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Appendix

Appendix 1 – Specific Laboratory Protocols

The methods here are organized according to the order of appearance in thesis.

RNA Isolation - TRIZOL METHOD

Materials

RNA later or Liquid Nitrogen

Trizol

Phenol-Chloroform

Ice

Chloroform

2-propanol (isopropanol)

RNA free tubes

RNA free water

Work Area

1. Set up work area for sterile work conditions. All working surfaces wiped over with 70% alcohol. This includes – pipettes, forceps, homogenizer, scalpel handles, bench area, scales, containers, etc.
2. NOTE- homogenizer may need to be treated with hydrogen peroxide solution prior to using
3. Work in fume hood
4. Use gloves at all times- change regularly when contacting non-clean areas.

Tissue Treatment

1. Tissues must be collected as quickly as possible from removal from patient. RNA begins to deteriorate immediately so speed is essential.
2. Tissues can be collected into liquid Nitrogen or into RNA later.
3. RNA later – Specimens don't need immediate refrigeration when using this product. Tissue size must be < 0.5 cm cubed and immersed into 5-10 the tissue volume of RNA later.
4. These samples can be then stored at room temp for at least a couple of days if necessary. NOTE – Do not freeze tissues in RNA later immediately. Store at 4°C for 24 hours before shifting to freezer for long-term storage.

Method - RNA Isolation

1. Remove tissues from RNA later with sterile forceps and shake of any excess solution – weigh out approx 100mg of sample
2. When using Trizol – use the fumehood as odor is very strong.
3. Place into 1ml Trizol and homogenize using sterile homogenizer or sterile mortar and pestle.
4. Place sample on ice and using new sterile syringe suck sample through small needle to further homogenise
5. Leave sample on ice for 5 minutes
6. Add 250µl of phenol-chloroform directly to sample NOTE – when removing phenol-chloroform from bottle ensure that pipette tip travels into the 2nd layer. Wipe tip edge on bottle rim to ensure water on outside of tip does not go into RNA sample. If it does it will dissolve the RNA and reduce the yield obtained.
7. Vortex making sure well mixed. If no lid then cover top with tissue to prevent contact with phenol chloroform
8. Spin at 13 000rpm for 10 minutes
9. Check sample now – should see 3 distinct layers (PHASES)
10. Top supernatant contains the RNA – remove gently in RNA free tube
11. Keep pellet and tube on ice until end of experiment
12. Add 250µl chloroform to each RNA supernatant sample. Weigh samples to check balance. Any differences make up with chloroform if needed
13. Vortex using tissue if required
14. Centrifuge 13 000rpm 10-15 minutes
15. Check phases again. RNA is in top layer so remove as much as possible to RNA free tube.
16. Add 250µl chloroform to new RNA supernatant again and spin again.
17. Again remove to RNA free tubes
18. Add 500µl of 2-propanol/ml of Trizol used.
19. Let sit on bench at room temp for 15-20 minutes. Occasionally upend tubes for gentle mix
20. Centrifuge at 13 000rpm 30minutes
21. Remove supernatant very carefully – THIS TIME WE NEED PELLET (RNA has been precipitated)
22. Draw around pellet on outside of tube then let sit and airdry. (Pellet becomes invisible when dry)
23. Re-suspend in 50µl of RNA free H2O
24. To quantify – RNA use spectrophotometer and make a 1:50 dilution

    98µl RNA free H2O

2µl sample

100µl

Control = 100µl RNA free water

25. Add 0.5µL RNA inhibitor to all samples
26. RNA sample can be stored in freezer now or go directly onto DNA Digestion

DNase Digestion of RNA

Used to eliminate the genomic DNA from the newly isolated RNA

1. Make up reaction volume as below in RNA free tubes

   X µL RNA 15µg RNA

   5 µL DNase Inhibitor

   4.5µL of DNase Inhibitor Buffer (10x)

   X µL RNA free H2O

   50 µL end volume

2. Incubate at 37°C for 40 minutes
3. Change to 70°C for 10 minutes
4. Sample can now be placed in freezer for storage until next stage

Purification of RNA

1. Used to remove the DNase enzyme and fragmented genomic DNA
2. Remove from freezer and add 200µl of 100% ethanol to each sample. This is to precipitate the RNA
3. Incubate for 1hour at -20°C for hour in freezer
4. High speed spin for 30 minutes at -4°C
5. Suck out ethanol and check pellet
6. Add 50µl of 70% ethanol. Re-suspend gently and spin immediately for 10 minutes at high speed
7. Dry at room temperature by keeping top open but gently covered with sterile foil to allow some air circulation. In order to see where pellet is draw around it on the outside of the tube. As pellet dries it becomes invisible.
8. When dry re-suspend in RNA Free H2O. Approximately 35µl (this can be increased or decreased depending on pellet size. Mix gently
9. Let sit on ice for 10-15 minutes
10. Check concentration with spectrophotometer as per step 24 of RNA isolation method
11. NOTE- It is normal to lose approximately half of the original concentration.

Reverse Transcription of Total RNA

Materials

RNA free tubes

Ice

70 °C tube incubator

42 °C tube incubator

Total RNA sample (between 1.5 – 2.5µg is ideal)

OLIGOdt

RNA Free water

RT Buffer (5x Promega)

dNTP 10mM (Promega)

RNase inhibitor 40U/µL

Method

1. DENATURING- Make up tube to contain

   X µL RNA sample

   2 µL OLIGOdt

   X µL RNA free water

   22 µL total volume

2. Incubate tube at 70°C for 10minutes
3. While sample is incubating make up reverse transcription mix as below. Always make up enough for 1 additional sample to allow for pipette errors.

   1x MIX

   8 µL RT buffer (5x) (Promega)

   2 µL dNTP 10mM (Promega)

   1 µL Rnase inhibitor 40U/uL (Promega)

   7µL RNA free water

   18 µL
4. Place directly onto ice to stop reaction for 2 minutes
5. After Ice centrifuge in mini-fuge for 1 minute
6. TRANSCRIBING - Sample into 42°µC and immediately add 18µuL of mix to each sample. Make sure pipette mixes sample gently. (Each sample volume = 22µL + 18µL = 40µL total so that RT buffer is now 1x)
7. Immediately add 1µL reverse transcriptase and incubate at 42°C for 1 hour
8. Transfer to 70°C to de-activate enzyme for 10 – 15 minutes

9. Immediately after this tubes can go into –20°C for storage and later use.

PCR

Methods for PCR are outlined in the tables listed in Chapter 3 – Follistatin Isoforms

Agarose Gel Electrophoresis

Materials

Super fine Agar

Ethidium Bromide solution (handle with care – gloves at all times)

Loading Buffer (Bromophenol blue/sucrose)

TAE Buffer (Tris Acetate EDTA buffer) 10x

48.4g Tris

11.42 glacial acetic acid

20ml of 0.5M EDTA (pH 8)

Make up to 1 litre with distilled H2O. Use as 1x TAE

Method

1. Place 1.5g super pure agar into 50ml of 1X TAE buffer into a 150ml conical flask
2. Wipe over gel mold with 70% ethanol and tape each end firmly with masking tape.
3. In order to dissolve, use microwave (SHARP) on high for ~2minutes. Check solution every 30 seconds and swirl gently each time. As solution becomes completely clear it is dissolved.
4. Add 1.25µl of ethidium bromide to gel mix and swirl to combine. Try not to create bubbles in solutions.
5. Pour into gel mold and place comb into gel
6. Leave to set in darkened room
7. Each well can be loaded with 10µl of sample
8. Samples are prepared with

   10µL of sample

   2µl loading buffer

   12µL total (only load 10µL into well)
9. DNA is loaded and runs from the black end to the red end of the electrophoresis setup

10. Gel is run at 120 V for ~ 1hour or until loading dye has moved down the gel to where it is needed.
11. In order to visualize the separation of bands – check under UV light
12. If insufficient, then continue electrophoresis. If appropriate, then take and photograph using the UV computer linked camera and Grab-It software.
13. When gel has been photographed dispose of in an Ethidium Bromide container for appropriate disposal

Affinity Chromatography Columns

Solutions

Homogenising Buffer

Benzamidine 5mM

EDTA 5mM

Sodium azide 0.02%

Sample Preparation

Placenta are weighed and then homogenized in homogenizing buffer at mass to volume ratio of 1:4

Patient placenta are identified as belonging to either spontaneous labour, induced labour or LSCS (nil Labour).

Placental homogenates are stored frozen as 2ml aliquots.

For the purpose of this study, 3 pools were created of 10ml placental homogenate using a number of patients for each pool. The pools are as listed in (2).

Con – A column

Solutions

Starting Buffer (2X)

0.04M Tris pH 7.4	2.42g Tris
1M NaCl	29.22g NaCl
2mM Mn	0.1979g MnCl2.4H2O
2mM Ca	0.1470g CaCl2.2H20

Make up to 0.5L with distilled water

Elution Buffer

Concentrations of 0.2M, 0.4M, 0.6M, 0.8M and 1M Glucose or

Concentrations of 0.1M, 0.2M, 0.3M, 0.4M and 0.5M α-D-methylmonnoside

Method – Con A affinity column

1. CLEANING – Wash column with 5ml of glucose 1.0M solution
2. Add 3X column volumes of starting buffer (1X)
3. STARTING- Make up 1ml of sample combined with 1ml starting buffer and add to column in 1ml aliquots.
4. Add 1ml aliquots of starting buffer collecting each 1ml fraction before adding another 1ml.
5. PROTEIN DETECTION- Test each fraction for the presence of protein using 20µL Bradfords reagent with 80µL of fraction sample. Blue colouration indicates presence of protein. Grade blue from 0 to 4+ for each fraction.
6. Continue to add starting buffer in 1ml aliquots until no more protein is detected.
7. ELUTION – Follow same procedure using first 0.2M then 0.4M glucose etc increasing concentrations till 1.0M is reached.
8. When no protein is detected in any of these concentrations then a new sample may be run through the column and procedure repeated.
9. STORAGE- If final sample has been eluted then column with 3X column volumes of distilled water, followed by 3X column volumes of distilled water with azide. Place cap on and refrigerate for next use.

Heparin Affinity Column

Solutions

Starting Buffer

0.05M Phosphate

0.15M NaCl (pH 7.5)

3.9g Na2HPO4

4.383g NaCl

Make up to 500ml and adjust pH as necessary.

Elution Buffer

0.05M Phosphate

1.5M NaCl (pH7.5)

3.9g Na2HPO4

43.83g NaCl

Make up to 500ml and adjust pH as necessary.

Wash Solution

0.1% Triton X-100

0.05ml Triton X-100 in 50 ml H20

Storage solution

20% Ethanol

20ml ethanol in 100 ml H20

Bradford Reagent

Method – Heparin Affinity column

1. Column should have been stored in 20% ethanol in fridge, therefore will require washing as follows.
2. Wash with 20ml of 0.1% Triton X-100 solution.
3. Wash with 50ml starting buffer
4. Add 10ml patient pooled placental sample
5. Discard the first 2mls of sample flow through. Collect as a pool the remainder of the flow through. This is known henceforth as the UNBOUND pool.
6. Add starting buffer to column collecting all flow through into the UNBOUND pool.
7. Periodically check for protein content of the flow through with Bradford's reagent. Use 80µl of flow through with 20µl of Bradford's reagent. The presence of any trace of blue colouring indicates protein. A reddish colour is negative for protein.
8. Continue to add starting buffer until the flow through is protein negative.
9. Elute using Elution buffer. The first 2ml of flow through can be collected into the UNBOUND pool.
10. All additional flow through is now known as the BOUND pool. Again periodically check for the presence of protein using Bradford's reagent.
11. When the flow through is clear of protein, add 10 mls starting buffer to the column. The first 2ml can be added to the BOUND pool.
12. The rest of the flow through can be discarded.
13. When the starting buffer has completely run through the column, then the UNBOUND pool is again run through. This ensures any additional protein has the opportunity to bind to column.

14. Repeat steps from step 4 using the UNBOUND pool instead of the patient sample pool.
15. Once repetition is complete, then column needs to be washed prior to next patient pool.
16. Add 10ml Triton X-100 to column.
17. If storing then add 20% ethanol so that column is completely immersed in ethanol.
18. NOTE – if column work is going to go overnight, ensure column is covered with whatever solution is currently being used, tap closed, lid on and placed in fridge.

Sepharose Coupling for Signal Amplification

Solutions

1mM HCl

55ul in 500ml distilled H2O

Coupling Buffer

8.41g NaHCO3

29.22g NaCl all in 1 litre distilled H2O

Mix and adjust pH to 8.3

Blocking Buffer

3g of glycine

Add to 200ml of coupling buffer, and adjust pH to 8

Washing Acetate Buffer

1.64g NaCH3COO

5.84g NaCl

Add to 200ml distilled H2O and adjust pH to 4

CNBr- activated Sepharose 4B (kept in fridge in dry container)

Method – Gel Preparation

1. Weigh out 1g of CNBr- Sepharose 4B – (NOTE 1g dry powder = 3.5ml gel)
2. Place in new 50ml tube and add 50 ml 0.1mM HCl solution

3. Mix gently and centrifuge at 3000rpm for 5mins
4. Decant or suck supernatant off gently and repeat 4x times
5. Wash gel in coupling buffer (approximately 5ml per gram dry gel), spin and remove supernatant
6. Immediately, add antibody/coupling buffer solution at gel:buffer ratio of 1:2. In this case CK20 pool antibody/coupling buffer was added at (400ul:6.6ml)
7. Retain 100ul of this mix before adding to gel (Pre-gel sample)
8. Mix antibody/coupling solution with gel on a rocker either overnight at 4°C or for 2 hours at room temperature
9. Spin and remove supernatant. Retain (Post-gel sample)
10. Transfer gel to blocking buffer either overnight at 4°C or for 2 hours at room temperature
11. Spin and remove supernatant – no need to retain
12. Wash with coupling buffer, spin and remove supernatant
13. Wash with acetate washing buffer, spin and remove supernatant
14. Alternate steps 12 & 13 until solution has been washed a total of 4 times by each
15. Final step – wash in coupling buffer, spin and remove supernatant
16. Gel is stored in PBS + azide in fridge

Sample-Antibody Amplification

1. Add 50µl of gel to 1ml of sample
2. Mix for 2 hours or overnight
3. Wash x4 with PBS, spin and remove supernatant – retain supernatant and label supernatant samples
4. Add 50ul sample buffer, 50µl of PBS and 10µl β-mercaptoethanol to each tube
5. Spin, remove supernatant – retain supernatant and label sepharose samples
6. SDS Gel/Western Blot -Gel is run with original sample, supernatant samples and sepharose samples
7. As per the sepharose samples all need to have sample buffer, PBS and β-mercaptoethanol added
8. Boil for 5-10 minutes
9. Run gel and western blot as normal

SDS Polyacrylamide Electrophoresis

Solutions Required:

4X Lower Gel Stock Solution (1.5Mtris/HCl + 0.4% SDS)

181.7 g Tris

4.0g SDS

1. Bring to 900ml with water
2. pH to 8.8
3. Bring to 1000ml with water
4. Filter through 0.45µm filter and store refrigerated.

4X Upper Gel Stock Solution (0.5M tris/HCl + 0.4% SDS)

60.6g Tris

4.0 g SDS

1. Bring to 900ml with water
2. pH to 6.8
3. Bring to 1000ml with water
4. Filter through 0.45µm filter and store refrigerated.

Acrylamide stock Solution

NOTE: Safety precautions necessary as acrylamide is a potent neurotoxin

90 g acrylamide

2.4g N,N'-methylene bis acrylamide

1. Bring to 300ml with water
2. Filter through 0.45µm filter and store refrigerated in a foil covered bottle.

10x Running Buffer

45.5g Tris

216g glycine

15g SDS

1. Make up to 1.5 L with water. Do not adjust pH
2. Store at room temperature.

Roeder's Stain

125mg Coomassie R-250

125ml isopropanol

50 ml Acetic Acid

1. Combine these ingredients and bring to 500ml.
2. Destain in 10% Acetic acid/20% methanol or just 7.5% acetic acid.

2x Reducing Sample Buffer

3.75ml 4xupper gel stock

3ml 100% glycerol

1.5ml 2-mercapto-ethanol

0.9g SDS

Bromophenol blue (small amount to colour samples)

1. Bring to 15ml with water
2. Store in freezer in aliquots.
3. Can be thawed and refrozen many times.

2x Non-Reducing Sample Buffer

5.6ml 4xupper gel stock

3ml 100% glycerol

0.9g SDS

225µL Nonidet P-40 detergent (NP-40)

Bromophenol blue (small amount to colour samples)

1. Bring to 15ml with water
2. Store in freezer in aliquots.
3. Can be thawed and refrozen many times.NOTE: Dilute with water for 1x

Separating Gel (for 2 gels) 12.5%

4x Lower gel stock	5ml
Acrylamide stock	8.35ml
Water	6.66ml
Ammonium Persulfate 10%(10mg/100µL)	100µL
TEMED	10µL

(NOTE: Each gel needs approximately 7ml gel)

Stacking Gel (for 2 gels)	(4.06% acrylamide)
4X Upper gel stock	1.4ml
Acrylamide stock	0.75ml
Water	3.45ml
Ammonium Persulfate 10%	30µL
TEMED	10µl

Method – GEL PREPARATION

1. All equipment, particularly glass and aluminium plates, must be spotlessly clean before beginning. Any dirt on plates causes gel to stick and tear.
2. Every half dozen runs spray the aluminium plates with a light coating of silicon. When dry wash off any greasy residue thoroughly.
3. Combs are also washed thoroughly and stored in ethanol when not in use.
4. Make sure casting plate is ready prior to adding final ingredients to separating gel solution.
5. Grease spacers (1.5mm) lightly with Vaseline and place in between glass and aluminium plates. The lower edge of these must be absolutely flat to minimize leakage. Lightly grease lower corners of combined plates. Place into casting plate.
6. Add a small amount of distilled water using a syringe to check for leakages and then drain.
7. Prepare separating gel by adding lower gel stock, acrylamide and water to conical flask. Swirl gently over very low heat. This is to remove oxygen from solution which will prevent the polymerization process. NOTE: Do NOT overheat solution as it will prevent it from setting!
8. To conical flask add ammonium persulfate and TEMED. This solution sets rapidly.
9. Using a syringe slowly add solution to mold ensuring no bubbles.
10. Overlay gel with very small amount of n-butanol taken from the top layer of the n-butanol bottle. A distinct surface will be apparent beneath the n-butanol.
11. This gel will take approximately 30minutes to set.
12. Mix stacking gel as described for the running gel ensuring TEMED and ammonium persulfate are not added until ready.
13. When running gel has formed, tip off the n-butanol, and wash gel surface with 3X distilled water.
14. Remove comb from the ethanol in which it was stored and dry thoroughly. Insert into casting mold.

15. Add TEMED etc to stacking gel and quickly syringe onto the surface of the mold. Ensure no bubbles around comb.
16. Let stacking gel form, then remove comb carefully with slow even force.
17. Flush gel and bottom of wells with running buffer to remove any unpolymerised gel out of wells.
18. Wells may need to be straightened following washing. This is also a good time to mark where the first well is to improve visibility.
19. Dissolve samples (25µL) in sample buffer (25µL) and boil 90-100°C for 12-15 minutes. Spin in microfuge for 1-2 minutes.
20. Apply samples (10µL) using pipette – ensure that samples do not contaminate other wells. When pipetting into well keep pipette tip straight to ensure wells are not shifted.
21. Place gel in electrophoresis apparatus and apply 30 mA/2 gels. After tracking dye has reached the running gel the amperage can be increased.
22. Run gel until tracking gel has reached the bottom.
23. Turn off current and remove gel from the apparatus.
24. Carefully remove the spacers from between plates and remove plates. Wash gel from plate surface using distilled water wash bottle. Try to minimize gel breakage.
25. Stain overnight in Roeder's stain. De-stain until background is absolutely clear.
26. TO DRY GEL: In a shallow dish add gel shrinking solution for approximately 15 minutes.
27. Take cellophane membrane and moisten in distilled water to soften.
28. Using frame and plate place cellophane membrane then gel then the additional layer of cellophane membrane on top.
29. Take second frame and clamp to form a sandwich.
30. Leave set up to dry over a period of 2-3 days.

Method for Protein Transfer

Solutions:

Transfer Buffer

0.025M Tris

0.2M glycine

0.01% sodium dodecyl sulphate

15% methanol

pH 8.2-8.4

Ponceau S

200mg ponceau so

3g trichloroacetate acid

3g sulfosalicylic acid

Mix in distilled water to 100ml

Tris Buffer 1M Stock

121.1g Tris (TRIZMA base)

In 1 litre distilled water , adjust pH to 8.0 using HCl

Use as a 1:20 dilution for wash

High salt ELISA Buffer (pH 8.0)

2.42g Tris (TRIZMA base)

2ml 10% sodium azide

29.22g NaCl

600uL 0.1M ZnCl2

600uL 1.0M MgCl2

25ml 20% Tween 20

1g BSA

Make up to 1 litre with distilled water. Filter if cloudy.

Method - PROTEIN TRANSFER

1. Remove gel from the elctrophoresis set up and remove from between the aluminium and glass plates using distilled water wash bottle.
2. Place gel into a shallow dish containing transfer buffer for 5-15 minutes. Transfer buffer contains methanol which will cause gel to shrink. The gel needs to have shrunk now rather than whilst in processor.
3. Whilst gel is equilibrating in transfer buffer, cut filter paper to same size as sponges (x4). Whilst doing so use gloves and minimise handling of filter papers.
4. After 5minutes in the transfer buffer measure the gel and cut nitrocellulose membrane to approximately 0.5cm larger than the gel. Use gloves and minimize handling the membrane.
5. Use shallow dish filled with transfer buffer to wet the nitrocellulose membrane. Membrane must be thoroughly soaked before assembling sandwich.

6. When assembling use a large pyrex dish approximately half full of transfer buffer. Open cassette and assemble the transfer sandwich as follows

White - Anode (+)
Sponge
Filter paper x2
Transfer membrane
Gel
Filter paper x2
Sponge
Black- Cathode (-)

7. Transfer gel across by tipping dish and gel gently onto sandwich set up in dish.
8. When membrane is added make sure there are no bubbles between membrane and gel. Smooth over surface carefully. USE GLOVES.
9. Close cassette gently.
10. The transfer unit needs to be connected at base to water which acts as a coolant for the unit whilst transfer occurs.
11. Arrange sandwich so that gel is closest to the cathode, which is the black lead of the unit.
12. Run at 40V and 298mA for 1 hour for follistatin transfer. (Hook up older transformer – Output 1, 0-500V. Adjust output 1 knob for mA and adjust the negative knob for voltage adjustment).
13. Remove membrane gently from unit and sandwich. Wash 5 times for 5 minutes a wash in tris buffer (20mM Tris, 0.15M NaCl, 0.1% Triton X 100, pH 8.2-8.4)
14. To confirm transfer stain membrane with Ponceau S in a shallow dish using a gently rocking action. Only stain for approximately 5 minutes just to determine if the proteins have been successfully transferred to the membrane. Once confirmed, de-stain in distilled water.
15. To block membrane use a small zip lock bag. Make bag slightly larger than membrane.
16. Check bag for leaks with the addition of distilled water.
17. Make up solution containing PBS buffer and 1% skim milk powder.

18. Slide membrane and solution into bag. Ensure minimal bubbles and heat seal bag
19. Block membrane for one hour. Bag is placed on rocker so as to distribute solution thoroughly.
20. Wash in tris buffer X 5 times for 5 minutes each.
21. PRIMARY ANTIBODY- Using new bags, incubate one gel in primary antibody in 20ml of 1% milk in PBS and the second gel in normal serum in 20ml of 1% milk in PBS (control).
22. Incubate on rocker overnight.
23. Wash in 5x tris buffer
24. SECONDARY ANTIBODY- Using new bags incubate both gels in 40µL anti-sheep biotin in 40 ml ELISA buffer (Enough for 2 gels).
25. Incubate on rocker for 2 hours.
26. Wash in 5x tris buffer
27. BAND DETECTION- Using new bags incubate both bags in 40µL strepavidin/alkaline phosphatase in 40 ml ELISA buffer (Enough for 2 gels).
28. Incubate on rocker for 1 hour.
29. Membranes were then developed using BCIP/NBT One Step (Pierce Chemical CO)

Immunohistochemistry methods

Method -Silanised Slide Preparation:

1. Dissolve 4 ml 3-aminopropyltriethoxysilane (APES) in 300mls acetone and place in large coplin jar
2. Dip slide rack into solution and drain thoroughly on paper towel.
3. Slide rack is then dipped into 300mls acetone and drained thoroughly on paper towel.

4. Finally dip slide rack into 300mls distilled water and place in oven for overnight drying at 56° C.

NOTE: 300mls is sufficient to cover a slide rack in a large coplin jar.

Solutions required:

0.1M Citrate Buffer Stock Solution (pH 6.0)

Weigh 2.0 g citric acid (MW- 210.15)
12 g tri-sodium citrate (MW- 294.10)

1. Dissolve in 500ml distilled water.
2. Use in immunohistochemistry for follistatin as a 1:10 dilution.

0.5M Phosphate Buffered Saline Stock Solution

Weigh 57.94g sodium hydrogenphosphate
12.93g potassium hydrogenphosphate
87.66g sodium chloride

1. Dissolve in 1 litre distilled water
2. Use in immunohistochemistry for follistatin as a 1:10 dilution.

Materials

Phosphate buffered formalin

Absolute alcohol

Distilled water

Paraffin wax

0.1M citrate buffer (pH 6.0) stock solution – to be diluted 1:10

0.5M phosphate buffered saline (PBS) stock solution – to be diluted 1:10

10% skimmed milk powder in PBS

Follistatin antibody JMCK20 1:10 glycerol stock solution – to be diluted 1:200 with PBS

Normal rabbit serum 1:10 glycerol stock solution – to be diluted 1:200 with PBS

Biotinylated animal anti-rabbit Ig 1:10 glycerol stock solution – to be diluted 1:800 with PBS

Alkaline phosphatase- conjugated streptavidin 1:10 glycerol stock solution – to be diluted 1:800 in PBS

Method - Immunohisotchemistry

Tissue preparation

1. Tissues are immersion fixed in phosphate buffered formalin for 6-18 hours.
2. The tissues remain in formalin until needed for processing.

Tissue Embedding-

1. Tissues were dehydrated using alcohol stages- (50% alcohol → 70%alcohol 80%alcohol → 90% alcohol → Absolute alcohol → Absolute alcohol)
2. This is followed by clearing stages in histolene and then a wax bath for tissue penetration until embedding.
3. Tissues are embedded in paraffin wax blocks. Sections are cut at 3µm with approximately 2 sections per slide.

Immunostaining

1. Tissues were de-paraffinised using Histolene I → 4min, Histolene II → 4 min, Absolute alcohol I → 2 min, Absolute alcohol II → 2 min, 80% alcohol → 2 min, 50% alcohol → 2 min.
2. Antigen retrieval was performed by placing tissues into 0.01M citrate buffer (pH 6.0) in a coplin jar. Plastic container is placed inside a beaker of water and cover with cling wrap to prevent the citrate buffer from boiling off.
3. Coplin jar was microwaved (Microwave –SHARP) on high for 16 minutes with regular checks to top up buffer when needed.
4. Wash slides gently in 0.05M PBS for 5 minutes
5. Places slides into 10% skimmed milk powder solution for 60 minutes at room temperature
6. Dip slides into 0.05M PBS to remove excess skimmed milk solution.
7. Circle each tissue section with wax crayon to prevent run off and mixing of solutions. Will require 3 drops per crayon ring.
8. Make up antibody solutions freshly
9. Primary antibody is placed onto slides in a humid closed environment at 4°C (refrigerated) overnight
10. Control slide – Normal rabbit serum 1:10 glycerol solution – to be diluted 1:200 with PBS
11. All other slides - Follistatin antibody JM19 1:10 glycerol stock solution – to be diluted 1:200 with PBS

12. Day 2- remove from closed environment and rinse in PBS gently
13. Biotinylated animal anti-rabbit Ig 1:10 glycerol stock solution – to be diluted 1:800 with PBS, is added to slides using 3 drops per crayon ring. These slides are then placed in a humid closed environment at 37° C for 60 minutes
14. Rinse gently in PBS
15. Alkaline phosphatase- conjugated streptavidin 1:10 glycerol stock solution – to be diluted 1:800 in PBS is added to all slides using 3 drops per circle. Place slides in humid closed environment at 37° C for 60 minutes.
16. Rinse gently in PBS
17. Add BCIP/NBT One step (NEN, Boston, MA 02118, USA) to each slide for 5-10 minutes
18. Rinse in distilled water
19. Slides were dehydrated using 50% alcohol → 2 mins, 80% alcohol → 2 mins, Absolute alcohol → 2 mins, Absolute alcohol → 2 mins, Histolene → 2mins, Histolene until ready to coverslip.
20. Coverslip slides using DPX adhesive (BDH Laboratory Supplies, Poole, BH151TD, England).

Total Protein ELISA

Materials

BSA – Bovine Serum Albumin

Bradfords Reagent

PBS 0.05M Phosphate,1.5M NaCl (pH7.5)

3.9g Na2HPO4

43.83g NaCl

1. Make up to 500ml and adjust pH as necessary
2. Add sodium azide (10%)

Method

1. Weigh out BSA and make up a stock solution of 10mg/ml in PBS. Allow it to dissolve in incubator for half an hour before use. Store unused stock as aliquots in freezer.
2. Make up a dilution series beginning at 2000 µg/ml, 1000, 500. 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.9, and 0 using PBS solution.
3. Samples are diluted 1/20, 1/80, 1/320 and 1/1250 with PBS
4. Add 160µl of standard or sample to each well in duplicate
5. Add 40µl of Bradfords reagent
6. Incubate on shaker for approximately 30 minutes
7. Turn on plate reader and edit plate layout etc. Use Bradford's protocol and the 620nm wavelength
8. After 30 minutes read plate

Follistatin Direct ELISA

Method

1. Coat plate with 100µl of standard or sample (fraction). When assessing fractions, place starting material only in a dilution series and in duplicate. Leave overnight.
2. Block with 1% milk in ELISA Buffer. 200µl per well is needed (approx 18ml for a full plate). This generally done for 1 hour but can be extended for a number of days if necessary. Ensure covered and refrigerated if longer than a few hours.)
3. Tip off and wash with 3X Tris Wash Buffer
4. Add primary antibody (CK20) 1:2000 (NOTE – CK20 stock is at 1:10, 55µl in 11 ml). 100µl per well and incubate for 3 hours.
5. Tip off and wash with 5X Tris Wash Buffer
6. Add biotinylated animal anti-chick at 11µl per 11ml of ELISA buffer, 100ul per well and incubate for 3 hours.
7. Tip off and wash with 5X Tris Wash Buffer
8. Add alkaline phosphatase – conjugated streptavidin 1:10 glycerol stock solution at 22µl per 11ml ELISA buffer, 100µl per well. Incubate for 1 hour.
9. Tip off and wash with 5X Tris Wash Buffer
10. Make sure plate reader and computer are switched on
11. Measure out 11ml of NPP substrate and add 100µl per well. If development is too slow and need to leave plate then cover and refrigerate.
12. Watch for colour development and read plate using 405nm.