RNA Isolation with Trizol Reagent

1 ml Trizol (using small amount of tissue) for 50-100 mg tissue or 10^7 cells. Sample volume shoμld not exceed 10% of the volume of Trizol.

RNA Extraction

1. Place cells/tissues in trizol and homogenate with pestle.
2. Allow 5 minute incubation at room temp.
3. Add 200 μl Chloroform/1 ml Trizol
4. Vortex
5. Let sit for 10 min at room temp.
6. Centrifuge at 11,300 rpm for 15 min @ 4 degrees C^o
7. Sample will separate in 3 layers- Phase separation
    a. Top layer clear aqueous phase = RNA
    b. Middle layer white cloudy phase = DNA
    c. Bottom layer red phenol phase = protein
8. Extract 80% of the RNA layer leaving 20% behind in the tube
9. Transfer the 80% RNA layer to a new, labeled tube

RNA Precipitation

1. add 0.5 ml isopropanol/1 ml Trizol
2. invert tubes 5X or vortex for 10 sec
3. let sit for 10 min @ RT
4. centrifuge at 12,000 rpm for 10 min @ 4 degree C.
5. Remove supernatant BY PIPETTE (Be carefμl not to disturb the pellet)

RNA Wash

1. wash with 1 ml cold 75% EtOH (25% DEPC water)
2. vortex pellet
3. Centrifuge @ 9,100 rpm for 5 min @ 4 degrees C.
4. Remove supernatant using pipette (Be careful not to disturb the pellet)
5. allow to dry @ RT for 10-15 min

Solubilization

1. turn on heat block (low setting) to 55 degrees C and put water in wells
2. Dissolve RNA pellet in 100 µl elution buffer and mix well by vortexing (15 sec).
3. Incubate 5 min @ 55° C in heat block.

DNAse Treatment

1. add 10 µl of 10X DNAse buffer from your box in freezer (DNAse buffer will be 10% of the final volume of RNA + elution buffer + DNAse buffer + DNAse I) (10% of Aqueous fraction).
2. add 1 µl of DNAse I from your box in freezer (1% of Aqueous fraction)
3. mix by pipette or vortex, then spin.
4. Incubate at 37°C in the incubator for 30 min
5. Add 11 µl of DNAse inactivation reagent FROM FREEZER (10% of Aqueous fraction) Incubate 2 min at RT while vortexing one after the other
6. Centrifuge at 11,000 rpm for 1 min (can put in for 5 min)
7. Transfer supernatant to a new, labeled microcentrifuge tube leaving behind white power pellet (takes out 90% to new tube, leave behind 10%)
8. You can toss the tubes with just the white pellet left in them

Reconcentrate RNA

9. add 1 µl of linear acrylamide (~1% of initial volume of supernatant from step 8 above)
10. add 10 µl of 5M ammonium acetate (~10% of initial volume of supernatant from step 8 above)
11. add 300 µl of 100% EtOH (1 part Aqueous: 3 parts EtOH)
12. Place in -20°C for 1 hr (can leave overnight)
13. Centrifuge at 14,000 rpm for 15 min @ 4 °C.
14. Remove supernatant, let dry 30 min.
15. Air dry pellet (30-40 minutes)
16. Dilute in 50µl elution buffer (depends on pellet size)
17. Mix by pipette, put on ice

Quantify the RNA

1. Prepare a blank in micro centrifuge tube (498 µl DNAse/RNAse free water + 2 µl elution buffer
2. Sample (498 µl water + 2 µl RNA (premixed))
3. Vortex sample

-Leftover RNA add 20 µl ammonium acetate, 200 µl EtOH 100%, store at -20˚

Take reading

Smart Spec 3000

1. Press DNA/RNA button :enter
2. yes: enter
3. turn on vacuum and rinse cuvette with distilled water
4. add 500 µl of blank in to cuvette and take blank reading {press read blank}
5. empty contents in to vacuum and rinse
6. add 500 µl of sample into cuvette and take a reading {press read sample}
7. empty contents into vacuum and rinse
8. print : 3 fµll report : exit assay : turn off
9. calcµlation: in excel

RNA concentration = A_{260} x 40x dilution factor

Purity = A260/A280, Purity shoµld be between 1.8-2.0

cDNA Synthesis

RNA + reverse transcriptase = cDNA

1. warm heating block to 70˚C put water in wells
2. obtain Fermentas kit
3. thaw tubes in hands
4. 11 µl water(DEPC) into new small tubes
5. 1 µl random hexamer
6. add 2 µl RNA
7. Mix by pipette, spin 1 min.
8. incubate at 70˚C for 3-5 min. (in heat block)
9. chill on ice (immediately) 2-3 min
10. spin
11. add 4 µl 5X reaction buffer
12. add 1 µl reverse transcriptase
13. 1 µl RNAse inhibitor
14. 2 µl dNTP 10 mm
15. vortex and spin

Mini cycler

1. Put in mini cycler 25˚C for 5 min.
   FERM-RT : run proceed : yes : heated lid proceed
2. 25˚C for 10 min.
3. 37˚C for 60 min.
4. 70˚C for 10 min.
5. chill on ice or at 6˚C

PCR

1. add 1 µl cDNA into new tubes
2. 1 µl Primer (F)

3. 1 µl Primer (R)
4. 10 µl Mastermix
5. 7 µl water

total volume 20 µl

6. vortex and spin
7. put in mini cycler overnight

Program actin

- Once reagents are used store on ice

cDNA synthesis for PCR

**Start warming heating block and put water in the wells

**Do procedures using RNA/DNA free tips, tubes, etc.

1. Use excel program to determine RNA/Water ratio. <30ug of RNA use 10uL RNA and no water (1uL UP water with new Reverse Transcriptase).
2. Add RNA (and water) to .5 microcentrifuge tube.
3. Add 1uL of Random Hexamers
4. Mix be pipetting
   - Put on heat block for 5 minutes at 70^{o}C
   - Put on ice for 3 minutes
5. Add Master Mix
   - 5uL 5x Rxn Buffer
   - 1uL RNAse Inhibitor
   - 2uL dNTP 10mM
   - 1uL Reverse Transcriptase (RT)

   =9uL (Total solution should be 20uL)
6. Spin down and lightly vortex

7. Put sample(s) in minicycler, program=**FERM-RT**… takes about 1hr and 15mins. Store at 4^{o}C.

PCR

1. Make Master Mix:
   - 1 uL Primer-F (check to make sure it is mouse or human)
   - 1 uL Primer-R (check to make sure it is mouse or human)
   - 10 uL Master Mix (from kit)
   - 7 uL UP water

   = 19 uL total
2. Add master mix to .5 mil PCR tube. Then add 1 uL cDNA
3. Vortex then spin down
4. Put in mini cycler. Takes about 1 ½ hours Program **ACTIN**

RNA Extraction/Quantification

**Use RNAse free tips, pipettes, tubes.. etc.

1. Prepare buffer: 1mL RLT and 10uL B-mercaptoethanol
2. Remove fluid from microcentrifuge, leave pellet.
3. Add buffer 350uL-600uL of RLT to cells for lysis (600uL is almost always used)
4. Vortex cells to make sure there are no clumps.
5. Add same volume as RLT of 70% ethanol to lysed cells (probably 600uL of 70% ethanol)
6. Vortex cell mixture.
7. Transfer lysate to RNA easy spin column with 2mL microcentrifuge tube. Transfer 700uL at a time. Then centrifuge 15 sec. at about 10,000 RPM, discard flow through. (make sure centrifuge is balanced)
8. Add 700uL of buffer RW_1, centrifuge for 15 sec at about 10,000 RPM, and discard flow through. (Get a 1.5mL tube per sample)
9. Add 500uL of buffer RPE, centrifuge for 15 sec at about 10,000 RPM, and discard flow through.
10. Repeat step 9
11. Place RNAeasy column to new 1.5mL microcentrifuge tube
12. Add 30-50uL of RNAse free water (add more depending on cells) Centrifuge for **2 minutes at about 10,000 RPM**… Pun on ice when done.

Quantify-

1. Add 498uL of UP water into a microcentrifuge tube
2. Add 2uL of RNA into the same tube
3. In another tube add 500uL of UP water, this is for the blank.
4. Make sure you mix well and vortex the samples
5. Clean the cuvette and add the blank solution, 500uL (Press DNA/RNA button, enter, enter, read blank)
6. Do the same for RNA and print the (full #3) report. (Press right arrow, read sample)
7. Store RNA in the -80^{o}C freezer

Agarose Gel Electrophoresis

Note:

Depending on the gel rig/tray use the appropriate amount of agarose. Prepare 1 to 1.5% gels

- Small – 30 ml total (Biorad)
- Large – 50 ml total (VWR)

1. Add 0.45 g agarose powder to a 100 ml Erlenmeyer flask (use larger flask is larger rigs is used)
2. Add 30 ml TBE 1X buffer
3. Boil in microwave for 60 sec, stir at 15 and 30 sec intervals
4. Put tape on ends of tray to seal, put in combs
5. Add 3 µl Ethidium Bromide to agarose
6. When gel is warm (not hot) pour into tray, let set 10 -15min
7. Add distilled water to flask and boil in microwave to remove any remaining gel
8. Pour waste in EtBr bin
9. Remove combs and tape
10. Add TBE 1X buffer to tank
11. Place tray in tank, make sure gel is covered with buffer
12. Add 6 µl DNA marker with dye
13. Load 6 µl of samples into each well
14. Plug in +red –blue at 80 volts for 1 hr or 125 volts for 20 min
15. Turn machine off, remove lid
16. Wipe off UV Tran illuminator
17. Remove tray, slide gel out
18. Use protective shield to view
19. Take a picture: Scion image: special: start capturing
20. Reuse running buffer
21. Remove gel with spatula and put in hazardous waste

Making Standards for Q-PCR (Qiagen)

1. Excise DNA from 1% TBE gel.
2. Weigh the gel.
3. Added 3X vol of QG buffer to gel weight
4. Incubate at 50° C for 10 min (until gel is completely dissolved)
5. Check the pH of DNA gel solution by color
6. Add 1 gel volume of isopropanol to sample & mix
7. Place a Q/A quick spin column in a 2 ml collection tube
8. Place DNA solution in QIA quick spin column, centrifuge for 1 min.
9. Discard flow thru
10. Add 0.5 ml of buffer QG to spin column, centrifuge 1 min.
11. Add 0.75 ml buffer PE, spin 1 min
12. Wait 1 min, then spin again at ~17,900xg to remove all residual EtOH
13. Place spin column in 1.5 ml tube
14. Add 50 µl EB buffer to center of spin column, spin for 1 min.
15. Let stand 1 min., spin again

Q-PCR

1:10 dilutions

A → B → C → D → E → F → G

Primer Normalization

1. Make cDNA standards A→ E
2. Select the cycle and fix the annealing temperature.
3. Run Q-PCR with standards and Blank
4. Check the melting curve, whether it has single product or not.
5. Check 3.3 CT value difference between 1:10 dilutions
6. Check whether blanks amplified or not.
7. Check whether it has non specific amplification.
8. Confirm the single product by running gel.

Cell Culture work

Split the cells

1. Start the biosafety cabinet and let it equilibrate for at least 3 min. Turn on UV light
2. Place the 1X trypsin into 37° C water bath for 3-5 min.
3. Label the cell culture flasks with cell line type, passage number, date, and initials.
4. Make sure the cells are confluent. (Complete growth of monolayer) and that there are no signs of contamination.
5. Remove the culture Media from flask using sterile pipette and dispose the media into waste beaker container.
6. Add 1x trypsin
    a. T25 flask - ~ 0.5-0.75 ml trypsin and swirl flask (do not bring media into the neck).
    b. T75 flask - ~ 0.75-1.0 ml of 1X trypsin for T75 flask.
    - Remove inactivated trypsin and repeat process at least 2 times.
    - The first trypsin volume removes the serum and remaining media. The second volume works to detach cells.
7. Quickly mix flask in order to cover entire cell monolayer with trypsin. Tapping on the bottom or side of the flask will help release any adhered cells.
8. Place flask on the microscope and check for cell detachment
9. Add approximately a 1:2 ratio of media to the flask to stop the reaction. (trypsin:media)
10. Use pipette to draw up and expel the media onto the side of the flask. (This step forces the adherent cell layer off the side of the flask). ****Note**: Be careful about splashing media onto sides of flask, as this will increase the amount of debris in the flask.** Do not splash media into the neck!!
11. Aliquot media + cell solution into number of flasks as needed.
12. T25 flask received a total of 3.0 – 3.5 ml (cells + media volume needed) while a T-25 flask = 9.0 - 10 ml
11. Incubate flasks at 37° C.
12. Record split and passage number in calendar or lab book
13. If needed, change media in flask after 24 hours if dead cell debris is significant.
14. Keep track of the passage number each time you split the cells. When cells reach P20, thaw a new batch to expand.

Freeze the Cells

1. Grow a large batch of cells preferentially in a T-75 flask.
2. Trypsinize each T-75 as described above.
3. Stop trypsinization with 2-4 ml of media.
4. Centrifuge at 1,000 rpm 4^{o} C for 8 minutes.
    - Rule: 1,000 – 1,200 rpm for most cells
    - *Toxoplasma*: 3000 rpm for 8 min
5. Remove supernant leaving as little volume as possible
6. Make the **freezing media** (see below)
7. Resuspend cells in desired volume of freezing media (~0.75 -1.0ml)
8. Aliquot 1.0 ml into precooled (-20° C) cryovials. Leave the tubes on ice until you fill all the freezing tubes with cells.
9. Put cryovials in -20^{o}C for an hour and move to the -80° C freezer.
10. 24 hours later, move to Liquid Nitrogen cryo storage and mark locations in the logbook provided.

DMEM Culture Media: 50 ml total

10 ml FBS
1 ml Non essential aminoacids
1ml antibiotic/mycotic
1m l HEPES
1.5ml Na Bicarbonate
33.5 ml DMEM

Freezing Media: 10 ml total

DMEM – 6.5 ml; FCS – 3.0 ml; DMSO – 0.5ml = 10 ml

Thaw and grow the cells

1. Remove a cryovial from Liquid Nitrogen storage (marking it off in the logbook). Put the vials in the ice immediately.
2. Keep the vial in the rack under the hood. Agitate gently, until thawed.
   Note: Thawing should be rapid as possible once the vial is removed or keep the vial on the dry ice or cold until ready to finish.
3. Wipe the outside of the vial with EtOH or the EtOH wipes, and uncap.
4. Remove cells with a sterile pipette, and place in a cell culture flask with media with FCS.
5. The treat cells as normal passage.

Purification of *Toxoplasma* from cells

23G, 25G and 27G needle

10 ml syringe

Toxo filter

50 ml tube -1

15 ml tube -1

1. Remove all the adhered cells from thee flask to the bottom of the flask(Why not trypsin? cell scrappers kills lot of cells, we are not going to maintain these cells!!)
2. Draw 10 ml of contents through syringe and 23G, 25G and 27G needles move forward and backward three times with all the needles and lyse the contents mechanically.
3. Fix the toxo filter in 50 ml tube. Pass the contents through the filter.
4. Push the rest of the contents with 5 ml of DPBS.
5. Centrifuge 3000 rpm for 5 minutes
6. Remove the supernatant.
7. Resuspend with PBS
8. Count in Hemocytometer.

Isolation of intestinal epithelial cells

1. Wash the intestine with Ca+ Mg+ free PBS
2. Open longitudinally, cut in to small pieces
3. Incubate with Ca+ Mg+ free RPMI-1640 with 5mM EDTA with 100 IU/ml of penicillin streptomycin, 1% fungizone, 50 IU gentamicin for 20 minutes (epithelial cells)
4. Incubate 25 mM EDTA with Ca+ Mg+ free RPMI-1640 with 5mM EDTA with 100 IU/ml of penicillin streptomycin, 1% fungizone, 50 IU gentamicin for 20 minutes (epithelial cells + intraepithelial lymphocytes)
5. Wash 5 times to remove the EDTA.

Isolation of LP lymphocytes

a. Isolation of intestinal epithelial cells

1. Wash the intestine with Ca+ Mg+ free PBS
2. Open longitudinally, cut in to small pieces
3. Incubate with Ca+ Mg+ free RPMI-1640 with 5mM EDTA with 100 IU/ml of penicillin streptomycin, 1% fungizone, 50 IU gentamicin for 20 minutes (epithelial cells)
4. Incubate 25 mM EDTA with Ca+ Mg+ free RPMI-1640 with 5mM EDTA with 100 IU/ml of penicillin streptomycin, 1% fungizone, 50 IU gentamicin for 20 minutes (epithelial cells + intraepithelial lymphocytes)
5. Wash 5 times.

b. Isolation of LP lymphocytes

6. Take the remaining portion of Intestine
7. Incubate RPMI-1640 with 10% FCS with 125 IU/ml Collagenase VIII, 2 hr at 37^{o}C.
8. Filter through the filter (like sieve)
9. Filter through glass wool column to remove the clumps
10. Magnetic beads isolation –

Magnetic cell separation of lymphocytes

1. Prepare single cell suspension
2. Remove the clumps by passing through 70 µm nylon cell strainer and finger flipping the tip of the tube
3. add Fc blocker 0.2 µg/ 10^6 cells , incubate ice for 15 minutes
4. Wash cells with MACS buffer and remove the supernatant
5. Vortex CD4 particles thoroughly, add 50 µl for every 10^7 total cells.
6. Mix thoroughly and refrigerate for half an hour.
7. Bring the volume up to 1-8 x 10^7 cells/ml with Mag buffer.

Isolation of cells by Magnetic columns

8. Fix the column
9. Fill the column by 500 µl buffer, let the buffer to flow through, discard the effluent, do not allow them to dry.
10. apply the magnetic labeled cells in 500 µl buffer - 10^8 total cells, 1000 µl buffer – 2 x 10^8 total cells

11. Rinse with 3x 500 µl buffer
12. Apply 1 ml buffer to flush out the positive
13. Rinse 2-3 x 500 µl buffer to wash the remaining
14. Positive minimum is 500 µl

Intestinal epithelial cells – primary cell culture

1. Flush the contents of the intestine with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) containing 2% glucose, 25 ng of amphotericin B per ml, 100 U of penicillin per ml, and 100 µg of streptomycin per ml.

2. Splice the intestine into small pieces and incubate for 15 min at 22°C on a shaker platform in Ca^{2+}- and Mg^{2+}-free HBSS containing 5mM EDTA, 2% bovine serum albumin, and 0.2 mg of soybean trypsin inhibitor per ml.

3. Take out the supernatant and wash with (DMEM), 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 5% fetal bovine serum (FBS)

4. Cells were cultured in 24-well plates, at a seeding density of approximately 2X 10^6 cells/well

5. One hour before plating cells, culture surfaces were coated with 40 µl of Matrigel (BD Biosciences) per cm^2 diluted 1:2 in phenol-red-free DMEM (Sigma).

6. Epithelial cells were cultured in epithelial cell medium (ECM) containing equal volumes of phenol-red-free DMEM and Ham's F-12 medium (Biowhittaker) with the following additives:
7. 5 µg of insulin (Sigma) per ml,
8. 5×10^8 M dexamethasone (Sigma),
9. 60 nM selenium (Sigma),
10. 5 µg of transferrin (Sigma) per ml,
11. 5×10^8 M triiodothyronine (Sigma),
12. 10 ng of epidermal growth factor (Sigma) per ml,
13. 20 mM HEPES,

14. 2 mM glutamine,

15. 100 U of penicillin per ml,
16. 100 µg of streptomycin per ml,

17. 0.2% D-glucose,

18. 2% FBS.

19. Cells were cultured in 5% CO_2 at 37°C with periodic supplementation of medium to maintain a volume of 2 ml per well.

FACS cell preparation

Ab conjugated with conjugate

1. Cells 1-5X 10^6 cells in PBS, 3% BSA/PBS in conical 15 ml tubes
2. Block Fc blocker (20µl/million cells)
3. Incubate on ice for 15 minutes, spin down (10 min 1500 rpm at 8°C and dump the supernatant.
4. add ab (CD4 -5 µl) (1µg) in 3% BSA or CD8 (2µl)
5. Incubate 20-30 min on ice
6. Wash wells 3 times 400 g for 5 minutes and resuspend in cold PBS.
7. After the last step centrifuge cells and remove the supernatant.
8. Add 0.5-1 ml of cold 0.5% paraformaldehyde and vortex immediately.
9. Store 4°C in dark.

Use primary and secondary Ab conjugated with conjugate

10. Add primary ab 0.1-10µg/ml
11. Incubate 30 mts on ice
12. Wash three times 400 g, 5 min, remove supernatant.
13. Dilute Sec ab with FITC conjugate in 3% BSA/PBS and add to the cells
14. 20 mts rp
15. Wash three times 400 g 5 min, remove supernatant and remove supernatant
16. , wash three times in ice cold PBS
17. add 0.5-1ml 0.5% paraformaldehyde 4°C in dark.

Protein work

Indirect Fluorescent antibody test (IFAT)

1. Place the antigen slides on the rack and let them dry.

2. Fix them with 4% Paraformaldehyde for 5 minutes and dry.

4% Paraformaldehyde

15 ml PBS – heat to about 55^{o}C.

0.75 g paraformaldehyde to PBS

0.1 N NaOH (Break Paraformaldehyde to formaldehyde)

Cool and adjust pH

Make up to 25 ml

3. Block them with PBS + 1% BSA for 10 minutes in a humidified chamber.

4. Wash the with TBST (TBS+0.02% Tween 20)

TBS (10X -1L)

Sodium chloride - 90 g

Tris base - 12.12 g

Make up to one liter

5. Using PBS + 1% BSA as a diluent, dilute the sera in 1.5 ml micro centrifuge tube (1:50, 1:100, and 1:200). Need to standardize while doing first time. Dilutions depend upon the concentrations of stock.

5. Place the diluted serum on antigen slides and leave in a damp chamber (humidified Chamber) for 40-60 minutes at 37°C.

6. Wash the slides three times with TBST (or TBS + 0.02% Tween 20) on a shaker for 5 minutes.

8. Air-dry the slides (no need to dry them completely)

9. Using PBS + 1% BSA as a diluents, dilute the secondary antibody with FITC

conjugate in 1.5 ml micro centrifuge tube The dilutions (1:100, 1:200, and 1:400). Need to standardize while doing first time. Dilutions depend upon the concentrations of stock.

9. Incubate the slides with the conjugate. This step should be done in dark place, cover the slides with box with aluminum foil.

10. Wash 3 times as before.

11. Without drying the slides, add one or 2 drops of Fluoromount-G and put cover glass, careful not to get any air bubble in between.

12. Observe the slides under a fluorescence microscope (X200-400).

Counter stain

Evans Blue

Evans Blue can be added to the conjugate for counter stain.

FITC conjugate 50 µl,
1% Evans Blue 30 µl
PBS 920 µl
Incubate for 30-60 minutes at 37°C in dark.

Methyl Green Solution (counter stain for Immunohistochemistry)

Methyl Green Solution (0.5%)

Methyl green - 0.5 g
0.1M Sodium acetate buffer, pH4.2 - 100 ml
Mix to dissolve.

0.1M Sodium Acetate Buffer, pH4.2:

Sodium acetate, trihydrate (MW 136.1) - - 1.36 g
Distilled water - 100 ml
Mix to dissolve and adjust pH to 4.2 using concentrated glacial acetic acid

Staining Procedure

1. Wash the sections with distilled water
2. Stain in methyl green solution for 10 minutes at room temperature

3. Rinse in distilled water (sections will look blue).

4. Dehydrate quickly through 95% alcohol (10 dips, sections turn green), 2 changes of 100% alcohol (10 dips each) (alcohol used for dehydration removes some of the stain).

5. Clear in xylene

6. Mount with DPX mounting medium.

SDS PAGE and Western blot

1. Wipe down the spacer plates (spacers attached) and short plates (BioRad) with

D.water, 70%ethanol to remove any adherent material, dry and clamp them together.

2. Solutions used:

a. 1.5 M Tris-HCl, pH 8.8

b. 0.5 M Tris-HCl, pH 6.8

c. 30% acrylamide/bisacrylamide

d. N,N,N',N'-tetramethylethylene diamine (TEMED).

This solution can be bought commercially and stored in fridge.

e. 10% SDS

Add 5.0 g SDS to a 100 ml bottle

Add 50 ml deionized water

f. 10% w/v ammonium persulfate, make fresh every month and store in fridge.

g. Running buffer: Take 100 ml of stock (10X Tris glycine running buffer) and 900 ml of

Distilled water and make up to one liter.

e. Sample buffer:

100 mM Tris, pH 6.8,

2% SDS,

5% ß- mercaptoethanol,

15% glycerol,

3. Separating gel (add the following recipe)

Percentage	14%	12%		10%		7.5%	
Total	40 ml	**10 ml**	**5 ml**	**10 ml**	**5 ml**	**10 ml**	**5 ml**
D.Water	10.33 ml	3.35 ml	1.68 ml	4.0 ml	2.0 ml	4.85 ml	2.43 ml
Tris buffer (1.5M, pH 8.8)	10 ml	2.5 ml	1.25 ml	2.5 ml	1.25 ml	2.5 ml	1.25 ml
Acrylamide : Bis acrylamide	18.67 ml	4.0 ml	2.0 ml	3.33 ml	1.67 ml	2.5 ml	1.25 ml
10% SDS	400 µl	100 µl	50 µl	100 µl	50 µl	100 µl	50 µl
10% APS	200 µl	50 µl	25 µl	50 µl	25 µl	50 µl	25 µl
TEMED	40 µl	15 µl	15 µl	15 µl	15 µl	15 µl	15 µl

3. First add the add D.Water, Tris buffer, Acrylamide: Bis acrylamide solution and 10% SDS.

4. Add 10% ammonium persulfate and gently mix the solution.

5. Add 15 µl of TEMED (stored in refrigerator), and gently invert to mix the gel components thoroughly (avoid introducing air bubbles as this can inhibit polymerization).

6. Immediately pour the mix in between the plates. Fill the space up so there will be enough room to form a stacking gel of 0.5 to 1 cm.

7. Overlay with 70% ethanol to a depth of a few millimeters.

8. Allow the gel to polymerize for 20 minutes.

9. After the running gel has polymerized, rinse the ethanol from the surface with D.water. Drain excess water.

10. Prepare the stacking gel. This is composed of 4% acrylamide

Stacking gel (add the following recipe)

Percentage	4%	
Total	**10 ml**	**5 ml**
D.Water	3.35 ml	1.68 ml
Tris buffer (0.5M, pH 6.8)	2.5 ml	1.25 ml

Acrylamide : Bis acrylamide	4.0 ml	2.0 ml
10%SDS	100 µl	50 µl
10% APS	50 µl	25 µl
TEMED	15 µl	15 µl

11. Add the stocking gel mix. Insert appropriate combs.

12. Polymerize stacking gel for 30 minutes.

13. By the time prepare the protein. If the protein is already in solution, add an equal volume of 2X sample buffer and boil in a boiling water bath for 5 min. Boil the markers if necessary (according the instructions by the company).

14. Carefully remove comb (add some drops of running buffer in between well and combs to do the job easy)

15. Rinse wells thoroughly with running buffer and assemble the gel in the electrophoresis rig.

16. Pour running buffer in the top and bottom chambers,

18. Load the samples in appropriate wells and add more running buffer in the top chamber

20. Run the gel. Use a constant Amps power supply (80 mA). It will take approximately 1-2 hours.

21. Coomassie staining your gel

Coomassie stain

a. Dissolve 2g Coomassie Blue R, 250 in 250ml water

b. Add 75ml of glacial acetic acid.

c. Add 500ml of ethanol

d. make up to 1000ml with water

22. Immerse gel for 1 hr in Coomassie.stain solution.

23. Destain gel

Distaining solution

a. Methanol -50%
b. Acetic acid - 10%
c. Distilled water -40%

24. Immerse the stained gel into the destain solution Change the destain solution when it becomes very blue.

Western blotting.

1. Cut a piece of PVDF membrane approximate to the size of the gel.

2. Soak them about 10 min in methanol at room temperature.

3. Soak the sponges and filter paper in the transfer buffer in a separate tray.

4. Once the gel run is over take the gel from PAGE set up carefully.

5. Assemble the blot in the following order Sponge- filter paper- gel- membrane- filter paper-sponge.

6. While assembling the membrane should be placed in transparent side and gel in black side

7. Transfer for 1 hr at 80 Volts in a tank. Place the cold pack inside the buffer tank. By using magnetic stirrer the buffer should be mixed. Change the ice pack after half an hour.

7. After one hour take out the set up and take the membrane.

8. Incubate the membrane in blocking buffer (5% not fat dry powdered milk in TBS) for one hour at room temperature or $4^{o}C$ at overnight.

9. Wash with 3 times 10 min with TBST (0.05% in TBS).

10. Incubate with primary antibody diluted in PBS for one hour at room temperature or $4^{o}C$ overnight.

11. Wash with 3 times 10 min with TBST

12. Incubate with secondary antibody diluted in PBS for one hour at room temp.

13. Wash with 3 times 10 min with TBST
14. Develop the blot using chemiluminescence (ECL) substrates.

Developing the blot

PIERCE Chemiluminescence substrates (Super signal).

1. Mix equal parts of "Enhancer solution and Peroxide solution in 15 ml test tube.
2. Mix and allow the solution to come to room temp (~ 5 minutes)
3. Incubate with the membrane for about 5 minutes.
4. Either view through Alpha-Innotech or develop the signal through X-ray film.

Develop through X-ray film

1. First add Developing solution, Fixing solution and stop solution into the appropriate Trays
2. Place the blot into the zip lock bag.
3. Cut all the edges of zip lock bag
4. Flatten the plastic bag, clear air bubbles
5. Tape down blot in plastic bag.
6. Turn off all the light except dark room light
7. Place a film over the blot
8. Place tape on the films edge to the table
9. Allow 12 minutes exposure time
10. Remove the film carefully
11. Place the X-ray film into the developer for 10 minutes and carefully see for the signal develop.

12. Stop immediately once see the bands. Put them into the stopping solution for 2 minutes.

13.Place them into the fixing solution for 2 minutes.

14.Finally wash the films with water.

15.View the bands.

Stripping the blot

1. Rinse blot off with TBST (0.05% Tween 20 in TBS).

2. Add 5 ml of 0.5 M NaOH solution and incubate the blot with 20 minutes

3. Rinse blot off with TBST

4. Block for about 1 hr with 5% BSA/Tween 20, or overnight with 3% BSA/Tween 20.

Lysis buffer for Protein

10% glycerol
1% Triton X-100
150 mM NaCl
50mM Hepes
1.5 mM MgCl2
2mM EGTA,pH8
Cocktail of protease inhibitor 1 tablet (Roche , Cat No. 1697478)
mix and cool to $4^{o}C$

MODE-K media		**500 ml**
DMEM	-	434 ml
10% FBS	-	50ml
10mM Hepes	-	5 ml
0.4g L L-glutamine	-	5 ml
50U/ml Penicillin	-	5 ml
50 mg/ml streptomycin		
100 µg/ml gentamicin	-	1 ml
50µM 2-Mercaptoethanol	-	0.862µl

PBS

NaCl	-	138 mM	-	8 g
KCl	-	2.7 mM	-	0.2 g
KH2Po4	-	1.2 mM	-	0.24 g
Na2HPo4	-	8.1 mM	-	1.44 g

10% neutral buffered formalin (100 ml)

37% formaldehyde solution	-	10 ml
Nacl		-0.8 g
Potassium phosphate monobasic	-	0.4 g
Potassium phosphate dibasic	-	0.65 g
Distilled water	-	90 ml

HFF/Macrophages media

DMEM complete

10% FCS

Gentamicin	-	0.2 ml/100 ml
Hepes	-	1 ml/100 ml
Na bicarbonate	-	1.5 ml/100 ml