 DNA extraction from agarose gels (Spin-column)

Contributed by Matt Lewis <hop2kin@yahoo.co.uk>

These are excellent for extracting DNA if you can afford them. They cost 1–2 US\$ each. Manufacturers include Qiagen, Sigma, Novagen

- Dissolve the gel-slice in 3 volumes of chaotropic agent at 50C for 10 minutes
- Apply the solution to a spin-column and spin for 1 minute (the DNA remains in the column)
- Wash the column by passing 70% ethanol through (the DNA remains in the column, salt and impurities are washed out)
- Elute the DNA in a small volume (30µL) of water or buffer, spin to collect.

This method is based, with permission, on an original protocol available [here](#).

1.9.15 DNA Extraction from Cheek Cells

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

A Salting Out Procedure for DNA extraction from cheek cells obtained by rinsing the mouth with 25mls of any commercial mouth wash solution available for about 30sec the first thing in the morning. It is important not to brush your teeth before collecting the specimen.

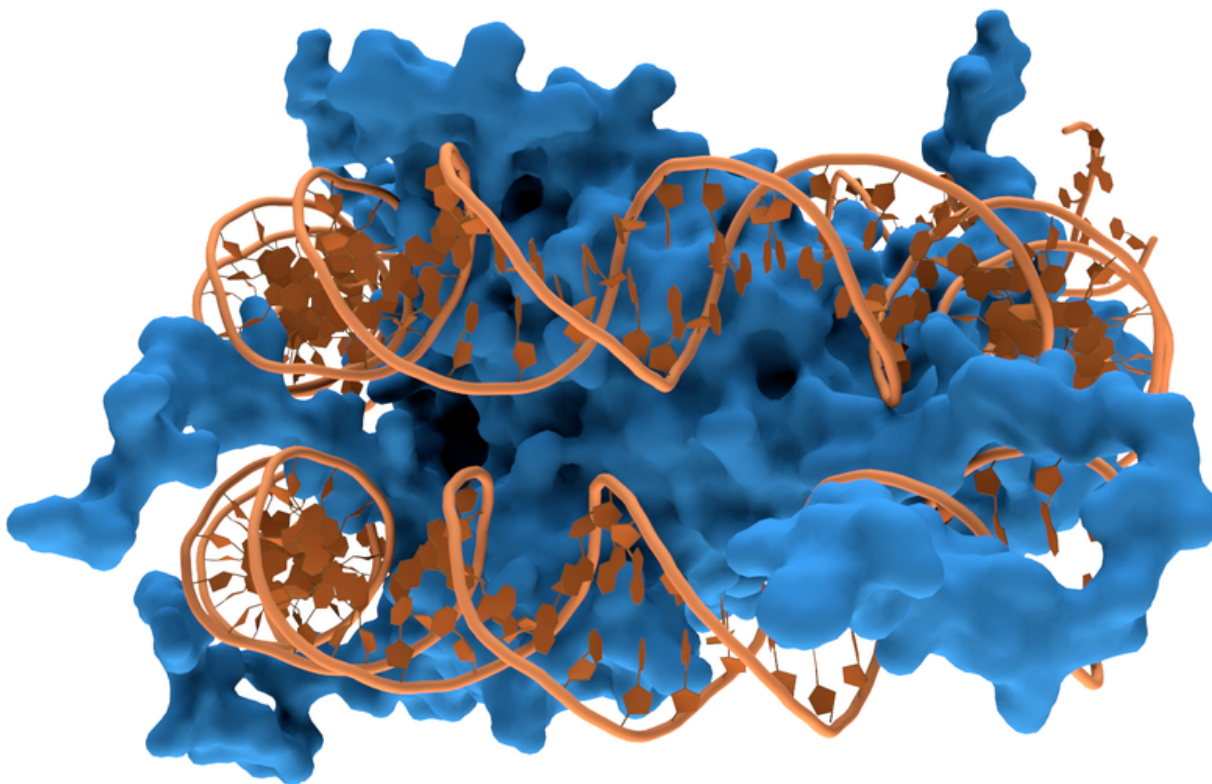
This method was evaluated at the DNA Laboratory, Medical School, Malta. (Mr. Joseph Borg). Histone image by Thomas Splettstoesser

Requirements

51ml TE buffer 2ml SE buffer containing 1ul proteinase K and 1% SDS 500ul 6M NaCl 2.5ml chloroform isopropan-2-ol 75% ethanol

Method

- Tube containing mouthwash is centrifuged at 4000 rpm for 15 minutes
- Supernatant is removed cautiously by means of a sterile pipette, without disturbing the pellet at the bottom
- Pellet is washed with 25ml TE buffer, by means of centrifugation at 4000rpm for 10 minutes
- Repeat steps 2 and 3
- The pellet is resuspended in 1ml SE buffer containing 1µl proteinase K and 1% SDS



- Incubate at 37°C overnight in a water bath
- Add 1ml SE buffer
- Add 500µl 6M NaCl
- Add 2.5ml chloroform
- Mix for 60 min in an over-the-top rotator
- Vortex for 20 sec
- Centrifuge for 10 minutes at 2000rpm, and set the slowest breaking force
- Transfer the supernatant to a clean tube
- Add an equal volume of isopropanol to the supernatant to precipitate the DNA
- If DNA IS NOT visible centrifuge the tube for 4 minutes at 11,000g and discard the isopropanol. Add 1ml of 75% ethanol.

If DNA IS visible, spool out the DNA and place in microfuge tube filled with 1ml of 75% ethanol.

- Centrifuge for 4 minutes at 11,000g and discard the ethanol taking care not to lose the DNA
- Add ethanol and repeat centrifugation 2 times. Discard ethanol after last centrifugation
- Evaporate ethanol by placing microfuge tube in oven (max 60°C) for about 20-30minutes
- Add 100µl of TE buffer to rehydrate DNA, and mix overnight on an over the top rotator.

This method is based, with permission, on an original protocol available [here](#).

1.9.16 DNA fragment isolation

Contributed by Ivan Delgado <ivanjdo@gmail.com>

Isolate a DNA fragment from Low Melting agarose

Requirements

Low melting agarose TBE buffer DNA fragment Phenol Chloroform Isoamyl alcohol Note 1: Most likely, this is your first step in the purification process right after a restriction enzyme digest. Use as much DNA as possible as a good portion of it (>50%) will be lost in the subsequent steps. >10 mg of DNA is my usual starting material. If other protocols are used to purify the DNA fragments (columns, ...) less DNA is necessary (each phenol extraction is only 50 to 70% efficient in recovering DNA) Note 2: If the digest will require an Alkaline Phosphatase (AP) treatment, it is good practice to treat with AP right before purification as well as right before phenol extraction to make sure all the fragment has been dephosphorylated. Therefore, if necessary, treat digest with Alkaline Phosphatase before starting this protocol

Method

- Make 100 mL of 1.0% Agarose in 0.5-1% TBE and let it solidify in gel apparatus with an appropriate comb
- Cut out portion of gel to be used for fragment isolation
- Prepare ~50 mL of 1% Low Melting Agarose and load on cut out region of gel. Let it solidify at 40C (alternatively, all the gel can be made of LM agarose)
- Load samples and separate bands by running gel at 40C (RT is also ok)
- Cut bands out (visualization in UV box) and place them in a 1.5 mL eppendorf tube
- Centrifuge samples for 10 secs and add 1X Vol (~200 µL) of TE buffer (can store agarose at -20oC if necessary)
- Incubate at 65oC for 5 minutes, vortex into solution, incubate for 5 more minutes and vortex again (the idea of this step is to completely melt the LM agarose and leave as fluid a solution as possible (addition of extra TE buffer may aid in this process))
- Alkaline Phosphatase treatment if necessary
- Add ~300-400 µL of TE-saturated Phenol, incubate at 65oC (2-3 minutes) if phenol was cold, vortex and centrifuge for 3 minutes
- Add ~300-400 µL of 24 Chloroform : 1 isoamyl alcohol, vortex until solution turns milky/cloudy (~30 secs) and centrifuge for 3 minutes
- Precipitate DNA (upper phase) with 0.8X Vol of 2-propanol, 0.1X Vol of 3M NaOAc, pH 5.2, by mixing and centrifuging at 40C for 30 minutes. Dry pellet in SpeedVac (5 minutes) and resuspend in 20 µL of water

This method is based, with permission, on an original protocol available [here](#).

1.9.17 DNA ligation

Contributed by Ivan Delgado <ivanjdo@gmail.com>

Ligation of a DNA fragment to a vector

T4 DNA Ligase is very unstable on ice (use as fast as possible and keep at -20oC). One unit catalyzes the exchange of 1 nmol of ATP in 20 mins at 37oC. This implies that ~1nmol of donor DNA can be ligated to an equivalent amount of acceptor DNA under these condition, in other words, use SMALL amounts of fragment and vector (otherwise there will be a lot of vector left without any fragment at the end of the reaction)

This protocol assumes that all necessary treatments to the DNA fragments (for example: DNA Fragment Isolation from LM agarose, Fill In/Chew Back of DNA overhangs, Alkaline Phosphatase removal of PO₄ from DNA fragments, Kinase addition of g-phosphates to 5'-OHs) have been performed by now and that the fragments are ready to be ligated to each other.

Following restriction digestion and dephosphorylation (the two steps that can be performed in the same reaction, one after the other, without the need of purification - DNA Fragment Isolation from LM agarose-), the dephosphorylated fragment (usually vector) can be used directly without the need of a purification step (this is specially true if the vector was only linearized by digestion with a single restriction enzyme. Since there is only one fragment in the reaction, there is not need to gel purify it. In order to do this, AP has to be HEAT inactivated (Alkaline Phosphatase removal of PO₄ from DNA fragments) prior to the ligation reaction

Requirements

5X DNA Ligase reaction buffer (commercial) (250 mM Tris HCl, pH 7.6, 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol (PEG)-8000. Store at -20oC. Vortex vigorously at RT when thawing)

Method

- Prepare reaction: Mix 4 mL of 5X Ligase reaction buffer, Vector DNA (up to 0.03 nmol, up to 0.1-1 mg -see below), Insert DNA (up to 0.1 nmol, up to 0.1-1 mg -see below), and 0.1 units of Ligase (for cohesive end ligations; or 1 unit for blunt end ligations). Final reaction volume (including vector DNA, insert DNA, and dH₂O) should come to 20 mL
- NOTE: the vector to insert ratio should always be at least 1:2. 1:3 is ok, but higher ratios will result in multiple inserts (if this is possible). Total DNA should not exceed 100 ng for cohesive end ligation and 1.0 mg for blunt end ligations
- Incubate at 15oC overnight*

Many different incubations can be performed. For blunt end ligations it is better to incubate at 15oC overnight. For cohesive end ligations, one hour at RT is more than enough. The bottom line is that some ligation will occur at RT regardless of the reaction (specially if enough ligase is present), so it is a matter of how much DNA was prepared and used for the ligation and the amount of the total reaction that will be used for the transformation. The more DNA the faster the reaction can be stopped and the least that needs to be used for the transformation (a portion the ligation can be taken out and used for transformation, while the rest can be left to continue and be transformed later). Cohesive end ligations specially hold true for the above criteria. As for blunt end ligations, it is better to let them go longer or use a lot more DNA

- Although not necessary, DNA Ligase can be inactivated at 65oC for 10 minutes
- Invitrogen recommends the use of up to 5 units of DNA ligase for blunt end ligations of up to 0.18 nmol of insert with up to 0.06 nmol of vector. This has worked for me. I would discourage “rapid ligations” that have incubations of 5 minutes; the same amount of Ligase can be used for the above reactions with good results (if lower camounts have proven unsuccessful)
- Another important step, although not directly related to the ligation per se, is the competent cells used for the experiment. Commercial competent cells are extremely good and I must admit, much better than the homemade kind (Preparation of competent E. coli cells using CaCl₂). Nevertheless, for most applications these homemade cells are more than enough. For important (hard) ligations, removing the uncertainty of this step by using commercial competent cells is recommended

This method is based, with permission, on an original protocol available [here](#).

1.9.18 Excision of Clones as pBluescript from IZAPII

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States

Excision of Clones as pBluescript from IZAPII

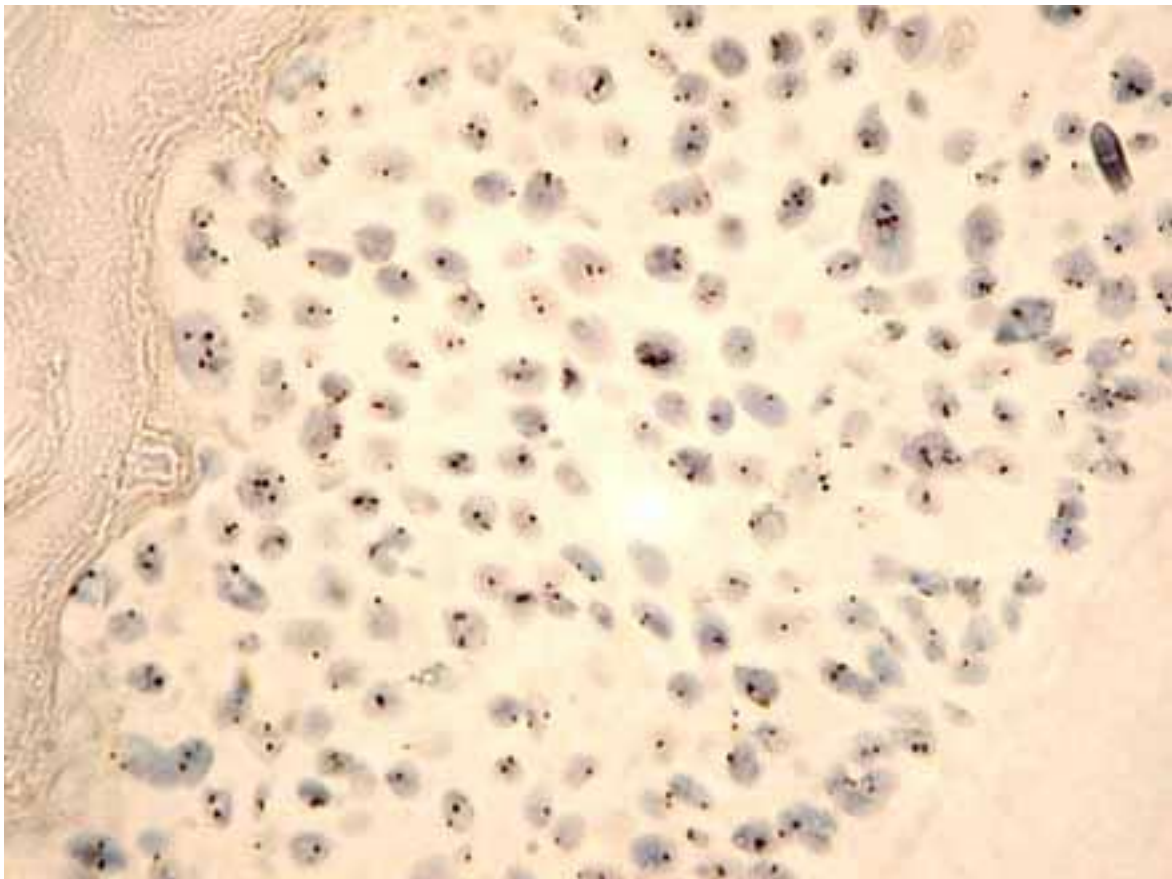
- Streak XLI-Blue MRF' from frozen stock on an LB-tetracycline plate.
- Incubate plate overnight at 37°C. Streak SOLR strain from frozen stock on an LB-kanamycin plate.
- Pick a single XLI-Blue MRF' colony and a single SOLR colony with a sterile toothpicks to each inoculate 10 ml of LB broth in 50 ml conicals.
- Grow overnight at 30°C.
- Warm water bath to 70°C
- Add 50 ml of LB to 0.5 ml of each overnight culture, and grow at 37°C for 2-3 hours in a 250 flask.
- Check OD600 at 2 hours and every 30 min thereafter.
- Wish to have XLI-Blue MRF' in mid-log phase at OD600 of 0.2-0.5. Allow SOLR to grow to OD600 of 0.5-1.0; before they reach OD600 ? 1.0, remove from incubator and let sit at RT on rotator.
- Spin XLI-Blue MRF' for 10 minutes at 2,766 rpm in a sterile 50 ml conical using Beckman GS 6R centrifuge.
- Carefully decant media and gently resuspend pellet at OD600 = 1.0 in 10 mM MgSO₄ (3-4 ml).
- For each clone, in a 50 ml conical, combine:
 - 200 µl of OD600 = 1.0 XLI-Blue MRF' cells
 - 250 µl of picked clonal plaque in SM/chloroform
 - 1 µl of #1065 ExAssist helper phage
- Incubate at 37°C for 15 minutes.
- Add 3 ml of LB broth and incubate for 2 hours at 37°C with shaking.
- Spin down cells for 15 minutes at 3,194 rpm in Beckman GS 6R centrifuge.
- Transfer supernatant to a Sartstedt 55.514 tube.
- Heat tube at 70°C for 15 minutes.
- Spin in a Sorvall centrifuge with a SA600 rotor at 5,262 rpm for 15 minutes.
- Decant the phage supernatant into a sterile tube. Add 200 µl of OD600 ? 1.0 SOLR cells into two 1.5 ml eppendorf tubes (2 tubes/clone); add 100 µl of supernatant to one tube and 10 µl to the other tube.
- Incubate at 37°C for 15 minutes.
- Plate 10 µl, 50 µl and 10 µl of 1/10 - 1/1000 diluted onto LB amp plates using a ethanol flamed glass spreader and rotator. Plate SOLR cells alone as a negative control.

This method is based, with permission, on an original protocol available [here](#).

1.9.19 In situ hybridisation to alpha satellite sequences

Contributed by Matt Lewis <hop2kin@yahoo.co.uk>

In this protocol a biotin or digoxigenin labelled DNA probe is detected using HRP-conjugated antibodies. The signal is visualised with diaminobenzidine (DAB). Normal, healthy nuclei show two spots. Aneusomic nuclei show 1, 3, 4 or more spots.



- Immerse the slides in 1M sodium thiocyanate at 80C for 10 minutes.
- Rinse in H₂O and allow to dry.
- Prepare 4mg/ml pepsin as follows:

Make up 0.2M HCl [50mls H₂O + 800ul conc. HCl], weigh out a little pepsin (Sigma P7012) into a universal and add the 0.2M HCl to make 4mg/ml.

- Place the slides flat in a humid chamber (eg a large square plastic petri dish) and drop the 4mg/ml pepsin gently onto the slide. Place in the 37C incubator for 30 minutes.

Pepsin digestion is the most crucial step. Too little and the probe will not be able to access the chromosomes; too much and the morphology will be disrupted.

- Rinse in H₂O, 2 x 5 minutes, and allow to dry.
- Prepare the hybridisation solution.

*A multispot slide with 13mm circular coverslip needs 6ul; a square 22mm coverslip uses 10-12ul. For larger numbers place slides face to face (acting as each other's coverslip) with 30ul solution each.

Description	Details	Quantity	Solution
Al6mls de-ionised formamide,	2mls 20x SSC,	2mls H ₂ O,	2g dextran sulphate. Store in a universal at room temperature
25ul De-ionised formamide	(Sigma F7508)	15 ul	Probel
Appligene/Oncor #CP5040-B.5 (biotin labelled) or label your own probe (~10ng/ul)	5 ul	Carrier DNA	Sheared salmon sperm DNA (10mg/ml)
0.5 ul H ₂ O	2 ul 20 x SSC	2.5 ul TOTAL	50 ul*

- Apply an appropriate amount of hybridisation solution onto the slide and cover with a coverslip.
- Incubate at 80C for 10 minutes to denature the DNA
- Transfer to a humid chamber and incubate overnight at 37C
- Dip the slides into a coplin jar containing wash solution, allow the coverslips to slide off. Immerse the slides in formamide wash solution for 20 minutes at 42C.

Wash can be done without formamide at higher temperature (try 0.25 x SSC at 72C for 5 minutes)

- Immerse briefly in PBS to wash.
- Lay the slides flat in a humid chamber and drop blocking solution onto them
- Incubate for 20 minutes at room temperature.
- Drain off the blocking solution and replace with mouse anti-biotin antibody diluted 1:100 in blocking solution e.g. DAKO antibody #M0743, 100-200ul per slide.
- Incubate in a humid chamber for 40 minutes at 37C.
- Wash with blocking solution for 10 minutes
- Incubate with rabbit anti-mouse HRP (e.g. DAKO P0260) antibody diluted 1:80 for 40 minutes at 37C
- Wash with blocking solution for 10 minutes
- Incubate with swine anti-rabbit HRP (e.g. DAKO P0217) antibody diluted 1:100 for 40 minutes at 37C
- Wash in PBS for 5 minutes
- Make 5ml of DAB solution and then carefully drop the DAB onto the slides. DAB is carcinogenic so take care. The signal takes about 10 minutes to develop. If you're lucky you may be able to see tiny spots at low power under the microscope (take care not to dip the lens in the DAB solution)
- Rinse in tap water, stain the nuclei using haematoxylin, de-hydrate through the ethanol series and mount in DPX.

*Duration of the haematoxylin stain depends on the haematoxylin and how much it has been used. Try 30 secs at first. Check under the low power microscope. *

This method is based, with permission, on an original protocol available [here](#).

1.9.20 Lacritin Stimulated Cell Proliferation Assay

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States

Lacritin Stimulated Cell Proliferation Assay with siRNA or Inhibitors

- For PKCa depletion, a pool of four PKCa-specific siRNAs or individual siRNAs (100 nM; Upstate LLC, Charlottesville VA) were transfected into HSG cells via Lipofectamin 2000 in Opti-MEMI Reduced Serum Medium (Invitrogen, Grand Island NY) in the absence of serum and antibiotics according to manufacturer's instructions.

*PKCa depletion was confirmed by Western blotting. *

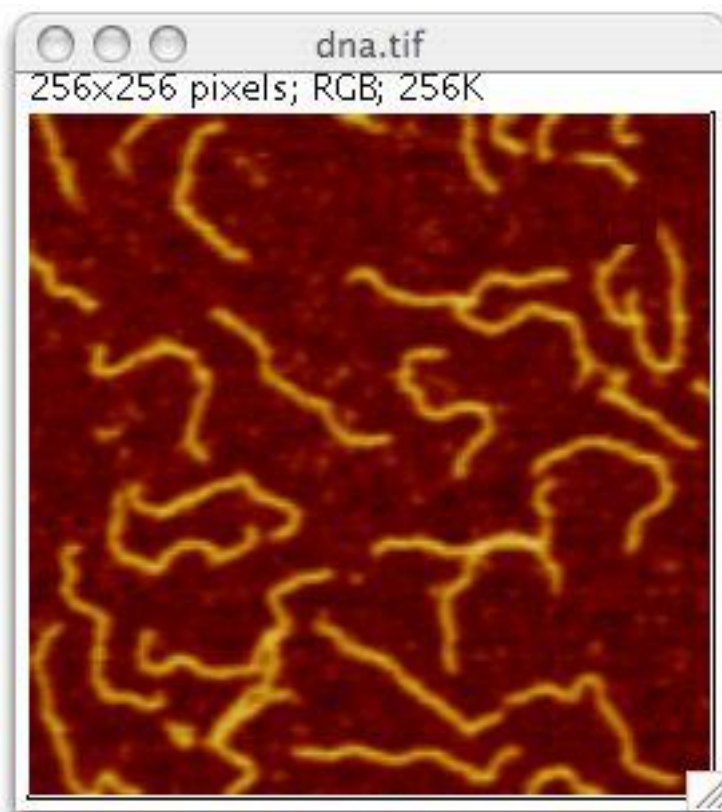
- Other cells were transfected with pools of NFATc1, TRPC1, mTOR, or STIM1-specific siRNAs. In negative controls, cells were transfected with a pool of four lamin-specific siRNAs (all 100 nM; Dharmacon Inc, Lafayette CO).
- Incubate transfection for 72 hours.
- Post-transfection, mitogenesis was assessed by co-addition of 10 nM lacritin and [3H]-thymidine (2 mCi/ml; Amersham, Piscataway NJ) for 24 hrs.
- For rapamycin and cyclosporine inhibition, cells that had been incubated in serum-free media overnight were treated with 1 μ M cyclosporine (Sigma Chemical Co., St. Louis MO) for 5 hours or with 100 nM rapamycin (Calbiochem) for 15 min, or both;
- Rapamycin/cyclosporin inhibitor cells were then stimulated with 10 nM lacritin in the same medium with 2 mCi/ml 3H-thymidine for an additional 24 hours.
- In syndecan experiments, incubate cells alone with lacritin or together with increasing amount of bacterial-expressed human SDC1 ectodomain (hS1ED) as a soluble inhibitor.
- Cells depleted of heparanase-1 or SDC1 were treated with lacritin in [3H]-thymidine 48 hours after siRNA transfection.
- To rescue heparanase depleted cells, ~1 mg heparanase enriched from HSG or HEK293 cells using heparin affinity column or 0.0001 units bacterial heparitinase (Seikagaku America) was added together with lacritin and [3H]-thymidine for 24 hours.
- [3H]-thymidine incorporation was stopped by placing on ice. Cultures were washed twice with ice-cold PBS, fixed with cold and then RT TCA (10%) for 10 min each
- Wash twice with RT PBS, collected in 1 N NaOH, neutralized with 1 N HCl, and then transferred to liquid scintillation vials for measurement.

1.9.21 Measuring DNA Contour Lengths with ImageJ

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

This is an example of how to measure the lengths of DNA contours on images acquired using an atomic force microscope (AFM).

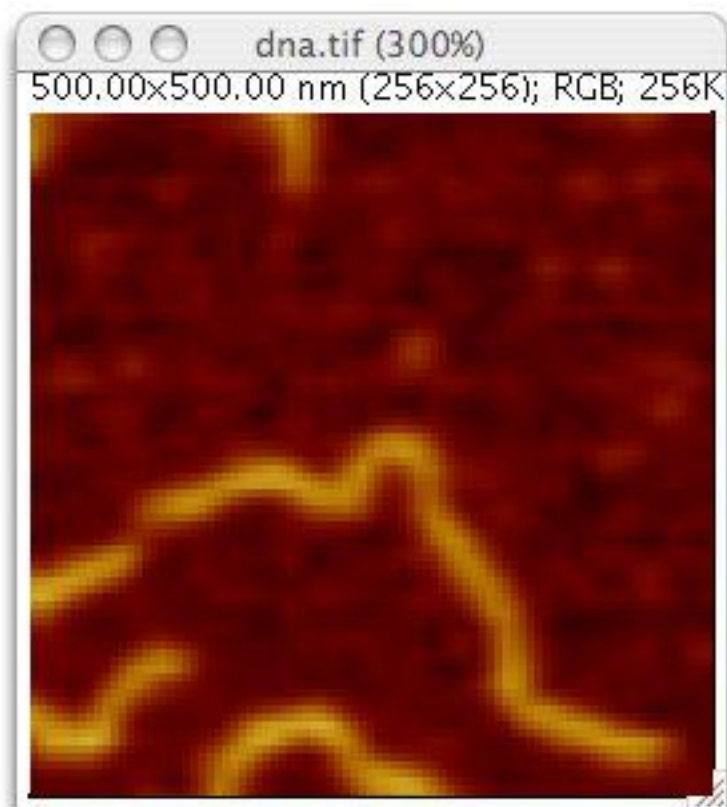
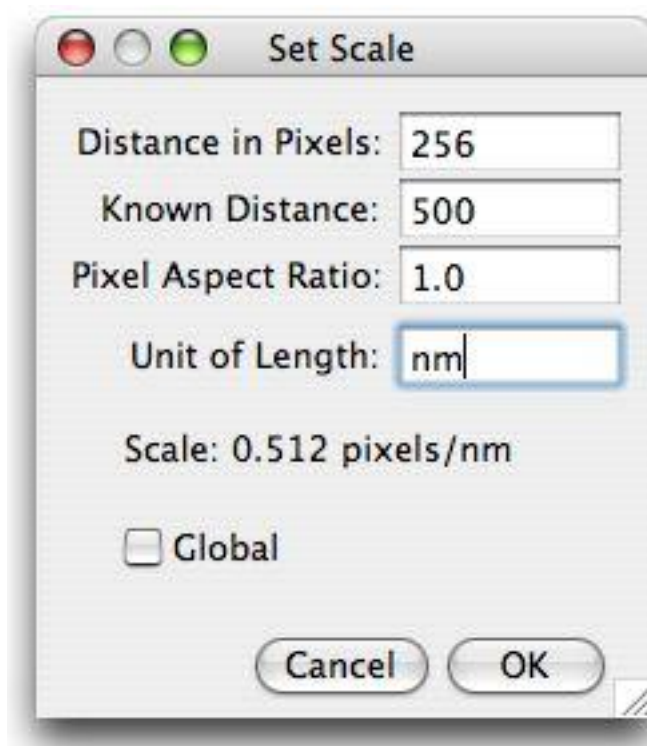
Requires ImageJ 1.30 or later.

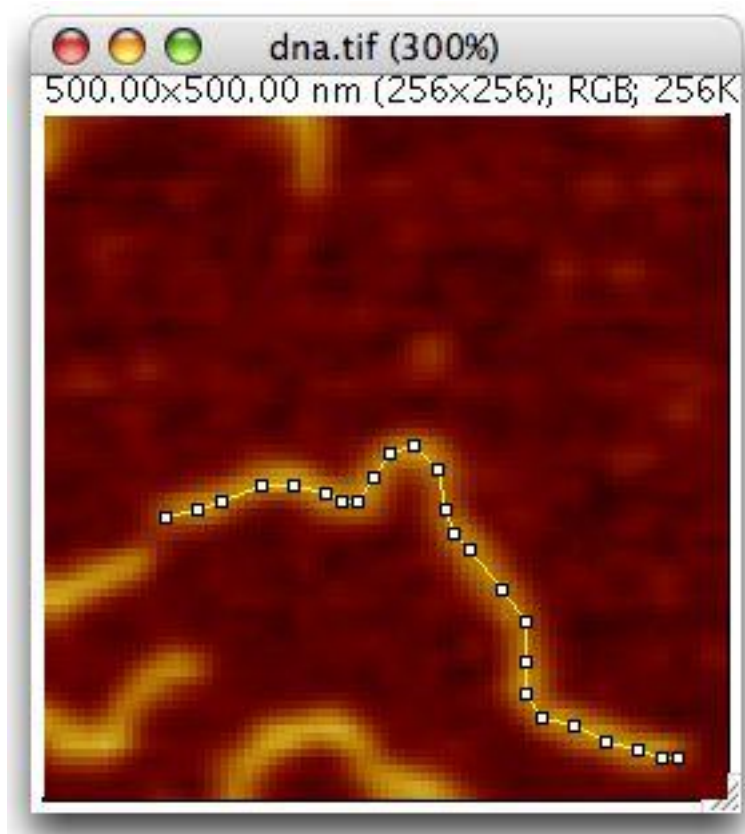
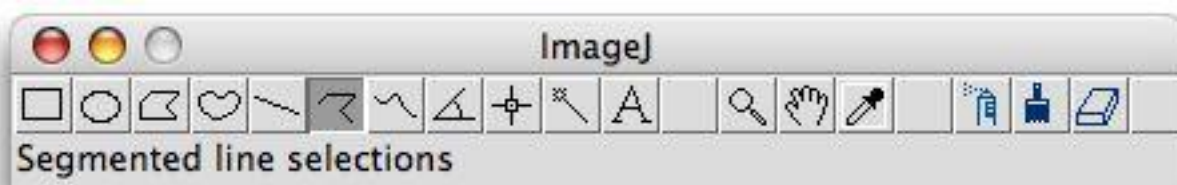


In this example, we measure the length of the DNA contour in the lower right corner. The field width of the image is 500nm. The image is from M. Lysetska, et al., “UV light-damaged DNA and its interaction with human replication protein A: an atomic force microscopy study”, *Nucleic Acids Research*, 2002, Vol. 30, No. 12 2686-2691 (nar.oupjournals.org/cgi/content/full/30/12/2686), used with permission.

Method

- Use the Analyze/Set Scale dialog to define the spatial scale. Enter the image width in the “Distance in Pixels” field, enter the field width in the “Known Distance” field, enter “nm” as the “Unit of Length”, then click “OK”.
- Use the magnifying glass tool to zoom in on the DNA contour to be measured, in this case, the one in the lower right corner of the image. To zoom out, right-click or alt-click with the magnifying glass tool.
- On the ImageJ toolbar select the segmented line tool (sixth tool from the left) to outline the DNA contour. The three tools on the right end of the toolbar are tool macros.
- Use the segmented line tool to create a line selection that outlines the DNA contour. To finish outlining, right-click, double-click or click in the box at the starting point. The line selection can be adjusted by clicking and dragging the tiny black and white “handles” along the outline.
- Finally, use the Analyze/Measure command to measure the length of the DNA contour, in this case 181nm. Measurements can be transferred to a spreadsheet by right-clicking in the “Results” window, selecting “Copy All” from the popup menu, switching to the spreadsheet program, and then pasting.





Results

	Mean	Min	Max	Length
1	101.685	71	124	180.908

The image shows the 'Results' window in ImageJ. It contains a table with the following data:

References

Lysetska M, Knoll A, Boehringer D, Hey T, Krauss G, Krausch G UV light-damaged DNA and its interaction with human replication protein A: an atomic force microscopy study. *Nucleic Acids Res* (2002) pmid:12060686

This method is based, with permission, on an original protocol available [here](#).

1.9.22 Metabolite extraction from supernatant cells

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Methanol/Chloroform extraction of whole cell small molecular weight metabolites from cells in suspension.

- Pre-chill methanol on dry-ice
- Transfer cell culture to 15ml tube to wash.
- Centrifuge at 1500rpm for 5 minutes to pellet. Add 5ml pre-warmed PBS, resuspend and repeat x2
- Centrifuge at 1500rpm for 5 minutes to pellet. Resuspend in 1ml pre-warmed PBS.
- Transfer cells to champagne flute glass vials. Centrifuge at 1200rpm for 5 minutes to pellet.
- Pre-chill centrifuge to 4°C.

Pre-chill chloroform and dH2O on wet ice.

- Completely remove supernatant PBS, then resuspend pellet in 400ul pre-chilled methanol. Place glass vials on dry ice to freeze rapidly.

OPTIONAL: Samples may now be stored at -80°C until required.

- Pipette 325ul dH2O via pipette onto each sample.
- Syringe 400ul chloroform via Hamilton syringe onto each sample (chloroform will digest plastic).
- Keep samples at 4°C for 10 minutes to allow phases to separate.
- Centrifuge at 1200rpm for 10 minutes at 4°C in swingout rotor.
- Transfer vials to the bench at room temperature for 5 minutes.
- Remove ~400ul upper (polar) layer into eppendorf using by pipette.
- Remove ~200ul lower (non-polar) layer into glass vials using Hamilton syringe.
- To dry samples for re-suspension in analysis buffer, place samples in vacuum dryer. Polar fraction will dry within 3-4 hours.

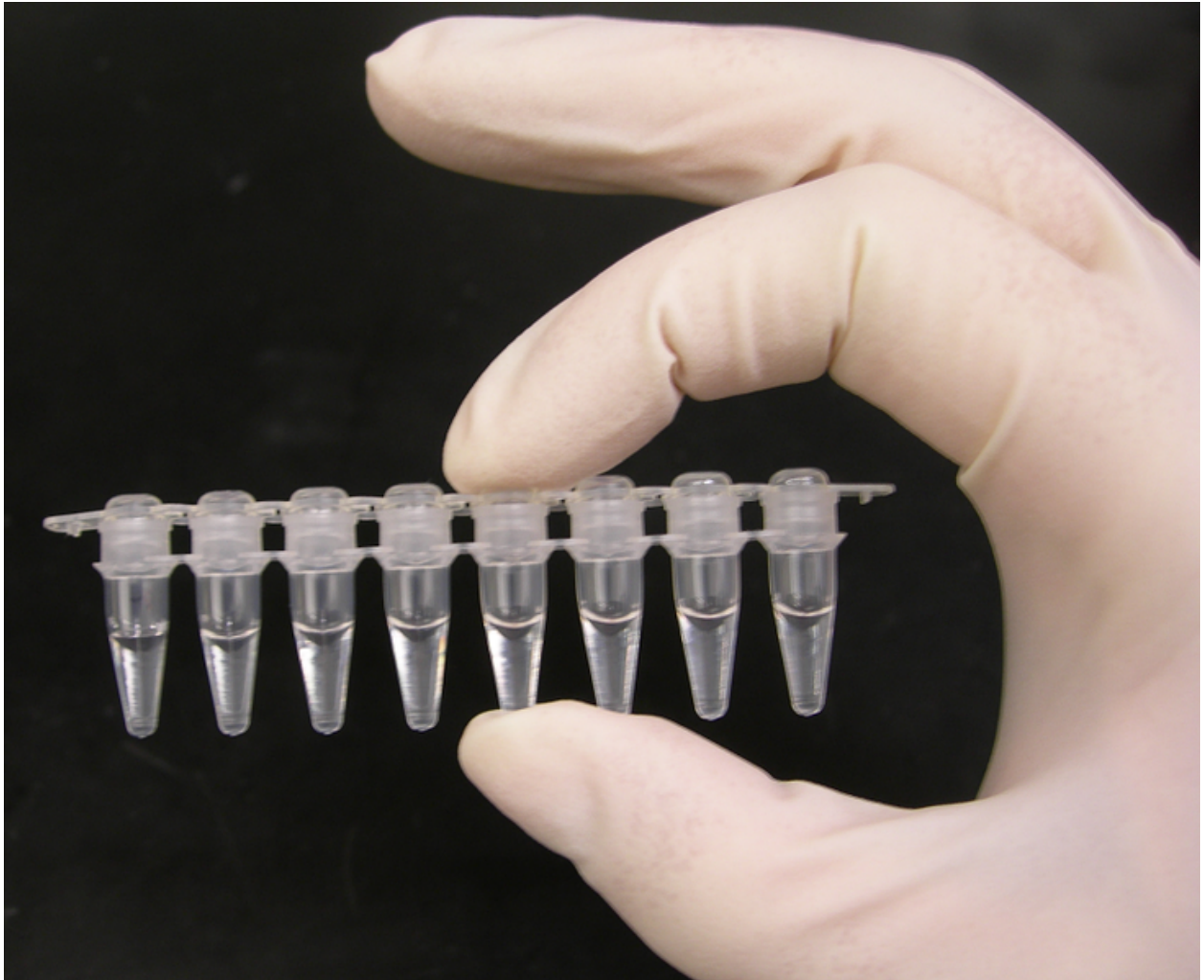
1.9.23 PCR mutagenesis

Contributed by Matt Lewis <hop2kin@yahoo.co.uk>

PCR mutagenesis is a method for generating site-directed mutagenesis. This method can generate mutations (base substitutions, insertions, and deletions) from double-stranded plasmid without the need for subcloning into M13-based bacteriophage vectors and for ssDNA rescue.

This method uses a proof-reading polymerase to read all the way around a plasmid and thus incorporate the primer as the new (mutant) sequence. Only a few (say 12) PCR cycles are performed on a relatively large amount of plasmid template to minimise the chance of expanding PCR sequence errors.

- You need two primers, complementary to each other, containing the new (mutant) sequence flanked by 20 bases on each side.



*For example, suppose you have the following sequence in some gene in some plasmid;

CTA CTT CCA GAG ACA ACT GAT CTC TAC TGT TAT GAG CAA TTA AAT GAC AGC GGG

And you want to change it to; *CTA CTT CCA GAG ACA ACT GAT CTC TAC GGT TAT GAG CAA TTA AAT GAC AGC GGG*

One primer will be;5' . . *CCA GAG ACA ACT GAT CTC TAC GGT TAT GAG CAA TTA AAT GAC AGC 3'*

and the other primer will be the exact complement; '*... .5' . . GCT GTC ATT TAA TTG CTC ATA ACC GTA GAG ATC AGT TGT CTC TGG 3'*.*

- In the PCR reaction most of these primers will be annealing to each other (and thus not extending - hence avoid normal Taq) whilst a few will be annealing to your target sequence with a small mismatch in the middle.

The primers must be FPLC, HPSF or PAGE gel purified (to avoid annoying n-1 primers).

Contrary to popular opinion there is no need to phosphorylate the 5' ends.

- Pre-heated the PCR machine to 94C.
- This PCR reaction requires a proof-reading polymerase such as Pfu polymerase. Make up your reaction mixture as follows:

5ul 10 x Pfu polymerase buffer (inc Mg) 4ul 10mM dNTPs (i.e. a mixture of all four at 10mM each) 0.2ul Primer 1 (primers are ~42 mers at 100pmol/ul) 0.2ul Primer 2 1ul Plasmid template (10ng) 37.6ul H₂O 2ul Pfu polymerase

Keep on ice until you put it into the PCR machine.

*Pfu polymerase contains a 3'-5' exonuclease and it will start to chew up single-stranded DNA (the primers) from the 3' end making them shorter and less specific. Therefore assemble the reaction on ice adding the enzyme last. *

- Run PCR programme as follows

Duration/Temperature/Cycles -|-| 60 seconds|94C|1 30 seconds|94C|12 30 seconds|55C|- 12 minutes|68C|-

- The extension temperature is 68C to minimize 'breathing' of the 5' end of primer. Then, when the polymerase has read all the way around the plasmid it is less likely to displace the primer and incorporate the 'old' sequence from the plasmid. The extension time is 2 minutes per kb of plasmid.*
- Cool the PCR reaction to room temperature and add 1ul of restriction enzyme DpnI.
- Incubate at 37C for 1 hour.

*DpnI is a 4-cutter which only cuts dam methylated DNA. The parental plasmid DNA will be cut to pieces whilst the nascent PCR DNA is left intact. All routine E.coli strains have an intact dam methylase system. Check in the back of the NEB catalogue if unsure. *

- Transform 5ml and 45ml of the reaction into competent E.coli.

*There is no need to carry out a ligation before the transformation. The PCR product is a 'nicked' circle with the nicks in opposite strands displaced by 42bp. This is identical to a classical de-phosphorylated vector plus insert transformation (with a 42bp insert). *

- Miniprep some colonies, check that they are the expected size and screen 2 of them by sequencing.

Once confirmed, subclone the gene out of the plasmid back into the vector in the classical way to avoid sequencing the whole vector for any PCR errors.

This method is based, with permission, on an original protocol available [here](#).

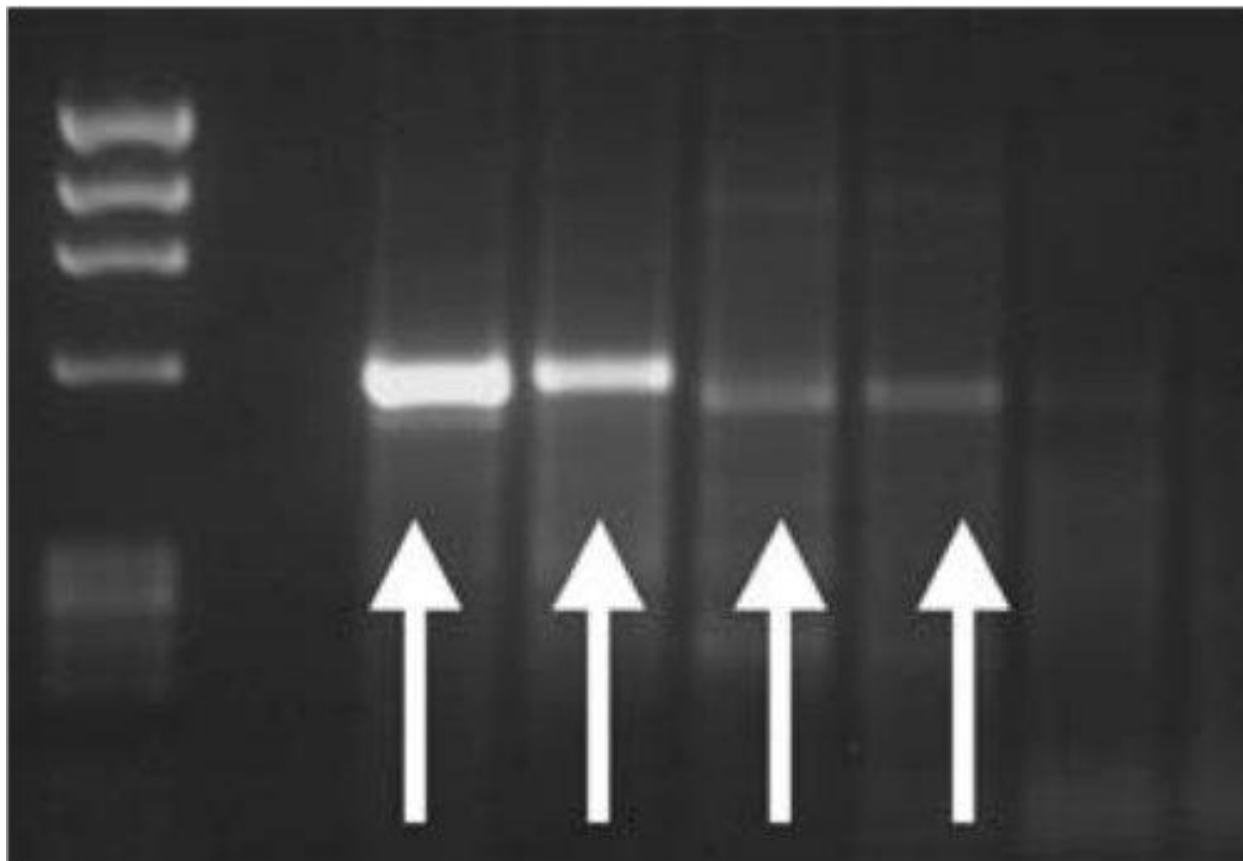
1.9.24 PCR product "clean-up" (SAP/EXO)

Contributed by [Paul Barber](#)

The purpose of this procedure is used to chew up excess primers and remove excess dNTPs from your PCR product. This procedure is necessary to ensure clean and readable DNA sequences. Work in HIGH DNA part of lab.

- Visualize your PCR products to make sure that your PCR worked and there are not multiple bands. A

strong amplification product (bright on the gel) will tend to sequence better. Good bands will yield good sequences, so spend the time to optimize your PCR.



Yes Yes NO NO

- Label enough 200 L strip tubes so that there is one tube for each PCR product to be sequenced. There is no need to sequence samples that did not amplify.

Put labels on the sides of the strip tubes, not the cap as the thermocyclers heated lid will lift writing on the caps. Also, put the date and initials somewhere on the strip of tubes.

- Using a HIGH DNA pipette, add 5 L of each PCR product into a 200 L tube, changing tips for each sample.

There is nothing magic about this volume - it provides template for two sequencing reactions if the product is strong. If you need more template than this (if you have weak bands) then scale up.

- Using NO DNA pipette, make a master-mix containing the following for each PCR product to be sequenced:
 - 0.5 L of shrimp alkaline phosphatase (SAP)





- 0.5 L of exonuclease I (EXO)

*So that you don't run out, make enough mastermix for 1 extra sample per 10 samples to be digested. Therefore, if you have 9 samples, you will need 5 L of SAP and 5 L of EXO. *

- Pipette up and down to mix the SAP/EXO master mix. Ensure that it is mixed well.
- Add 1 L of master mix to each tube of template. Pipette up and down to get all SAP/EXO out of the tip.

Change tips for each sample.

- Check tubes for bubbles. If necessary, spin tubes briefly at HIGH in centrifuge.
- Set the thermocycler to SAPEXO program (if available). This program will incubate the reactions at 37 °C for 30 minutes, then kill the enzyme with 80 °C for 15 minutes, and then cool the reaction to room temperature, 25 °C. Place all samples in the machine, close the heated lid and run the program.



- The products are now ready for sequencing and can be stored at room temperature. Use 1-2 L in a 10 L sequencing reaction, depending on how bright the band was.

This method is based, with permission, on an original protocol available [here](#).

1.9.25 Phosphatase Removal of PO₄ groups from DNA

Contributed by Ivan Delgado <ivanjdo@gmail.com>

The removal of phosphate groups from DNA using Alkaline Phosphatase

1 unit of Alkaline Phosphatase (AP) can hydrolyze 50 pmol of 5' terminal phosphorylated DNA fragments (3' recessed, 5' recessed or blunt-ended) when incubated at 37°C for 1 hour. This implies that 25 pmol of DNA are dephosphorylated (at both ends) in one hour by 1 unit of AP at 37°C

Requirements

10X Dephosphorylation buffer (0.5 M Tris-HCl, pH 8.5, 1 mM EDTA) Alkaline phosphatase

Method

- This protocol takes into account the prior preparation of a DNA fragment, such as one isolated from low melting agarose
- Phosphatase treat appropriate samples (for example: if digested vector can religate, i.e. digested by a single enzyme): mix 20 mL of 10X dephosphorylation buffer, 10 uL of Calf alkaline phosphatase (1U/uL) (contains 50% glycerol, so the volume of AP used should never be more than 10% of the final volume), and ~170 uL of Vector + dH₂O (fragment) (assuming ~0.5 ug) (200 uL total volume)
- Incubate at 37°C for 1 hour
- Add 0.1X Vol of 200mM EDTA/EGTA (in this case, 20 uL) and HEAT inactivate at 65°C for >10 mins. The DNA is ready for downstream applications

NOTE: the EDTA/EGTA should not interfere with ligation reactions if only 0.1X Vol of the AP reaction is used in the ligation reaction (no purification step in between, see DNA Ligation), in which case the EDTA/EGTA would have been dropped down to 1 to 2mM) NOTE: Note: when determining the amount of DNA to use: 1) determine concentration by spectrophotometer or better 2) run an aliquots (1, 5, 10 uL) on a gel. Determine the amount that is barely visible (EtBr has a visibility limit of about 100-200 ng on agarose gels) on a THIN gel (thick gels will mask some of the signal, specially at low DNA concentrations). Use two to three times more for the AP reaction. For example: if the aliquot that could be detected in the gel was 5 uL, then use 10 to 15 uL and dilute with dH₂O to the appropriate volume (in the example above, to 170 uL with 160 to 155 uL dH₂O)

This method is based, with permission, on an original protocol available [here](#).

1.9.26 Preparation of Competent XLI-Blue MRF' Cells

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States

Preparation of Competent XLI-Blue MRF' Cells (ref. p. 1.76 of Maniatis)

- Streak XLI-Blue MRF' (#1059; in Box 1A of Locator) cells from frozen stock onto Super Optimal Broth (SOB) agar plate. Incubate overnight at 37°C.
- Transfer 4-5 colonies (1-2 mm diameter) into 1 ml of SOB containing 20 mM MgSO₄.
- Disperse by vortexing at moderate speed, then dilute culture in 100 ml of SOB containing 20 mM MgSO₄ in a 1 l flask (use flask reserved for preparation of competent cells).
- Grow to OD₆₀₀ of 0.45 -0.5 (equals approx. 108 cells/ml; check OD₆₀₀ every 30-60 min).
- Dispense cells into (2) 50 ml prechilled conicals. Cool cultures for 10 minutes on ice.
- Spin for 10 minutes at 2,000 rpm (Beckman GS6R centrifuge; 4°C).
- Pour off supernatant, then leave inverted for 1 minute to remove all supernatant.
- Resuspend pellets in 20 ml/tube of ice-cold transformation buffer by gentle vortexing. Place on ice for 10 minutes.

- Spin for 10 minutes at 2,000 rpm (Beckman GS6R centrifuge; 4°C).
- Pour off buffer. Invert 1 min to remove all buffer.
- Resuspend in 4 ml/tube of ice-cold transformation buffer by gentle vortexing.
- Add 140 µl/tube of DMSO. Mix gently by swirling and store on ice for 15 minutes.
- Add an additional 140 µl/tube of DMSO. Mix gently by swirling and place on ice.
- Quickly dispense 300 µl aliquots into prechilled sterile 1.5 ml screw top tubes. Immediately snap freeze by immersing in liquid N₂.
- Store until needed. Before use, thaw one aliquot to check for transformation efficiency using pUC18.

References

Tom Maniatis [Molecular cloning: A laboratory manual](#) Cold Spring Harbor Laboratory 9780879691363

1.9.27 Preparation of Sonicated Salmon Sperm DNA

Contributed by Gordon W. Laurie, School of Medicine, University of Virginia, United States

Preparation of Sonicated Salmon Sperm DNA

- Use Pharmacia #27-4564-01. With clean flamed scissors and forceps, weigh 0.25 g/50 ml conical and add 50 ml/conical of 0.02 M Tris, pH 7.6. Allow to dissolve over several days at 4°C.
- Vortex. Draw up into a 20 or 30 ml syringe. Add a 25 gauge needle and shear DNA by squirting through needle into fresh 50 ml conicals at 15 ml/conical.
- Add 0.9 ml/conical of 5 N NaCl.
- Boil in water bath (95 - 100°C) for 20 minutes.
- Cool in ice water.
- Vortex and then add 1 drop (from a standard transfer pipet) of 1.2 N HCl (concentrated HCl diluted 1/10) to neutralize.
- Add two volumes of 100% ethanol, let stand for 20 minutes on ice.
- Spin for 10 minutes in the Beckman GS-6R at 2500 rpm (4°C).
- Pour off the supernatant, let dry for 5 min
- Add 15 ml/tube of 0.02 M Tris, pH 7.6. Vortex.
- Allow to dissolve for several days at 4°C.
- Determine OD₂₆₀ of a diluted aliquot to check concentration (1 OD₂₆₀ = 50 µg DNA/ml), then store at -20°C.

This method is based, with permission, on an original protocol available [here](#).

1.9.28 Proteinase K treatment

Contributed by [Martin Fitzpatrick](#), University of Birmingham, United Kingdom

Basic proteinase K treatment

- Aliquot an appropriate volume of 10mM Tris-HCl
- Add 1mM EDTA (ethylenediamine tetraacetic acid)

- Add 1% v/v Tween 20
- Add 0.1mg/ml proteinase K
- Mix solution thoroughly then adjust to a pH 8.0 as appropriate with ,magnetic stirrer
- Incubate sample overnight at 37°C.

Longer incubations and higher concentrations of proteinase K will improve DNA quality and yield from fixed tissue sections.

- Boil sample in solution for 8 minutes to inactivate the proteinase K. Sample is now ready for PCR.

1.9.29 Retroviral infection of PHKs

Contributed by Matt Lewis <hop2kin@yahoo.co.uk>

Retroviral infection of primary human keratinocytes (PHKs)

PHK Key Points

PHKs must be actively dividing to be successfully infected. It is important not to allow cells to become confluent as this will prevent trypsinization (even if you do force them all off only 10% will re-attach in the new flask).

PHKs must be allowed to 'get going' after resuscitation from freezing, try to infect at the 4–16 cell cluster size (3–4 days?).

PHKs differentiate in response to serum and/or Ca⁺⁺. Apparently they are able to de-differentiate if the exposure was not too long/strong.

Packaging cell points to bear in mind

Packaging cells grow very fast especially PT67s (12-hour doubling time). Overconfluence is not so bad (titres remain OK)

Half-life of virus is about 4 hours in media at 37°C. Supernatants are useful at anything from 6 hours to 24 hours. More than this then defective particles may become a significant factor although at our low titres it is not such an issue.

- Resuscitate frozen PHKs into T25s or T75s in suitable media. Remember to include controls for mock infection (T25s or a 6-well plate)
- Incubate overnight at 37°C
- Refeed cells with appropriate media.
- Incubate overnight at 37°C
- Check PHKs and determine if you can currently see plenty of 4-cell clumps - if so tomorrow will reach 8-cell stage (infection day). If not, wait a day.
- Resuscitate packaging cells into DMEM
- Resuscitate J2-3T3s into a 6-well plate in DMEM (for titres)

*Its best to do a titre for each virus. If doing lots of different infections then set off the J2-3T3s earlier and trypsinize today into 6-well plates. They should be 20–80% confluent at infection time. *

- Check packaging cells. These should be 50–100% confluent now. If not then consider (a) resuscitating more and adding on top, or (b) delaying infection until day 4.
- Refeed packaging cells with KGM. Not the ideal medium for them but the titre is OK
- Incubate all cells overnight; tomorrow is infection day.
- Remove supernatants from packaging cells (put to one side) into universals and refeed cells with DMEM.

- Filter supernatants through 0.45µm filters into fresh universals

If you don't filter then loads of fibroblasts come through and spoil the experiment.

- Refeed PHKs with a 1:1 ratio of infectious supernatant and fresh [KGM + polybrene (12µg/ml)] and put back in the incubator to allow infection to occur for 6–8 hours.

*for T25 use 2mL supernatant + 2mL KGM for T75 use 5mL supernatant + 5mL KGM

Polybrene is hexadimethrine bromide, Sigma H9268, you want a final conc. of 6µg/mL. Store polybrene stock of 3mg/mL in PBS at 4°C.*

- Allow infection to proceed all day.
- Refeed with fresh KGM.
- Incubate overnight to day 4 to begin selection.
- Selection:

G418 only: use 150µg/mL in KGM. Check each day. Puro only: try 1µg/mL G418 + Puro: try 50µg/mL G418 + 0.4µg/mL puro.

This method is based, with permission, on an original protocol available [here](#).

1.9.30 Retrovirus titration

Contributed by Matt Lewis <hop2kin@yahoo.co.uk>

Titration of retrovirus lines.

Note, target cells must be dividing to be infected. As the viral RNA/protein complex cannot enter the nucleus entry must wait for nuclear membrane to dissolve at mitosis.

- The day before infection set up J2-3T3s in 6-well plates.

J2-3T3s are recommended as they provide discreet colonies after selection

Some retrovirus cannot be titred on rodent cells such as 3T3. For example, packaging lines using the gibbon ape leukaemia virus (GALV) env protein must be titred on human cells.

- Check cells are 20-80% confluent on the day of infection
- Prepare 6ml/titration of [DMEM + 9ug/ml polybrene] in a 50ml universal tube e.g. for 3 titrations prepare 18ml.

Polybrene is hexadimethrine bromide (Sigma H9268). Final concentration should be 6ug/ml. Store polybrene stock of 3mg/ml in PBS at 4C.

- For each titration set up 6 Eppendorf tubes each containing 800ul of DMEM, labelled 1-6.
- Add the 200ul of supernatant to Eppendorf no.1 and mix by inverting several times
- Transfer 200ul from Eppendorf 1 to Eppendorf 2 and mix by inverting.
- Continue through all 6 tubes producing a serial dilution.
- Refeed the J2-3T3s with 1ml/well of [DMEM + 9ug/ml polybrene]
- Add 0.5ml from each Eppendorf to each of the wells 1-6.
- Incubate cells overnight at 37°C to start infection. Select the following day.
- For G418; use 600ug/ml in DMEM, it takes around a week to get the result.

For Puromycin; use 12ug/ml in DMEM, it can take several days to get the result.

To avoid counting colonies down the microscope remove media and let the plate dry overnight. You can then score colonies visually.

This method is based, with permission, on an original protocol available [here](#).

1.9.31 RNA-isolation (TRIZOL method)

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

Isolation of RNA from whole worms using TRIZOL reagent (Gibco-BRL)

- Wash worms from a 60 mm plate with 1 ml DEPC-treated water.
- Spin down worms at 4000xg in microfuge for 1 minute.
- Remove excess water and add 1 ml Trizol reagent. Vortex and invert tube. Let sit at room temperature for 10 minutes (should see stringy-like material).
- Spin tubes in microfuge at 14K for 10 minutes at 4° C.
- Remove supernatant into fresh RNase free eppendorf tube and add 200 µl of chloroform.
- Vortex for 15 seconds and let sit at room temperature for 3 minutes.
- Spin in microfuge at 12K for 15 minutes at 4° C.
- Carefully remove the top layer (clear) into a new RNase free eppendorf tube and add 500 µl of Isopropanol. Invert to mix. Let sit for 10 minutes at room temperature.
- Spin in microfuge at 12K for 10 minutes at 4° C. (RNA will be seen as a nice white pellet).
- Wash pellet with 100 µl of 75% ethanol (made by diluting into DEPC-treated water).
- Spin in microfuge at 7500 xg for 5 minutes at 4° C.
- Remove supernatant and air dry pellet for 10 minutes.
- Dissolve pellet in 25 µl of DEPC-treated water. Heat for 10 minutes at 60° C to help dissolve RNA.

This method is based, with permission, on an original protocol available [here](#).

1.9.32 T-A cloning Vectors

Contributed by [Ian Chin-Sang](#), Queens University, ON, Canada

A method for direct cloning a PCR product, by the T-vector technique. This is cheap and easy way to clone PCR products with A 3' overhangs.

- Making the T-vector:

Digest 5 ug pBluescript II with EcoRV (blunt cutter).

- Heat kill the enzyme or gel purify using Qiagen column elute in 50ul EB.
- Add 10 ul of 10X PCR buffer, 2 ul of 100 mM dTTP, 37.0ul of distilled water and 1.0 ul of Taq DNA polymerase. Incubate at 72 C for 2 hours.
- Purify the T-vector by phenol/chloroform extraction and ethanol precipitation or purify over a Qiagen column. Resuspend/elute the prepared T-vector in 100 ul of water or TE. We use about 2- 5ul in a ligation reaction.
- Cloning the PCR product:

Purify the PCR product over a Qiagen column (Gel purify if necessary). Elute in 50 ul EB. Use 5-10 ul of this PCR product in a ligation reaction e.g.

- 10ul PCR insert
- 5ul T-Vector
- 4ul 5X Ligase buffer
- 1ul T4 Ligase
- Ligate 15min RT(rapid ligation kit) to overnight (15C).
- Transform into E. coli (XL1Blue).
- Plate on Amp (75ug/ml) X-gal (spread 60ul of 2% solution on plate) Tet (15ug/ml) IPTG (0.1-1mM)
- Pick white colonies to prep.

References

Marchuk D, Drumm M, Saulino A, Collins FS [Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products](#). *Nucleic Acids Res* (1991) pmid:2020552

1.9.33 Transformation of plasmid DNA to E. Coli

Contributed by Matt Lewis <hop2kin@yahoo.co.uk>

Transformation of plasmid DNA to competent E. Coli cells

- Thaw competent cells on ice. 20–200µL per tube
- Add max. 20µL of a ligation reaction and mix _very_ gently

When transforming purified plasmid into competent cells add just 1ul plasmid DNA solution.

- Incubate the tubes on ice for 30 min
- Heat shock the cells at 42°C for 45 sec to 2 mins
- Place the tubes immediately on ice for a further 2 minutes
- Add 800µL SOC medium to each tube
- Incubate for 1 hour at 37°C shaking vigorously
- Spin down at 1200rpm for 5 minutes and remove supernatant
- Resuspend cell pellet in 200ul SOC medium by pipetting up and down
- Plate out the suspension on a LB agar plate containing the appropriate antibiotic.

When transforming purified plasmid into competent cells plate out only 10–20µL bacterial suspension to the plate instead of all.

- Incubate the plates overnight at 37°C

This method is based, with permission, on an original protocol available [here](#).

1.9.34 Transformation of XLI-Blue MRF' Competent Cells

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Transformation of XLI-Blue MRF' Competent Cells

- Before starting, get water bath at 42°C.
- Add MgCl₂ (10 ml/l of 1 M) and MgSO₄ (10 ml/l of 1 M) to an aliquot of Super Optimal Broth (SOB).
- Prepare and prewarm SOC (SOB with Catabolite repression) medium to 42°C. Place LB-amp plates at RT.
- Prechill 2059 tubes by placing on ice. Thaw XLI-Blue MRF' competent cells on ice.
- Gently disperse cells then dispense into bottom of chilled 2059 tubes at 100 µl/tube using plastic 1 ml pipet. Keep on ice.
- Add 1 µl/tube (0.1-50 ng) of plasmid from miniplasmid prep. to cells. Include a no plasmid control. As a positive control, use 1 µl of pUC18
- Incubate on ice for 30 minutes.
- Put tubes in 42°C water bath for exactly 45 seconds.
- Remove and place tubes immediately on ice for 2 minutes.
- Remove from ice and add 0.9 ml/tube of 42°C SOC medium.
- Incubate for 1 hour (37°C) shaking at 225-250 rpm.
- Plate on LB-amp plates using 5, 50, 200 µl/plate. Use glass spreader and rotating platform.
- Incubate overnight at 37 °C.
- Pick colonies using grid plate method (LB-amp plate grid + Miniplasmid Prep.).

CHAPTER 2

Indices and tables

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