- **Bonding PAGE gels to glass plates using Bind Silane**

- This procedure is used to immobilise gels that will be scanned and subjected to robotic spot picking.

- Clean glass plates with 50% ethanol and Kimtech tissues thoroughly.

Bind Silane working solution

8 ml Ethanol

200 ul glacial acetic acid (under the hood)

10 ul Bind Silane (e.g. Amersham)

dH_20 to 10 ml.

- Pipette 1 ml of the above solution on the whole surface of the plate and wipe it over with a lint-free tissue until it is dry.
- Put the tissue on a rack and cover the plates with a lint-free tissue to prevent dust contamination and leave on the bench (2 hours/overnight) for excess bind-silane to evaporate and dry up.

Note: If the bind-silane is not left to dry sufficiently before the glass plates are assembled for casting, the solution will evaporate off the treated plate and coat the facing glass surface. This will cause the gel to stick to both plates when it sets.

- The gel will stay attached to bind-silane treated glass during electrophoresis, staining procedures, scanning and storage.
- From the template, attach the white stickers on front of back plate once bind-silane dries up.

- **Preparing the gels:**

Castor for 6/12 gels

Back plate

Spacer

Front plate

Front plate

Order of arranging plates

Spacer

Front plate 2nd Set

Back plate

Spacer

Front plate 1st Set

Back plate

Displacing Solution (not for 6 gel caster)

100 ml of Displacing solution Tris (1.5 M, pH 8.8) - 25 ml

(Need about 70 mls) Glycerol (87% w/v) - 57.5 ml

H_2O - 17.5 ml

Bromophenol blue - few grains

12.5% Acrylamide gel (1L)

(Need about 900 ml) Leave some in a falcon tube or bottle to check when it sets. Use the bottles for acrylamide gels on rack above.

Acrylamide - 312.5 ml

Tris (1.5M, pH 8.8) - 250.0 ml

10% SDS - 10.0 ml

APS - 1.0 g in 10ml H_2O

Water - 417.5 ml

TEMED - 138.0 ul

* Add TEMED last after filtration.

- No need of Displacing Solution for small gel casters.
- For 12 gel casters, casters can tilt back an angle on stand, so plates and

separators can be slotted in.

- Once plates are in, fill up the gap with thick plastic fillers and then

Separators until almost flush, leave only 1 mm of space for the gel to expand.

- Put the front (or face) and screw in. Let this all sit on a tray in case of leakage. Use disposable plastic filters. Attach to tap.

- Add TEMED and mix and invert slowly to avoid bubbles.
- Pour into funnel on top of gel caster and fill up to 3 mm down from bottom glass plate.
- Pour in displacing solution (~ 70 ml), just enough to sit at bottom and so that it doesn't go into gel casing.

(We use displacing solution so that we don't want acrylamide in tubing)

500ml Acrylamide gel is enough of 6 gel caster with the block

Displacing solution sits at bottom under casting

- Add saturated butanol to the top of gel (~1 ml on top of each gel)
- When gel is set, take butanol off. (Can pour off the butanol by holding the

entire caster).

12.5% acrylamide gel

12.5% gels

500 ml

600ml

700 ml

800 ml

900 ml

1L

Acrylamide 40%

156.25 ml

187.5

219

250

281.25

312.5

Tris(1.5M, pH 8.8)
125 ml
150
175
200
225
250

10% SDS
5 ml
6
7
8

9

10

APS

0.5g

0.6

0.7

0.8

0.9

1.0

TEMED

69µl

83

96

110

124

138

Water

213.75

***Add TEMED last after filtration.**

- Better to dissolve APS in water first.
- While filtering, let the water run first and then add your solution into the filter.
- Once the gel is set, dismantle and wash/rinse the gel upside down so that water doesn't enter inside between the plates.
- Wipe the plates with a tissue and wrap the plates with cling film to let them not dry out. Store in fridge till use.

Sample Preparation

Drosophila Heads

- About 100 drosophila heads in a cryo vial (dissected dry and then put in Liquid Nitrogen and then -70°C).
- Put 400 uls of DIGE lysis buffer into the cryovial and then transfer it to an Eppendorf.
- Homogenise with a blue probe.
- Sonicate for about a second and then put on ice to cool. Repeat the same 3-4 times.

Note: DIGE lysis buffer contains urea and if the protein sample is sonicated too long, it heats up urea which converts into isocyanate which can modify protein by carbamylation.

- Centrifuge for 10 minutes at full speed.
- Transfer the supernatant into a new eppendorf (Keep the pellet too, incase).

Drosophila Midgut and Tubules

- About 600-800 midgut/tubules into 400µls of DIGE Lysis Buffer. Dissect about 6-12 flies and transfer immediately midgut/tubules into DIGE Lysis Buffer (DLB) (with 1ul Protease Inhibitor/100µl DLB).
- Sonicate for about a second and then put on ice to cool. Repeat 3-4 times.
- Centrifuge for 15 minutes at 9000 rpm at 4°C.
- Transfer the supernatant to a cryovial, if to be stored at -70°C.

Protein Precipitation

(Removes salts, lipids, polysaccharides, etc.)

1. **1.** Add 4x sample volume of cold acetone (-20°C) acetone to protein suspension.

(If you have 400 µl of sample, divide into two 200µl samples and add

200x4=800 µl of acetone into each.)

2. Vortex tube and incubate for 30 minutes at -20°C.

3. Centrifuge at 13000g for 30 minutes.

4. Decant supernatant and wash pellet with 4x cold 80% acetone. (~800 ul). Disrupt the pellet with a closed tip.

5. If warms up, put in -20°C for half an hour. Can keep it longer if needed.

6. Repeat steps 3, 4 and 5.

7. Centrifuge for 30 minutes at maximum speed, decant supernatant, centrifuge briefly (2-3 minutes) at maximum speed and remove residual supernatant

 with pipette.

8. Airdry 2 mins. At RT. Do not overdry.

9. Resuspend pellet in DIGE lysis buffer 50 µl. (If larger pellet, 100 µl)

10. Vortex hard. Pipette up and down to dissolve the pellet. Vortex for 20-25 mins. on shaker.

11. Centrifuge at maximum speed for half an hour.

12. Just take the supernatant and put them at -20°C. (Keep the eppendorf with the pellet till you measure the protein concentration).

Bradford Assay

- Biorad reagent (in the fridge).
- Standards-BSA dilutions (3 replicates of each of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mg/ml)

Standards

- 5 µl DIGE Lysis Buffer (DLB)+ 5 µl of BSA dilutions (3 replicates of each of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mg/ml) + 200 µl Biorad reagent.

- Control-10µl DLB + 200 µl Biorad Reagent.

Samples

- 5 µl DLB + 5 µl Sample (Dilute the sample till the blue colour is in standard range).

Note: Read within half an hour of adding Biorad reagent.

Graph and Calculations

- Take out average for samples and standards and blank reading and subtract blank value from samples and standards average reading.

- Put Standard concentration and absorption values in 2 columns, go to graph (XY Scatter), Next, Columns, Next, Finish.

- Go to Chart, Add Trendline, Option, Format trendline, tick on Display equation and R^2 value.

- You will get an equation y=mx+c and we need to find x.

Therefore, x=(y-c)/m and y is the absorption value for the respective sample.

Multiply x with number of times you have diluted your sample and that is the concentration of your protein.

□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□

□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□

IsoElectric Focusing (IEF):

CyDye labelling:

- After protein assay, adjust protein concentration to 5 mg/ml, and take 10µl of protein and therefore, 50µg/10µl.
- On ice, add 10µl (50 µg) protein to 1µl aliquot of 400pmol CyDye (2,3,5) and mix immediately by pipette strokes.
- Incubate on ice for 30 minutes in dark.
- Add 1µl 10mM lysine to stop reaction.
- Keep on ice for 10 minutes in dark. (labelled samples can be stored at -70ºC).

Unlabelled Samples:

- Take atleast 250 µg and up to 500µg of protein
- A smaller quantity and concentrated amount works better than a larger volume and less concentrated amount of protein. E.g. 10µl of 350µg protein works better than 50 µl of 70 µg protein.

Setting up IEF:

- Best to start morning or midday as it takes 24-26 hours to run.

- pH 4-7; 24 cm IPG strips.

- Strip holders are in maroon boxes on shelf. Clean strip holders with tap water/ or preferably with bottle of strip holder cleaner at sink and use toothbrush, then rinse with dH$_2$O.

- Get rehydration buffer from freezer (2nd fridge, freezer middle drawer), thaw and add 10 mg (0.01g)/1 ml of DTT (end conc. of 65 mM).

- Make up your samples to 500µl with rehydration buffer.
- Mix and leave sitting on bench for 10 mins.
- Centrifuge at full power for 30 minutes.

- Take only 450µl of the supernatant and pour onto the strip holder and remove the bubbles.

- Use tweezers to take IPG strip out. It consists of a thick plastic strip with gel on it and another thin plastic strip attached to it. Remove the thin plastic strip with no barcode on and put the thick strip in the strip holder with barcode side towards the pointed end of holder and sticky gel side down facing the rehydration buffer with the sample in it.

- Pipette about 1 ml of mineral oil on the top, enough to cover and put lid on.
- Place strips on electrode bed on IPGphor system-(small room (fume hood)), with pointy end at the top. Close lid.

- Protocol-DIGE 9 on right machine. Set up on the machine.

IEF parameters. DIGE 9. Steps. Start. Number of strips. Use up or down arrow key. Start.

For a 24 cm strip, pH 4-7

10 hours @ 30V (rehydration step) (Can run from 10-15 hrs-longer than

2 hours @300V 10 is better)

1 hour gradient to 600V

1 hour gradient to 1000V

3 hours gradient to 8000V

8 hours @ 8000V

- Strips should accumulate between **70-80000 volts** on IPGphor. Don't leave strips on without any current as the proteins can diffuse away from IEF.

- The above protocol will take **25 hours** to run.

SDS-PAGE

- Have gels out of fridge. Rack for 1 hour before use**.**
- Have agarose sealing solution in heating block melting at 90°C. (for 10-15 mins. before use.

1% agarose sealing solution

1x SDS Buffer	100 ml
Agarose	1 g
Bromophenol blue	few grains

- Check there is enough 2X Running Buffer.

For 2X,	Tris	30.2g
	Glycine	144.0g
	SDS	10.0g
	dH_2O	make it to 5L

- Before strips go onto gel, they must be equilibriated with SDS Equilibriation Buffer (SEB)-(stored in 20 ml aliquots in the freezer in the room with fumehood).

10 ml is enough for 1 gel.

add DTT to 1st-0.2g

add iodoacetamide to 2nd- 0.5g

Make sure powders dissolve.

- Take strips out of holders and put into plastic tubes (on shelf-rinse with water before using). Place strips in tubes with plastic backing against tube and gel facing out.

Can stop here and freeze the strips if needed

- Add SEB+DTT enough to cover strip (about 10 ml) and rock gently for 15 minutes. (large flat rocker next to sink).
- Pour off and use bung to stop strip falling out, add SEB+IOA (about 10 ml), rock gently for 15 mins.

- Meanwhile, have the gel tank ready. Use blank cassettes (wet first) to fill up unused spaces. Put gels in front in the tank so they are visible.

Make sure tap at back of tank is closed.

Pour in some running buffer (1x) -7 to 7.5 litres to fill up the bottom chamber. Depending on number of gels being run, amount of 1x buffer needed will change.

- Put gel plates down on bench, take strip out of tube and lay down in gap, overlap of glass. Barcode end goes on left and gel side facing up. Push down till it touches the SDS-PAGE gel.

- Fix strips with 1% agarose sealing solution from heating block. Pipette along the length of the strip. If there is a leak, wait till it settles and add more to the top.
- Put gels into tank, lubricate with running buffer before pushing them down. Fill with 2x running buffer to lines on the tank.
- For o/n run, run at 2W per gel. Can go up to 10-20 Watts per gel.

Don't let gel sit without any current.

Scanning

- Once the gel has run enough (blue colour goes off), turn power off at Ettan

  II Dalt, take lid off and turn on the tap at back and buffer will drain out.

  (Gel takes longer to run at the bottom).

- Use metal flat hook (from the drawer) to hook bottom of gels and pull up.
- Close tap at back and refill with tap water and then open the tap and let it drain out.

Note: If somebody's gels are running and you need to take off your gels, some of the 2x buffer will mix with 1x buffer at the bottom, so its better to drain the buffer and fill it up with 1x in the bottom and 2x at the top after filling gaps with cassettes.

Typhoon (for fluorescent scanning) (For 2D gels):

- Use the metal plate holders to hold the plates firmly.
- Put gels in barcode down left hand side.

barcode down

- Turn on typhoon half an hour before scanning.
- Pull gels upside down in the rack and let it lie flat on tissue and wash with water and wipe with blue roll.
- Wipe the surface on the glass plate on the typhoon and the surface of the rubber part under the lid, before and after use, with ethanol.
- If the gel residues or anything else stuck doesn't come off, try with 30% hydrogen peroxide.
- Only use Kimtech tissues for wiping and not the blue roll.
- While scan is been performed, try not to move the scan window to a different position or open a new window as this can crash the scanning.
- Turn off typhoon after use.

Settings

For CyDye2

Laser- blue2 (680nm), PMT-600 V (volts).

Go down on PMT if it appears too saturated. (red).

For Sypro orange,

Laser-green (580 nm), PMT-600V,

Typhoon (for fluorescent scanning) (For Western Blots):

- After a clean wipe with ethanol, pour some water on top of scanning bed to avoid the membrane/gel drying out.
- Protein side of the membrane should face down.
- Settings-

Cy5-red filter, PMT-600V

Cy3-green filter, PMT-600V

Common settings

Tray-DIGE-Ettan DALT (For 2D gels)

For Western blots- Select the area to scan manually by dragging the cursor from the left bottom corner.

Fluorescence/Focus plane- 3 mm/Do not click on 'Press Sample'.

Low resolution- shorter scan (to see if there are spots)-1000µ (~2 mins.)

High resolution- longer scan (for better picture)- 100-200µ (~10 mins.-1 hr.)

STAINING

Colloidal Coomassie Staining

Coomassie Stock

5% Coomassie Brilliant Blue G-250 in water

Colloidal Coomassie dye stock (normally 500 ml per gel)

50g ammonium sulphate (along with other chemicals)

6 ml 85% phosphoric acid (fume hood)

Make up to 490ml with dH_2O.

10 ml Coomassie stock.

*Shake before use.

Colloidal Coomassie Stain (prepare fresh)

200 ml colloidal Coomassie Dye Stock (Can also use Coomassie G-250 solution as Dye Stock).

50 ml Methanol

Perform these steps with gentle agitation on a rocker or shaking platform.

*Fix gel for 1 hour (or o/n) in 40% ethanol, 10% acetic acid.

*Wash gel 10 minutes in distilled water, repeat.

*Stain gels in freshly prepared colloidal Coomassie stain o/n (can do for several days up to 1 week).

*Rinse gels in several changes of distilled water.

*Cover the tray with cling film.

*Scan with a normal scanner.

SyproOrange Staining

- Fix gel 1-2 hours 7% acetic acid/10% methanol. If gel has been stored or fixed for a longer period, incubate 1 hour in 0.05% SDS. (fixed o/n or till it is completely destained.)
- Stain gel 2 hours in freshly prepared SyproOrange stain diluted 1:10000 in 7% acetic acid.
- Rinse gel briefly (~2-4 minutes) in 7% acetic acid. Dry back plate and scan on typhoon using green laser, emission 580nm.

For **300ml fixing solution**,

7% acetic acid/ 10% methanol/ 0.05% SDS

21 ml / 30ml / 0.15 ml (150 ul) + 249ml dH2O.

To make up 0.05% SDS, add 1ml 10% SDS in 200ml H2O.

SyproOrange 1:10000

To make up 250 ml, add 0.025 ml (25 µl) syproorange stain to 250 ml 7% acetic acid. (For 300ml 7% acetic acid, 21ml acetic acid + 279ml dH2O).

Picking up spots on Ettan Spot Handling Workstation

- **Creating a pick list on DeCyder**

DeCyder Software

Username-SHWFGF Password- DeCyder1

- Open Image Loader. Add the required .gel file. Open Gel Image.
- Edit gel image. Contrast and brightness Adjustment.
- Crop. Right click. Perform cropping.
- Save as an edited file. Close Image editor.
- Open Differential In Gel Analysis.
- Create workspace. Go to folder. Create. Gel (name).
- Imageview. Process Gel Images. Number of spots. 1000.
- Autodetect reference markers.
- Exclude filter. (e.g. Slope-1.3. Number reduced from 712 to 588).

(Spike-noise/slope-protein)

- File. Save work.
- File. Export pick list. Open in excel. Save.

- **Setting up Ettan Spot Handling Workstation**

Buffers (don't need to make up every time)

Digestor 1: 50mM AmBio in 50% MeOH. 3.953/1L. Made up 2L.

Digestor 2: 50% ACN (Acetonitrile), 0.1% TFA. Made up 2L.

Digestor 3: 20mM AmBio in H2O. 1.58g/1L. About 500mls.

Digestor 4: 75% ACN in H2O. Made up 1L.

Matrix

(same quantity every time)-goes in the bottom well and top left side of the rack.

- Dissolve 20mg Cyano-4-Hydroxycinnamic acid in 1 ml Digestor 2 (50% acetonitrile solution) in an eppendorf.
- Vortex hard for 1-2 minutes and centrifuge for 2 minutes at full power.
- Take 900ul supernatant (90% saturated) in a vial (in a white box besides the workstation), add 100ul of 50% acetonitrile solution (Digestor 2).

MS plates and 96 well plates

- Clean MS plate with gel and then wipe it with a tissue till it is really clean.

1 MS plate can take 192 samples.

- 96 well plates (not the flat bottom ones)-need 2 for up to 96 samples.
- E.g. for 230 spots to be picked, arrangement will be 1 2 MS plate 1

1. 4 MS plate 2

(1,2,3,4,5,6 are 96 well plates) 5 6

- ∅ **Setting up the protocol on the computer**

Ettan spot Handling

Username: Service Technician

Password: passwd

For Calibrating the reference markers. (don’t need to do everytime)

Go to System Config.-Camera Alignment

Click on confirm the marker

Start.

Do the same thing 3 times. Make sure you close down System Config.

Go to Batch config.-Stdjuly04

Batch name – eg. Rujuta 181109……

MP ID – tubules 181109

(Open pick list. Save as C: Amersham Biosciences, Data, Picklists, Rujuta)

Get the pick list up from the required folder.

Tray ID: Scan the 1st barcode i.e. barcode of the gel tray.

Backing: Glass

Target type: ABI 4700 192

Layout: Use this one

Click on ADD button between ‘Backing’ and ‘Target type’.

Check the number of spots on the right are same as on the Pick list.

Click on ‘Next’.

Scan the barcodes on the paper attached on the robot machine. Viz.

Enzyme …. 4718….

Matrix…… 4717….

On the desktop, open the file ‘Calibrant A’. Copy the barcode and paste in ‘Enter Barcode’.

This will tell the amount of trypsin needed.

Start priming. Make the trypsin ready.

Trypsin

(20 ug aliquots in small freezer on the top)- Trypsin goes in right side of top well.

- Resuspend with 1 ml of Digestor 3.
- 1 vial approximately is enough for about 60-70 spots.

Note: Add Trypsin only after priming is done. Wash well with dH_2O.

Once trypsin is added, click on next. Tick all the boxes. Enter volume on the fourth last box. i.e. Matrix – 500

- 100

Tick boxes again.Start.

Analysing results of Mass Spec

- Once Mass-Spec is run, get a sample tracking report on SHW computer (besides the robot) and save it as a *html file on the memory stick.

If the robot crashes, then there will be no report generated and the report is not too important.

- On GPS computer (one on left), to get the Mascot results.

Open GPS explorer. Username. Password.

On left side, open Rujuta. New project. Rujuta.e.g. 02/11/09.

New sample set. Gel-based. Select spot sets.

4700pa+RB-rujuta 3 and 3b.Add. Add. OK.

Select [rujuta]. New analysis. Yes. Type- combined (MS+MS/MS).Select job runs.

4500pa. Add. OK.

Analysis Settings. Open template analysis.RB1.Database search. Drosophila.

Precursor tolerance 100ppm. Save search template (Dm). Save settings.

- Results. Refresh for results. Click on minus. Open up analysis.

To get mascot results page, select a significant hit and go for DB results.

O- analysed (not significant)

O- light green analysed (significant)

O- blue not analysed.

O- yellow-just below significant.(take it as non-significant)

O- dark green- wrong numbers given while set up, so MS analysis done on something which is not there.

- Double click on light green ones for results. Get the MASCOT MALDI results on the bottom by going to DB results. Copy Mass, Score, Accession, Description of protein and Maldi plate well number on an Excel sheet.

- To generate a PDF Report, go to Reports, click on your file on left. OK. File. Export. Memory stick.

Table View of Spot Number, Max slope, Area, Max Peak Height and Max Volume

- On the computer on right, Open DeCyder. Differential Gel Analysis.

Open DIA file. “Rujuta”.

Properties. Table View. Display only picked spots. Untick rest. Apply.

Organise by spot number. (i.e. Click on the column in the table which says

Spot number). Edit. Copy Table View. Open Excel. Paste.

- Put spot no., max slope, area, max peak height, max volume, maldi plate no., accession, mass, cut off score and description together on an excel file which are the final mass-spec results.

In Gel trypsin digestion of silver or coomassie stained proteins

- Cut the gel band into equal sizes and transfer them into 1.5ml eppendorfs.
- If gel slices are quite big-chop them up with scalpel blade**.** Use a glass plate (clean with dH $_2$O, then methanol). Clean glass and blade in between uses.

- Remove if there is any liquid present in the eppendorfs.
- Wash gel pieces in 500µl of 100mM ammonium bicarbonate. Put on shaker, RT for 30min-1 hr. Discard wash.

- Wash gel pieces in 500µl of 50% acetonitrile/100mM ammonium bicarb. (30min-1 hr). Put on shaker. Discard wash.
- Not necessary for 2D gels.

To reduce, add 150µl of 100mM ammonium bicarb. and 10µl of 45mM DTT. Incubate at 60ºC for 30 mins. (heating block)

Make up 1 ml then chuck after use.

1M = 154.2 g/l

45mM DTT= 6.9 g/l

=0.0069g/ml

=6.9 mg/ml

- Not necessary for 2D gels.

To alkylate, cool to room temperature – 10 mins.

Add 10 µl of iodoacetamide. Incubate in the dark for 30 mins. (drawer)

Make 1 ml of 100mM iodoacetamide. Chuck after use.

1M – 184.96 g/l

100mM- 18.496 g/l

- 0.0185 g/ml.

- Discard solvent and wash gel pieces in 500µl of 50% acetonitrile/100mM ammonium bicarb. with shaking for 30min-1 hr.

- Discard wash. Add 50µl of acetonitrile to shrink gel pieces. After 10 mins. remove solvent and dry gel pieces completely in vacuum centrifuge.

Vacuum centrifuge- remember to balance it.

Leave lids of tubes open.

Make sure tap at bottom is closed (up).

Turn switch on at bottom-refrigeration unit.

Don’t need temp on.

Check samples after ½ hr- should look very dried out. Touch with tip to see if rigid. Put back in if not dry.

- Start by adding 20µl trypsin and if completely absorbed, add additional trypsin until gel band appears fully rehydrated and swelled. Finally add sufficient 25mM Amm. Bicarb. to just cover the gel pieces.

(0.02ug/µl Trypsin- take a new vial each time from bottom shelf 2nd freezer outside and add 1000µl 25mM amm. bicarb.)

- Allow the protein to digest o/n at 37ºC.
- Briefly centrifuge the tube to pellet gel pieces. Transfer all liquid to clean 96 well plate (V bottom). Don't transfer any gel pieces. (Put samples in across the way A1-A12).

- Add 20µl of 5% formic acid (or sufficient to cover pieces) and incubate for 20 minutes.
- Add 40µl of acetonitrile and incubate for a further 20 minutes.

- Briefly centrifuge the tube to pellet gel pieces. Transfer all liquid to the same well as used for the first extract. Don't transfer any gel pieces.

- Dry down the combined extracts completely in the Speedvac.

Change rotor to 96 well plate and balance.

Start with 1 hour, may take longer (stop and check).

Once dried, put a lid, cover with a foil and store at -20C till LC-MS machine is free.

Tubule Membrane Preparation & 1D LC-MS

- **Sucrose Density Gradient**

Buffers used for Sucrose Gradient

- **Drosophila Lysis Buffer (DLB)**

Tris Cl (50mM pH 7.5); KCl (150mM); Sucrose (0.25M);

DTT (0.1M); PMSF (1mM); Leupeptin (2ug/ml); Pepstatin (2ug/ml)

- **2.5M, 2.0M and 0.5M sucrose**

- Make up 2.5M sucrose (42.79g/50ml TKMD) previous day. Add only little TKMD while dissolving sucrose and warm up sucrose by putting into a beaker with hot water in it.
- To make up 2.0M sucrose (3 ml), add 0.7ml of 0.25M sucrose (TKMD) to 2.3 ml of 2.5M sucrose.
- To make up 0.5M sucrose (2 ml), add 1.8 ml of 0.25M sucrose (TKMD) to 0.2 ml of 2.5M sucrose.

- **TKMD**

For 450 ml

Tris Cl (50mM pH 7.5) 22.5 ml (1M)

KCl (150mM) 67.5 ml (1M)

MgCl2 (5mM) 4.5 ml (0.5M)

+ 355.5 ml H2O

- **Na2CO3 (0.1M, pH 10.0) - Make up regularly.**

MW=105.99 and so, 2.12 g/200 ml

- **Laemmli buffer (2X)**

4 ml 10% SDS; 2 ml glycerol; 1.2ml 1M Tris-Cl (pH 6.8)

H2O 2.8 ml; Bromophenol blue 0.02% w/w

Day 1

- Thaw the tubules on ice. Add 25 µl of Drosophila Lysis Buffer (DLB).

Mix up and down with pipette.

- Homogenise with a blue probe. Centrifuge for 10-20 seconds.
- Pool all sample together. ~ 500µl into 1 eppendorf.
- Sonicate at 3000 rpm for 10 minutes at 4ºC.
- There might be no lipid layer on the top as it might have settled at the bottom with the pellet.
- Take out supernatant into an ultracentrifuge tube.

150µl 0.5M sucrose

250µl 2M sucrose

200µl supernatant

800µl 2.5M sucrose

Mark with a pen.

Mix

- Centrifuge at 100,000g for 2.5 hours at 4ºC. (48,000 rpm for TLA120.2 optima ultracentrifuge).

(Or, used the big ultracentrifuge in cell culture lab-100,000g=23,700 rpm for 4 hours-book in

advance).

- Switch on machine ½-1 hr earlier.
- Press display/enter once everything is set up.
- When it shows vacuum on screen that is when you can open the door. Press on ‘door’.
- Take the liquid out from the required layer into an eppendorf and add 1ml of Drosophila Lysis Buffer. Mix well.
- Centrifuge at 30000g (26,300 rpm for TLA120.2) for 30 mins. At 4ºC.(cell culture lab-24,000rpm)
- Remove the supernatant and keep at -70ºC.
- The membrane pellet can be analyzed further or stored at -70ºC.

Day 2

Carbonate Wash

- · Resuspend into 0.1M Na_2Co_3 (pH 10), 0.5 ml.
- · Put on ice for 30 minutes.
- · Centrifuge at 26,000 rpm for 30 mins. at 4C. (cell culture lab-24,000rpm)
- · Save the supernatant.
- · Resuspend the pellet in water. (10µl if very small pellet).

- · Do a **Bradford Assay** to check the protein concentration.

- · **Run all on a Biorad precast gel** at 50V for ½ hour and at 100V till the dye-front reaches bottom. (On 9µl samples, added 9µl loading dye-(2x Laemmli Buffer +1/20 B-mercaptoethanol).

Fix
with 40% ethanol, 10% methanol for 1 hour. (50 ml).
Wash
2 times (10 minutes) with dH
2
O.
Stain
with Coomassie Blue 50 ml (40 ml G-250 + 10ml Methanol) till you see bands.
Wash
2x10 minutes with dH
2
O. Keep in water and covered till trypsin in-gel digestion and LC-MS.

BIOTINYLATION

Pierce Cell Surface Protein Isolation Kit -89881

8 reactions

Kit contains:

- EZ-link Sulfo-NHS-SS-Biotin, 8x12 mg vials
- Quenching solution, 16 mls.
- Lysis Buffer, 4.5 ml.
- Neutravidin ™ Agarose, 2.25 ml settled gel supplied as 50% slurry (4.5ml total volume).
- Wash Buffer, 34 ml.
- Column Accessory Pack, 10 spin columns with bottom caps and 20 collection tubes.
- No-Weigh ™ Dithiothreitol (DTT), 8x7.7 mg microtubes.
- BupH ™ Phosphate Buffered Saline Pack, 2 packs, each pack results in 0.1M sodium phosphate, 0.15M NaCl;pH 7.2 when reconstituted with 500ml of ultrapure water.

- BupH Tris Buffered Saline Pack, 1 pack, results in 0.025M tris, 0.15M NaCl; pH 7.2 when reconstituted with 500 ml of ultrapure water.

Material Preparation:

Phosphate Buffered Saline (PBS)

Dissolve the dry-blend buffer with 500ml of ultrapure water. For long term storage of excess buffer, sterile filter the solution and store at 4C.

Tris Buffered Saline (TBS)

Dissolve the dry-blend buffer with 500ml of water. Same as PBS for storage.

Procedure for Cell Surface Biotinylation:

A. Biotinylation

- · Dissections done in Schneider's (Canton S).
- · Transfer all tubules in 500µl Schneider's (with 5ul Protease Inhibitor).
- · Remove Schneider's and wash tubules twice with 500µl ice-cold PBS. Quickly remove the PBS.
- · Do not allow PBS to remain in contact with tubules for more than 5 seconds to prevent rounding and detachment of tubules.
- · Dissolve the contents of one vial of Sulfo-NHS-SS-Biotin in 48ml of ice-cold PBS. Add 1 ml of the biotin solution to the vial (frequent changes).

- · Add 100µl of Quenching Soln. to each flask to quench the reaction. Gently tip the vials for even coverage and mixing.
- · Centrifuge at 500g for 3 minutes and discard supernatant.(2294 rpm)
- · Add 1 ml TBS to the pellet and gently pipette up and down. Centrifuge at 500g for 3 minutes and discard supernatant.

B. Cell Lysis

- · Add protease inhibitors to 500µl of Lysis Buffer and add it to the tubule pellet.
- · Pipette up and down.
- · Using low power to prevent foaming (e.g. 1.5), disrupt pellet by sonicating on ice using five 1-second bursts.
- · Incubate tubules 30 minutes on ice, vortexing every 5 minutes for 5 seconds.

Note: To improve solubilisation efficiency, perform additional sonications during incubation.

- · Centrifuge lysate at 10,000g for 2 minutes at 4ºC (10,258 rpm).
- · Transfer classified supernatant to a new tube.

C. Isolation of Labelled proteins

- · Insert a column into a collection tube.
- · Gently swirl the bottle of Neutravidin Agarose to obtain an even suspension. Add 500µl of Neutravidin Agarose slurry to the column and cap the column.
- · Centrifuge 1 minute at 1000g and discard flow-through. Reuse the collection tube through Step C11 (3,244 rpm).
- · Add 500µl of Wash Buffer to the gel, centrifuge for 1 minute at 1000g and discard flow-through. Repeat this step twice.
- · Apply bottom cap to column, add clarified lysate to the gel, and then apply top cap to

column.

- · **Make sure top and bottom caps are tightly in place.**
- · Incubate for 60 minutes at Room Temperature on a rotator.
- · Remove top cap and then bottom cap from column. Place column in the collection tube and replace top. **Remove top cap before bottom cap to prevent lysate from leaking from the bottom of the column** .
- · Centrifuge column for 1 minute at 1000g (3244 rpm) and discard flow-through.
- · Add protease inhibitor to 2.5ml of Wash Buffer.
- · Return column to the collection tube and add 500µl Wash Buffer. Cap the column and mix by inverting the column.
- · Centrifuge for 1 minute at 1000g (3244 rpm). Discard rinse and remove top cap. Repeat this step 3 times. Replace bottom cap on column.

D. Protein Elution

- · Puncture the foil covering on one No-Weigh DTT Microtube with a pipette tip and add 50µl of ultrapure water to yield 1M DTT.
- · Add 23.7µl of the DTT solution to 450µl SDS-PAGE sample buffer to make a final concentration of 50mM DTT.
- · Add 400µl of the Sample Buffer containing the DTT to the gel and cap the column. Incubate the reaction for 60 minutes at RT on a rotator. (Alternatively, heat at 95ºC).
- · Remove the column's top cap first and then the bottom cap. Place the column in a new collection tube and replace top cap.
- · Centrifuge column for 2 minutes at 1000g (3244 rpm).
- · Samples from various days of Biotinylation can be combined and then protein can be precipitated like in the protocol 'Protein precipitation'.

E. Carbonate Wash

- Resuspend into 0.1M Na_2Co_3 (pH 10), 0.5 ml.
- Put on ice for 30 minutes.
- Centrifuge at 26,000 rpm for 30 mins. at 4C. (cell culture lab-24,000rpm)
- Save the supernatant.
- Resuspend the pellet in water. (10µl if very small pellet).

- · Do a **Bradford Assay** to check the protein concentration.

- · **Run all on a Biorad precast gel** at 50V for ½ hour and at 100V till the dye-front reaches bottom. (On 9µl samples, added 9µl loading dye-(2x Laemmli Buffer +1/20 B-mercaptoethanol).

Fix
with 40% ethanol, 10% methanol for 1 hour. (50 ml).
Wash
2 times (10 minutes) with dH$_2$O.
Stain
with Coomassie Blue 50 ml (40 ml G-250 + 10ml Methanol) till you see bands.
Wash
2x10 minutes with dH$_2$O. Keep in water and covered till trypsin in-gel digestion and LC-MS.