CELL COUNTING PROTOCOL

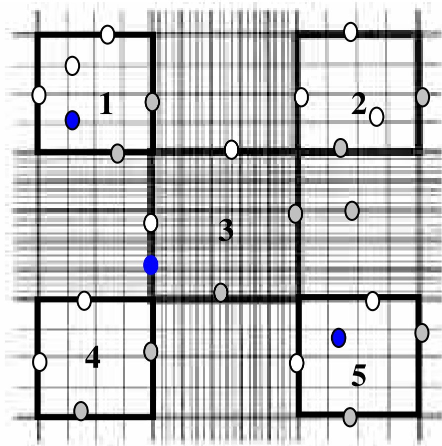
Time Period – 10 min

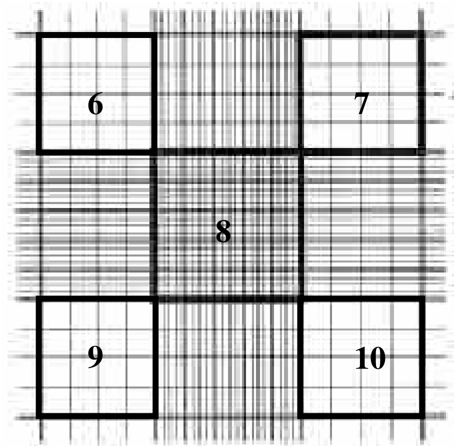
Formulation of Trypan Blue Solution

1. Obtain 100 ml bottle of trypan blue powder (StemCell Technologies Inc.)
2. 0.4% Trypan Blue - Mix 0.4 g of trypan blue with 100 ml of Phosphate Buffering Solution (PBS).
3. Will remain stable for two years while stored at room temperature.

Viable Cell Counting

1. Dilute Cells 1:1 in Trypan Blue. Add 10 µl of trypan blue to a microcentrifuge tube. Using sterile pipette tips and pipette aid add a 10 µl of the sample, taken from the center of the 15 ml conical tube, to the trypan blue. *Sterilize the pipette with alcohol and let dry before taking aliquot from sample*. Make sure that that the sample comes in contact with the dye. Allow the solution to sit for 5-15 min. Trypan blue stains non-viable (dead) cells. Be sure to thoroughly mix Trypan Blue with sample in microcentrifuge tube using pipette.
2. Hemocytometer preparation. To clean, rinse the chamber with distilled water and then alcohol and air dry. Position the cover slip over the chambers.
3. Place 10 µl of the mixture in both wells of the hemocytometer. To do this, center the cover slip on the hemocytometer, hold the cover slip at its sides with two fingers, lay the pipette tip as parallel to the ground as possible, and inject the mixture in the well underneath the cover slip. Make sure the entire surface of the rectangular grid of the hemocytometer is covered. It is important not to under fill or overfill the chambers.
4. Count viable cells using 100x that overlay the grid of the hemocytometer at the corners and at its center. If cells are on the border outlining each square, count only the cells on the top and left border of the square as shown below.
5. Count both sides of the hemocytometer using the hand counter while looking through the microscope. Count 5 of the 10 sub squares on each side of the hemocytometer. See example below.
6. Use the following calculations to determine the number of cells per milliliter.





Hemocytometer Chamber Grid Pattern: showing ten squares to be used to count viable cells as well as what constitutes a viable cell.

- ○ Count - Viable Cell
- ● Do Not Count – Viable Cell on Wrong Border
- ● Do Not Count – Dead Cell (Stained Blue)

Calculations:

Average Number of Cells

$$\frac{Total\#ofcells}{10squares(mm^2)} = Average_\#ofcells/mm^2$$

Dilution Factor

$$\frac{Volume_of_TrypanBlue + Volume_of_cell_suspension}{Volume_of_chamber_alloqoite} = \frac{10\mu l + 10\mu l}{10\mu l} = 2 = Dillution_Factor$$

Number of cells per milliliter

$$\frac{Average_\#ofcells}{mm^2} \bullet \frac{1mm^2}{0.1mm^3} \bullet \frac{0.1mm^3}{1\times10^{-4}cm^3} \bullet \frac{1\times10^{-4}cm^3}{1\times10^{-4}mL} \bullet Dilution_Factor = Avg.\#cells/mL$$

$0.1mm^3$ = volume of 1 square

10^{-4} $cm^3 \approx 10^{-4}$ mL

Simplified Calculation

$$\boxed{\#ofcells/mm^2 \bullet 2 \bullet 10^4 = cells/ml}$$

CELLULAR PLATING PROTOCOL FOR FLUID FLOW EXPERIMENTS

Time Period – 1 ½ hours

Necessary Materials

- Growth Media: 44% DMEM/F12
  10% FBS
  2% P/S
- Trypsin
- PBS

11 ml/dish containing a slide + 25 ml for procedure.

4. Place necessary materials into water bath for ~ 10 min to bring to 37 ˚C. If media is not brought to body temperature it will shock the cells and may result in cell death.
5. View dish to be passed and plated from under 100X. Be sure dish is 100% confluent.
6. Suck off media using sterile glass pipette tip and vacuum pump.
7. Wash cells with PBS and suck off. (X 2)
8. Add 2 ml (just enough to cover surface area for bottom of dish) of trypsin to Petri dishes to detach cells from surface.
9. Incubate for 10 min. Check if cells are floating under microscope. If they are not, incubate for a few more minutes. After 15-20 min, if cells are still attached to dish use cell scraper to release them into the media.
10. Add 5 ml of media to stop reaction. Brings total volume to 7ml in dish.
11. Place mixture into 15 ml conical centrifuge tube. Use pipette to "wash down cells" that may still adhere to the surface of the Petri dish before collecting it in the pipette for transfer.
12. Centrifuge at 1000g (or 1500 rpm on the Beckman centrifuge in Rm. 407 Chem Sci.) for 10 min. Be sure that centrifuge is balanced with another 15 ml conical centrifuge tube filled with water of same volume directly across from it.
13. Suck off supernatant being careful not to suck up pellet. It is better to leave a little supernatant than to lose the pellet. Use same sterile glass pipette tip to suck

off supernatant. Be sure glass pipette tip does not come in contact with anything between uses.

14. Resuspend pellet by mixing with pipette in 1-3 ml of media depending on the size of the pellet. If a pellet is not formed after centrifuging, check to see if cells are still on dish. If they are, begin again at step 3. Be sure there are no floating chunks in media before continuing, and be sure to thoroughly mix the cell suspension before beginning cell counting.
15. Count the cells. Need 150,000 cells/ml on each slide.
16. Must also have enough cells to pass to a new dish. This requires an extra 500,000 cells/dish being passed. Must account for this when determining cell distribution over and above what is necessary for cell plating onto slides.
17. Mix cell suspension before plating to obtain a uniform distribution in the solution. Make a new vial of the mixture to allow 150,000 cells to be contained in 1 ml. Place 1 ml of the mixture onto each slide.
18. Be sure that the 1 ml mixture remains only on the slide. If it falls off, suck it back up and try again, or prepare a new slide if enough mixture is available.
19. View slides after plating. This is a precautionary check for cell confluency.
20. Place the slides, without causing the mixture to fall off of the slide, in the incubator for at least 1 hour, and not more than 3 hours. Check slides to be sure that cells are beginning to adhere.
21. Add 10-12 ml of media to each slide. When adding fluid, do not add directly to slide, but on the area of the dish surrounding the slide. This is a precautionary measure that is taken so that the force of the media coming out of the pipette does not shear off any cells that are just beginning to adhere to the slide.

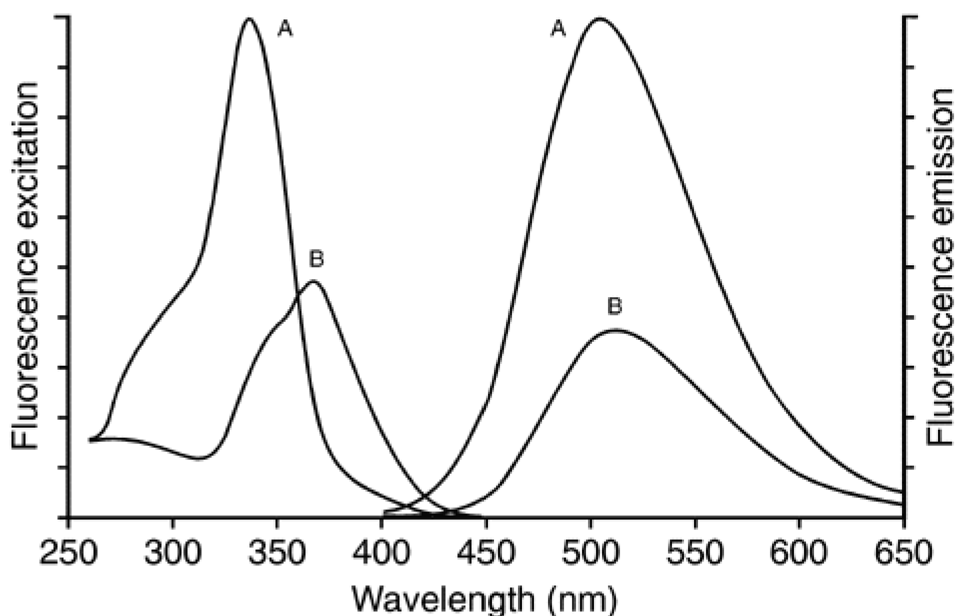
Chemical Hydrolysis of Fura2 AM Esters for Imaging System Testing

1. Dissolve 50 µg Fura2 AM in 50 µl DMSO water-miscible solvent. 1mM Fura Solution
2. Add equal volume of methanol; 50 µl.
3. Add 25 µl of 2M KOH/ dH_2O. If dye is not in solution at this point add more methanol.
4. Wait one hour at RT.
5. Adjust pH to ~ 7 by adding HCl. Between pH of 7-8 is optimal for Fura2 AM. **Spectra results shown below are specific for a buffer pH of 7.2.**
6. Test for fluorescent response by diluting 5 µl dye solution in 100 µl dH_2O and add separately to high calcium buffer and low calcium buffer. **Spectra results shown below are specific for a buffer pH of 7.2. Use PBS pH 7.2.**
7. If dye does not respond properly, add more KOH/methanol to the dye solution and repeat steps 6 and 7 until satisfied.

Not to be used for calibration!!

Spectra — Fura-2/Ca^{2+}

Fluorescence excitation (detected at 510 nm) and emission (excited at 340 nm) spectra of Ca^{2+}-saturated (A) and Ca^{2+}-free (B) fura-2 (F-1200) in pH 7.2 buffer.



These spectra relate to the following products: F-1200 · F-1201 · F-1221 · F-1225 · F-3029 · F-6799 · F-14174 · F-14175 · F-14176 · F-14177 · F-14178 · F-14179 · F-14180 · F-14181 · F-14185

Acquiring Fluorescence Excitation Spectra

Pg. 74-75 Imagemaster; Ratio Fluorescence imaging system reference manual from Photon Technology International (PTI).

1. In the **Acquisition Setup** dialog box, choose **Wavelength**.
2. Click on **Mono 1**.
3. Enter starting (**250nm**) and ending (**650nm**) wavelengths and the step size **(1)** in the appropriate fields.
4. Check the **Acquire** and **Display** buttons.
5. In the **Acquisition Control** dialog box, check **ROI Photometry**.
6. In **ROI Setup** choose the regions of the image to be measured; ~ one square inch near the fringe of the circular field display is sufficient.
7. Press **Free Run**. The excitation spectra of the sample in the ROI windows will be scanned with the camera as your fluorescence detector. The spectrum will be plotted on the **Data Window** and can be saved after the scan is complete.

Additional troubleshooting and spectra analysis curves presented on pg. 75 of manual.

1,9 Dimethylmethylene Blue Total Proteoglycan Assay (DMB)

1,9 Dimethylmethylene Blue Preparation

1. 16 mg DMB stirred with 5ml ethanol.
2. Add 2g sodium formate and 2 ml of formic acid.
3. Add dH2O up to 1 liter.
4. Store reagent in brown bottle at RT.

OR Preferred

1. Add 3.04g of glycine and 2.37g NaCl to 95 ml of 0.1 M HCl.
2. Add 16 mg DMB and raise temperature slightly.
3. Bring volume up to 1 L with dH2O
4. Stir overnight.
5. Store in brown bottle at RT.

Gives solution with pH 3.0 with A_{525} 0.31.

Digestion Buffer Preparation

Add all quantities to 70 ml of distilled water (dH_2O):

1. 17.54 ml of 0.5M NaH_2PO_4 (sodium phosphate monobasic)
2. 2.46 ml of 0.5M Na_2HPO_4 (sodium phosphate dibasic)
3. 87.82 mg L^+-Cysteine HCl
4. 186.12 mg Disodium Ethylenediamenetetraacetate (EDTA)
5. 0.1 M HCL (acid), or 1 M NaOH (base) as necessary to reach a pH = 6.2.
6. Bring final volume to 100 ml with dH_2O.

DMMB Assay Protocol

A. **Preliminary Work**:
1. Obtain cooler full of crushed ice
2. ~~Raise standard temperature of water bath to 60 ˚C. Overtemp setting must be above 6 to allow such a high temp.~~
3. Remove samples from -80 ˚C freezer and place in cooler of crushed ice to thaw; may take up to a few hours for samples to completely thaw. Occasional vortexing of samples will reduce this time.
4. Place DMMB Dye on stir plate to mix. The dye has a tendency to precipitate out, and can cause difficulty when added to samples.

A. **Standard** – Chondroitin Sulfate C Sodium Salt (Stored in -20°C refrigerator dessicator). Sigma Aldrich Cat #: C4385; Lot #: 042K1434.

B. Standard Protein Solutions – Make up 6 different standard protein solutions in microcentrifuge tubes. Microcentrifuge tubes should be labeled according to solution

letter designation. Media used to make up standard consists of 50/50 DMEM/F12; 2 ml is sufficient.

Solution Standard	Solute		Solvent Quantity (µl Media)	Final Concentration (µg/ml)
	Type	Quantity (µl)		
Stock	1 mg/ml	10	90	100
50 ug/ml	Stock	100	100	50
A	50 ug/ml	100	100	25
B	A	100	100	12.5
C	B	100	100	6.25
D	C	100	100	3.125
E	D	100	100	1.536
F	E	100	100	0.768

~~D. Sample/Standard Digestion Procedure~~

1. ~~Take 75 µl of each sample and place them in correctly labeled microcentrifuge tubes.~~
2. ~~5% papain is added to the digestion buffer immediately before it is added to the samples and standards. An equal volume of digestion buffer must be added to each sample and standard. An example of the calculation necessary to make up the digestion buffer is as follows:~~

$$DigestionBufferVol. = 75\mu lDB \bullet \# S\tan dards + 75\mu l \bullet \# Samples$$

~~5% of this volume must be papain, the rest is the digestion buffer.~~

3. ~~Once an equal volume of digestion buffer has been added to the samples and standards, vortex and centrifuge each to be sure they are thouroughly mixed and all liquid is at the bottom of the tube.~~
4. ~~Place standards and samples in water bath at 60 ˚C for one hour to aid digestion.~~

~~**Note:** 75 µl volume is used instead of 50 µl volumes which is added to the microplate wells because of the evaporation that occurs during the digestion at 60 ˚C.~~

E. Preparing Microplate Solutions – 96 well microplate is labeled in rows (A, B, C, etc…) and columns (1, 2, 3, etc…). To speak of a specific well just put the correct number with the correct letter such as A1.

1. Locate Blanks, and Standard solutions in columns 1 and 2. Standards and samples should always be done in duplicate.

   - A1, A2 – Blanks (0 µg/ml) each
   - B1, B2 - 50 µl Solution F (1.536 µg/ml) each
   - C1, C2 – 50 µl Solution E (3.125 µg/ml) each
   - D1, D2 – 50 µl Solution D (6.25 µg/ml) each

- E1, E2 – 50 µl Solution C (12.5 µg/ml) each
- F1, F2 – 50 µl Solution B (25 µg/ml) each
- G1, G2 – 50 µl Solution A (50 µg/ml) each

2. Locate Samples – place 50 µl of each untreated media sample in subsequent columns as necessary. Be sure to create documentation sheet of which sample is located where on the microplate before beginning.
3. Add 200 µl of DMMB Dye (Gives a 1:4 dilution of sample to dye-this has been found to be optimum for post flow culture media meniscus samples) to each well using multichannel pipette. To rid dye of floating precipitate:
   - Pour necessary amount in a 50 ml conical tube.
   - Place a 70 µm Falcom filter over another 50 ml conical tube.
   - Pour the DMMB dye into the filter, allow an air gap between the filter and the tube opening.
   - Place the filtered dye into a Petri dish or channel to allow the use of a multichannel pipette.

Gently mix all standards and samples before placing in microplate wells by pipetting.

F. Reading Microplate

1. Open software and default file named DMMB with an associated date, and prepare it for the sample at hand.
2. Set microplate reader to read absorbance at 525 nm.
3. Place microplate in microplate reader on more than 1 min after dye has been added. Shake with auto shake twice before reading.
4. Depress the READ button on the SOFTmax Pro window to read the absorbance of the well mixtures.

G. Microplate Reader Software – *A basic understanding of the software is necessary before beginning. Read sections of the manual that are pertinent to the desired assay being performed, and run through the tutorial file provided electronically in the SOFTmax Pro folder.*

1. Open the shortcut on the desktop to SOFTmax Pro, the microplate reader software package.
2. Under the File menu choose Open, and select the file named Bob.
3. Choose a file labeled DMMB with an associated date. This is a default template that will need to be modified for each trial, but the basic outline of the file will be used in all cases. Modifications that may be needed:
   - Number of experimental samples and control samples in the Template window.
   - The concentration values for the standards in the template window. If a different standard is being used, this will need to be modified.
   - Functions that are used to give desired output. Right now they indicate absorbance values, concentration values.

4. To further understand the software, open the generic tutorial file provided by the company. Then construct a new outline and template accordingly.

H. Interpreting Results – The results obtained are subject to change if you are not using the TotalProteinQauntDefault file.

Standard Curve:

1. Values for the slope and y-intercept will be given below the standard curve. These values can be used to make a function that will determine the concentration in each sample.
2. The R^2 value below the graph indicates the accuracy of the line to fit the data. A value above 0.75 is legitimate. A value above 0.9 is very good.

Sample Data:

1. The Values column indicates the absorbance at 525 nm of each well.
2. Any average column takes the average of the duplicate trials
3. The Concentration column has a function to determine the concentration of protein (μg/ml) using the standard curve data. The duplicate values should be in close agreement.

Appendix:

Sodium Phosphate Buffer (Sorensen) Stock Solutions

.

x: 0.2M solution of monobasic sodium phosphates (27.8g in 1000 ml)

y: 0.2M solution of dibasic sodium phosphate (53.65g of Na_2HPO_4 *$7H_2O$ or 71.7g of Na_2HPO_4 *$12H_2O$ in 1000 ml)

For each pH indicated, the ml volume of x indicated, added to the ml volume of y indicated should be prepared (Up to here it is 0.2M PO_4) and then diluted to the final

volume of 200 ml by adding an additional 100 ml distilled water.

Result: 0.1M PO_4 Buffer of desired pH

x	**y**	**pH**	**x**	**y**	**pH**
93.5	6.5	5.7	45.0	55.0	6.9
92.0	8.0	5.8	39.0	61.0	7.0
90.0	10.0	5.9	33.0	67.0	7.1
87.7	12.3	6.9	28.0	72.0	7.2
85.0	15.0	6.1	23.0	77.0	7.3
81.5	18.5	6.2	19.0	81.0	7.4
77.5	22.5	6.3	16.0	84.0	7.5
73.5	26.5	6.4	13.0	87.0	7.6
68.5	31.5	6.5	10.5	90.5	7.7
62.5	37.5	6.6	8.5	91.5	7.8

56.5	43.5	6.7	7.0	93.0	7.9
51.0	49.0	6.8	5.3	94.7	8.0

http://www.uprm.edu/biology/profs/navas/specimen.htm

References:

Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986 Sep 4;883(2):173-7.

Wyre RM, Downes S. An in vitro investigation of the PEMA/THFMA polymer system as a biomaterial for cartilage repair. Biomaterials 2000 Feb;21(4):335-43.

Spector, Myron: mspector@risc.bwh.harvard.edu Email regarding Total Proteoglycan Assay.

mRNA SAMPLE ISOLATION & STORAGE

(Time: 20 min)

Post Oscillatory Flow:

1. Incubate slides containing cells in 8 ml fresh post flow media for 6-24 hr in a new Petri dish. 8 ml of media should be used at all time points. When 1 ml was placed on the slides for short duration incubations, there was a tendency for the media to fall off of the slide leaving the cells dry causing cell death.

Cell Isolation:

2. Dump culture media to one side of dish and aspirate with vacuum being sure to remove all excess media surrounding and on the slide without placing vacuum tip directly on the slide.
3. Place 1 ml trypsin on slide for 10 min or until cells released. View cells after 10 min incubation to ensure that they have detached from the slide.
4. Pool cell mixture to one side of dish and collect with 1 ml pipette. Wash down slide in long direction (X2) using pipetted mixture.
5. Place cell mixture into cryogenic vial (polypropylene with silicone washer seal and internal threads VWR Cat #: 66008-251, 1.2 ml volume)

Flash Freezing:

6. Position vials in aluminum holder.
7. Place aluminum holder in liquid nitrogen tube.
8. Place tube in liquid nitrogen container until shipment.
9. Place samples in cooler containing dry ice and send overnight UPS.

PROTEIN PRODUCTION EXPERIMENTS

Time Period –	Set-Up	1 hour
	Run Time	1 hours
	Clean Up & Cell Prep.	1 hour
	Total	3 hours

Equipment List:

1. Flow cart with all components
    a. Computer w/ Linmot Transonic and WinDat Transonic Acquisition software
    b. Transonic Flow Probe Meter
    c. Actuator w/ Plunger accessories
2. 2 syringes (large opening)
3. 5 pieces of tubing-2 w/ bulbed ends
4. 1 valve (3 way w/ Φ 1 cm fittings)
5. Flexcell Flow Chamber w/ fittings
6. Overflow container (100 ml small mouth bottle w/ sealed top)
7. 1 sterile 250 ml container
8. Tape
9. Small Fan

View cells under 100X, need at least 80% confluency to perform test with success.

A. Make Flow & Growth Media

Flow Media: 48.5% DMEM/F12, 2%FBS, and 1%P/S
Post Flow Media: 48.5% DMEM/F12, 2%FBS, and 1%P/S

2 % FBS used to avoid saturation of the media during flow. It is again used in the post incubation culture media to stay constant with flow conditions, and to ensure that any synthesis that occurs is doe primarily to the shear stress induced by fluid flow, and not by culture environment.

1. Place all necessary ingredients into water bath to warm them to 37˚C for ≥ 10 min.
2. Mix Medias:
    - 250 ml flow: amount to fill 2 60ml syringes, tubing, and 30 ml for flow chamber, 30 ml for overflow beaker.
    - 75 ml growth media: 10 ml for each of 6 dishes.

B. Tubing Assembly (Done in coordination with Filling of the System)

1. Attach tubing to syringes, streamer fittings, and valves using the number scheme given in diagram. The bulbed end tubes should be attached to the syringes, shortest to the driving syringe (#1). Use an adjustable pipe clamps to rigidly fix tubing #1 to the driving syringe and the valve fitting. The high pressures that exist at this interface require assistance in maintaining hold.
2. Attach 3-way valve to syringe tubes, and to the longer piece of tubing (#2) leading to flow probe.
3. The flow probe will connect to another short piece of straight tubing (#3) that leads to the inlet fitting that attaches to the flow chamber.
4. Attach another long piece of tubing (#5) to the outlet fitting of the flow chamber, and have it empty into overflow container.

C. Filling System with Fluid (Must be done in Hood!) *This will help keep system ste*ril*e*, *and rid the tubing of unnecessary bubbles.*

Before Chamber is in Position, and after all tubing and valves hooked up in proper sections:

1. Fill inlet tubing not yet attached to 3-way vavle with media from hood, and allow media to flow directly into tubing from the pouring lip of a beaker. Be sure to connect all tubing together and stop one end with one way valves fitting that connects with the streamer. Fill tubing up to inlet fitting (end of Tube #3), and then attach the inlet system to the 3-way valve.
2. Attach the purging syringe and tube to the 3-way valve, fill the purging syringe and associated tubing, and work air pockets out of 3 way valve. Then with the inlet one way valve depressed, fit the plunger into the purging syringe. Be sure the 3-way valve is closed off to the driving syringe outlet.
3. Fill overflow tube in same way as number 1 above, but after it is filled submerge the open end into the overflow container with 20 ml media and seal the container as much as possible to eliminate contamination with airborne particles.
4. Bring all above assemblies to the flow cart and incubator and place as shown in the diagram below, and connect the remaining 3-way valve fitting to the driving syringe tube.
5. Fill the driving syringe and tube #1 with valve remaining closed off to it after it is affixed to the clamping fixture. Be sure there are no air pockets between the plunger head and media, otherwise large %Amps will be needed to maintain desired flow rate (shear stress).
6. Bend the tube inside of the incubator and tape it down to incubator to ensure that the mouth of the tube remains submerged in the overflow container.

D. Setting up Flow System (See Diagram Below) (**Done in coordination with filling of the system)**

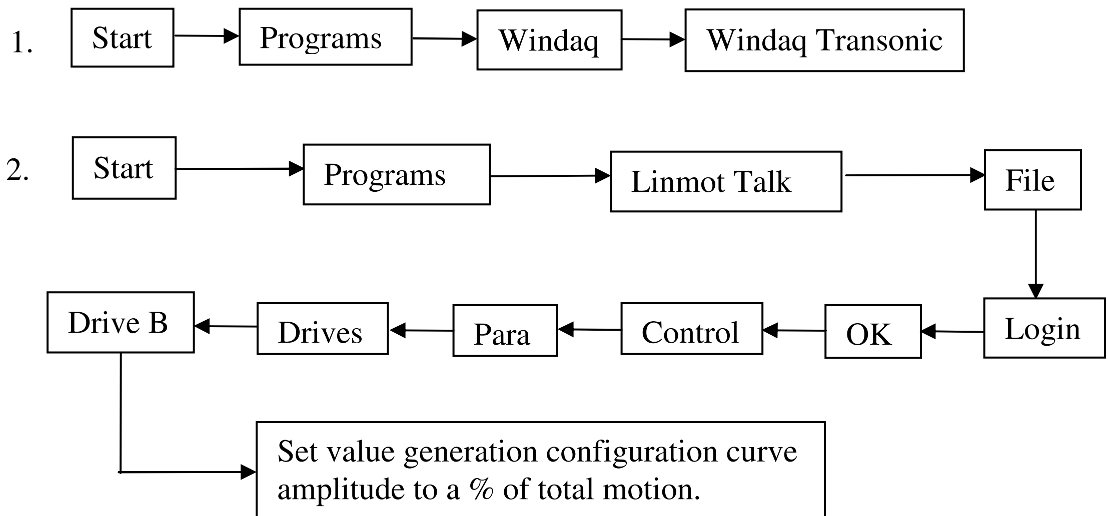
1. On Flow Cart
   - Slide driving syringe into bottom plate of bracket and hold in place by fastening top plate to bottom plate with syringe sandwiched in between.
   - Place driving plunger in between platens on actuator rod, and be sure that the plunger remains in vertical plane and is in line with linear actuator rod and syringe tube.
   - Affix purging syringe to Velcro patch.
   - Place overflow container on top shelf of incubator along with overflow tubing. Currently a 100ml screw top container is used with tape sealing the opening.
   - Make connection between male and female ends of coaxial cable of flow probe.

To start with, attach all components leaving out the flow chamber until ready to run experiment. Then place slides into chamber and attach it to flow system as last step.

The fittings that attach to the inlet and outlet ports of the flow chamber are one-way valves. The tubing should be attached to these fittings prior to being attached to the flow chamber, then they just click into the chamber ports when all else is ready.

E. Flow Computer Set-Up:

- Turn on computer. Follow flow chart below for setup of software.
- Turn Linmot cutoff switch on before turning on master power switch, located above master power switch on cart. Allows system to recognize the actuating mechanism.



By following these steps the LinmotTalk window will display the sinusoidal flow rate given by the actuator detected by the flow probe. The flow rate can then be used to determine the shear stress seen by the cells using the chart at the back of the streamer manual.

The % amplitude should be set to a value that gives1450 ml/min flow rate on the Windaq Transonic waveform, which corresponds to 40 dynes/cm^2 of shear stress according to the tables located at the back of the flow chamber manual. (between 20-40% amplitude gives this flow rate as of this date.)

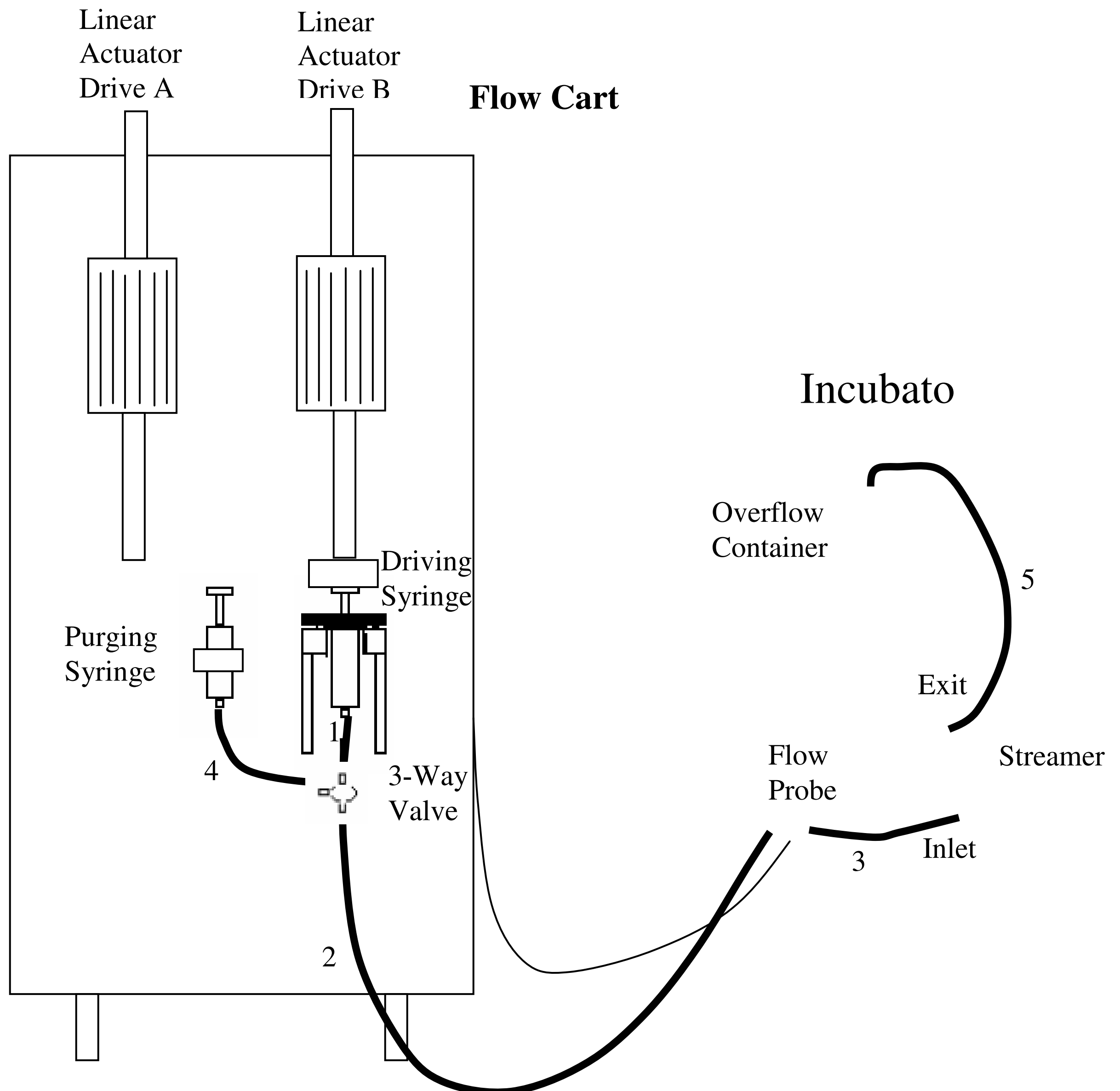
- Windaq Transonic
  - Follow the instruction in the flow system manual to calibrate the Windaq Transonic system and probe.
  - To find Peak to Peak amplitude of wave corresponding to Peak flow rate, go to Set Limits, and change values to those desired such as 1450 and -1450 respectively for a shear stress of 40 dynes/cm^2. *Because of non-ideal flow condition relating to the immediate effects near plunger, the pk to pk amplitude will not be centered about zero, in this case take the average of the high and low number which centers the amplitude about zero to determine the operating shear stress. This is seen as normal thus far.*

F. Flow Chamber (Done in Hood!)

1. Attach bottom of flow chamber, making sure all gaskets are aligned, and tighten Allen screws.
2. Fill chamber from top with media and check for leakage from bottom plate. If there is leakage, repeat process.
3. Remove slides from Petri dishes with forceps or gloved hand being sure not to touch surface with cells on it.
4. Place slides in flow chamber with cell side toward flow channel.
5. Close top, and seal with Allen screws, and check for leaks.

When inserting or taking out slides be very gently, DO NOT FORCE, and be sure to remain in the vertical plane the entire time. The slides break and snap easily and can become lodged in the flow chamber.

6. Attach flow chamber to inlet and outlet ports of flow system.



G. Removing Air Bubbles from System & Troubleshooting

1. Requires careful timing and adding of media.
2. Push media from purging syringe through tubing and flow chamber.
3. Tap tubing to displace bubbles to outflow tube. **Remember, air travels to highest point in system!**
4. Jerk chamber fairly vigorously and/or rotate chamber about central axis fairly rapidly until air trapped in chamber is removed.
5. Tap outflow tube to get them away from chamber. Also remove bubbles from inflow tubing in much the same way.
6. Close valve to syringes, and open the actuator plunger to the system of tubing. Push plunger slowly until reach about one inch from mouth of

syringe. This will help remove bubbles from chamber as well as ready the system for actuation.

7. Run trial runs to remove air bubbles. Repeat steps above while running, and after stopping actuation on the down stroke. Trial runs will also need to be conducted to find the correct % amplitude that corresponds to 1450 ml/min peak amplitude, or the flow rate necessary to cause desired shear stress.

H. Run Experiment

1. Once % amplitude is found, and all bubbles are out of system, place portion of flow system containing flow probe to overflow chamber in incubator that is set at 37˚C, and set to run for 1 hour.
2. Place fan to run on HIGH so that it blows directly on actuator to help from overheating.
3. Monitor the flow rate, there is a tendency for the flow rate to decline with time due to changes in lubricity between the syringe tube and plunger because of non-ideal contact and drying of media on syringe walls between the two. Adjust the linear actuator amplitude as necessary to maintain the correct flow rate.

I. Post Flow Incubation

1. Once flow experiment is terminated, disconnect the flow chamber from the inlet and outlet hoses.
2. Place 10 ml Post Flow Media in Petri dishes labeled Post flow. **Must be done before slides are taken out of chamber, otherwise they will dry up!**
3. **In Hood, while wearing sterilized gloves**, carefully remove the slides from the chamber and place individually in new Petri dishes labeled Post flow.
4. Place the dishes in incubator for designated time periods. (1,2,3 days)
5. Perform protein and media isolations as necessary. Will need to consult protocol for specific isolation under study.

J. Run Control Experiment – Leaving the tubing setup the same.

1. Place new set of slides **using sterilized gloves** in flow chamber, and reconnect to tubing system in incubator.
2. Let sit for designated time period (1 hours) with **NO FLOW** occurring.
3. Post flow incubation same as in part I above.

K. Clean-Up

1. Take flow chamber apart, and rinse with distilled water, alcohol, distilled water and place on open bench to dry. **Be sure not to lose gaskets, they fall out easily!**

2. Disconnect all tubing and bring as one unit to sink. While at sink take tubing system completely apart.
3. Flush tubing, valves, syringes, and fittings first with distilled water, then alcohol, and then water again. *To clean one way valves, depress the valve to allow fluid to flow through it as well as to dry the valve.* Use air to dry tubing and then hang long tubing on rack to dry further, and lay out all other components on open bench.
4. Flush probe with distilled water, alcohol, distilled water, and let dry on bench.
5. Before next trial it may be beneficial to soak the flow chamber, flow chamber accessories, 3-way valve and fittings in alcohol for a few hours to ensure that no bacteria will be present. **Do not soak flow probe**, this piece if equipment is very expensive!!
6. The flow chamber and all associated parts should be autoclaved in the pressurized chamber following the autoclaving protocol. All tubing and syringes can be autoclaved in the dry autoclace using a temperature near 215°C for 30 min, temperatures in excess of 250°C will degrade the tubing and syringe. **The 3 way valve and fittings and flow probe should not be autoclaved.**

QAURTZ SLIDE CLEANING PROTOCOL

If glass slides should reach the point where they need a thorough cleaning, they should be discarded and new slides purchased. They are relatively inexpensive compared to quartz slides used for calcium imaging studies. **Quartz slides should never be discarded!**

1. Follow slide washing protocol as described in the Short Term Calcium Studies Protocol before thorough cleaning is performed. Briefly, rinse slides with distilled water, wash slides thoroughly with Sparkleen powder detergent soap, rinse with distilled water, and rinse with ethanol. Let dry on paper towel, or begin thorough cleaning immediately.

2. Rinse slides thoroughly with deionized water, and place the slides in a bath sonicator in acetone.

3. Sonicate for 15 min, allow to stand 1 hr, and repeat sonication.

4. Rinse thoroughly with water, and Immerse slides in 0.1 M KOH and sonicate for 15 min.

5. Allow the slides to stand for 4 hr in 0.1 M KOH and repeat 15 min. sonication.

6. Rinse thoroughly with deionized water, and Immerse in ethanol and sonicate for 15 min.

7. Transfer slides to a clean ethanol solution and store submerged in ethanol.

RABBIT ISOLATION

Dissection/Rabbit	½ hour
Meniscus Digestion	6-8 hours
Cell Isolation	½ hour
Total	7 hours

Supply Check List:

- Collagenase (located in the 4˚C refrigerator)
- 2 - 50 x 9 mm (small) Petri dish/ leg
- 1 - large 100 x 10 mm Petri dish/ leg
- Dulbecco's Modified Eagles Medium (DMEM)
- Ham's F12
- Fetal Bovine Serum (FBS)
- Penecillin/Streptomyicin (P/S)
- Sterile Dissection tools—scalpel blades, tweezers, scissors, etc…
- 1 - 70µm Falcon filter/rabbit

Isolation Media: 49% DMEM/Ham's F12
2% P/S

Growth Media: 44% DMEM/Ham's F12
10% FBS
2% P/S

1. Place necessary solutions in water bath for ~ 10 min, or until reaching 37 °C.
2. To mix media, tip container back and forth without agitation. This will reduce the number of bubbles formed.

A. Rabbit Dissection

1. Rabbit legs received next day delivery via UPS from Mayo Clinic, Rochester Minnesota. Specimens are to be picked up from The Shipping Shop at 10:30 A.M. the day after animal sacrifice, and brought back to lab.
2. Sterilize hood by wiping with alcohol, then lay down diapers.
3. Place all necessary equipment in the hood including dissection tools, scalpel blades, and 2 - 50 x 9 mm Petri dish.
4. Sterilize gloves by washing with alcohol. **Be sure not to get alcohol on living tissue!**
5. Using scalpel, remove excess meat, fat, and other tissues surrounding knee until reaching synovial membrane immediately encapsulating the knee joint.
6. Wash gloves with alcohol and let dry. With new scalpel blade, that has not touched fur or skin of specimen, begin to severe the patellar tendon and the collateral, and cruciate ligaments. While doing so, be sure not incise the meniscus. To do this, choose the extremes of each attachment that are located close to or over bone.

7. Once cut, if femur and tibia remain connected, twist and pull the two bones apart until they snap and release. The menisci should be laying flat on the tibia.
8. Cut away additional attachments of the meniscus to the tibia and trim the circumferential or peripheral attachment.
9. Place the menisci in ½ of the small Petri dish containing 3 ml of PBS, and remove excess tissue that is not meniscal tissue.
10. To estimate cell #/gram of wet weight in the meniscus, weigh both the lateral and medial menisci together. Be sure to do this sterily by keeping menisci in PBS in a covered Petri dish. Weigh the dish with PBS first to zero scale, then measure again with menisci enclosed.

B. Meniscus Digestion

1. Mix the **isolation media** if not already done so. **There is no FBS in this media!**
2. In a new 50 x 9 mm Petri dish, place 3 ml of **Isolation Media**.
3. Measure out 4 mg collagenase per ml of media and add it to the Petri Dish. *When transferring collagenase into hood, cover with similarly sized weighing boat so that it does not blow away. When adding collagenase to media, turn off blower for the same reason.*
4. Swirl Petri dish to mix.
5. Place the meniscus into the new dish and cut into very small pieces. The smaller the pieces, the faster the digestion. Should be smaller than 3 mm cubes.
6. Place in incubator at 37 °C for ~ 6 hours, or until digestion is complete and no chunks of meniscus remain.

C. Cell Isolation

1. Place digested fluid slurry through 70 μm Falcon Filter into a 50 ml conical tube. The filter will fit the opening of this size tube only. To aid in the filtering process lift the filter slightly above the tube so that air is able to enter into the tube. Agitating the filter in an up and down motion will help get the final remains to travel through.
2. Take the liquid that passed through the filter and centrifuge it at 1500 rpm for 10 min (1000g).
3. After centrifuging, aspirate the collagenase supernatant using glass pipette tip connected to a vacuum source, and resuspend the pellet in 3 ml of **Growth Media**. *Most likely a pellet will not be seen, but that doesn't mean that one doesn't exist. Treat it with caution, and do not place tip of pipette near bottom of tube. Do not want to suck up pellet even if it is not readily observed.*
4. Mix the 3 ml solution and pellet well, scraping bottom of tube with pipette if necessary.
5. Transfer mixture from tube to ½ of 50 x 9 mm Petri dish. Then place this smaller dish half in a larger Petri dish. *The smaller dishes have airtight seals. Placing half of a small dish in a large dish aids in gas transfer with the cells.*
6. Wait ~ 6 days for dish confluency.

Note: When cells placed in large 100x20 mm Petri dish, the dish should be confluent within 7-8 days.

Determining Shear Stress for Calcium Imaging Studies

Assumptions: Cells are enclosed in a parallel plate flow chamber and they themselves cause no disturbance to fluid flow as the media passes over them. The plates are assumed to be unmoving.

Shear Stress Equation:

$$\tau = \frac{6Q\mu}{bh^2} = 1.579 \bullet Q$$

τ = shear stress (dynes/cm^2)
10 dynes/cm2 = 1 Pa
μ = viscosity of fluid = 0.921E-3 kg/ m·sec (water)
Q = flow rate (ml/min)
b = width of flow area = 8.0 mm
h = height of flow area = height of gasket = 0.27 mm

Table of values for Shear Stress Given a Flow Rate calculated from the above equation.

Flow Rate (ml/min)	Shear Stress (dynes/cm2)
40.75	64.3~65
32.6	51.5~50
24.45	38.6~40
16.3	25.7~25
8.15	12.9~15

The approximate values of shear stress were used when documenting the shear stress used for calcium studies for Rabbit Specimens 1-11.

The viscosity of water varies significantly even over a small temperature range. During experimentation with Rabbit 1 through 11, the temperature of the room, and/or media was not recorded. Thus, an average was used here assuming the media and the air temperature were at 75 °F. In future studies, air and media temperatures should be monitored and recorded to more accurately describe the shear stress-flow rate relationship stated in the above equation.

Table showing the relationship between temperature, viscosity, and their influence on the shear stress-flow rate equation.

Temperature		Viscosity	Shear Stress-Flow Rate Coefficient
°C	°F	N•s/m^2 E^{-3}	τ = A•Q (ml/min) dynes/cm^2
20	68	1.003	1.72
24	75	0.921	1.579

The dimensions of the chamber, which in the case of rabbits 1-11 was controlled by gasket dimensions, are also very influential in the coefficient used in the shear stress-flow rate equation. These dimensions should be checked periodically, and recorded if changed at any time. Throughout Rabbit 6-11 tests, the dimensions of the gasket were cut to assume those listed above, and were correct as an average value over the length of the chamber. Gaskets were cut to size with either a scissors or a scalpel blade, and thus were only as straight and uniform as the hand that holds them.

The shear stress equation above was taken from: Saunders MM, You J, Trosko JE, Yamasaki H, Li Z, Donahue HJ, Jacobs CR. *Gap junctions and fluid flow response in MC3T3-E1 cells*.Am J Physiol Cell Physiol. 2001 Dec;281(6):C1917-25., and is widely accepted by the scientific community.

TOTAL PROTEIN ISOLATION
(Urea)

Time Period – 1 hour

Stock Solutions:

Urea Buffer:

- Distilled deionized water (ddH2O)

The following all added directly to ddH2O

- 22.823 g Urea giving final concentration of 3.8 M Urea
- 0.606 g Tris Ultra Pure giving final concentration of 50 mM Tris pH 8.5
- Bring final volume to 100 ml with ddH2O.

Inhibitor Aliquots:

1. Phenylmethylsulfonyl fluoride (PMSF) 176 mg/ml isopropanol – make 1ml solution and divide into 50 µl aliquots placed in a 1.5 ml microcentrifuge tube. Label the tubes, seal with parafilm, and place in 4˚C refrigerator.

The inhibitor will only be added to the Urea buffer directly before use. The inhibitor is used to block proteases from destroying any proteins that may exist in the culture media.

Protein Isolation:

1. Mix Extraction buffer
   a. Place 0.5 ml of Urea buffer per dish in 15 ml conical tube (only need enough to cover surface area of slide, and don't want too much because microcentrifuge tubes only hold 1.5 ml).
   b. Add 5 µl of PMSF (10 µl/1ml Urea Buffer)
2. Remove media from Petri dish, measure volume, and place in 15 ml conical tube for future analysis. Label sufficiently. Volume of media is measured for use in normalizing data for presenting and comparing to literature.
3. Wash once w/ PBS to get rid of all of media.
4. Add Extraction Buffer
5. Use cell scraper to vigorously scrape cells from dish (will begin to see debris floating around). **Working on Ice**.
6. Wash debris to one side of dish by tipping and scraping slide and dish with cell scraper, and place dish at an angle.
7. Using 21 gage needles to shear DNA, lyse the cells by sucking all of media into needle.
8. Transfer the lysate to microcentrifuge tube, and mark the tube.
9. Put microcentrifuge tube on ice for 30 min. (Snow in 8" X 12" cooler from outside works beautifully).

10. Place microcentrifuge in 4˚C refrigerator to allow the centrifuge temperature to equilibrate.
11. Microcentrifuge cell lysate at 13,000 rpm (16,000 g) for 20 min @ 4˚C.

Conversion from gees to rpm's

$$gees = RCF = 11.18 * \left(\frac{n}{1000} \right)^2 * r$$

r= radius of centrifugation = 8.5 cm for Biofuge Pico
n=rpm's

12. Relocate supernatant (being sure not to such up pellet or any solid material) to new microcentrifuge tube, wrap in perafilm, label, and freeze @ -80˚C.

Save until used in Protein quantization and Western Blotting. Try to do these on same day.

TOTAL PROTEIN QUANTIFICATION

(Lowry Technique)

Time Period – 1 hour

Purpose: To get concentration of protein in sample that is to be used to determine the necessary

Aliquots for individual channels used for Western Blotting analysis.

Necessary Items:

- Bio Rad DC Protein Assay Kit
  - Protein Assay Standard II Lypholized BSA
  - Reagent A
  - Reagent B
- 50 µl dH2O
- Samples
- 96 well Microplate
- Microplate Reader, Software, and computer
- 5 Microcentrifuge tubes
- Pipettes including multichannel pipette

A. Obtain Styrofoam cooler filled with ice. Remove samples from - 80˚C freezer and place in cooler to allow controlled thawing. Samples must be completely thawed and vortex before use.

C. Standard Protein (SP) – Protein Assay Standard II Lypholized BSA (bovine serum Albumin) 1.38 mg/ml stock (Stored at 4°C refrigerator). Case Number: 9048-46-8

D. Standard Protein Solutions – Make up four different standard protein solutions in microcentrifuge tubes. Microcentrifuge tubes should be labeled according to solution letter designation.

Solution	Solute		Solvent Quantity	Final Concentration
	Type	Quantity (µl)	(µl dH2O)	(mg/ml)
SP	1.38 mg/ml	10	0	1.38
A	SP	10	10	0.69
B	A	10	10	0.345
C	B	10	10	0.1725

Sample concentration may be necessary for specific testing purpose, if it is, use portion with strikethrough below.

~~**I. Concentrate Samples** – Place sample quantity in 15ml tube containing concentration filter (Millipore Amicon Ultra 10,000 MW with a 4 ml volume); centrifuge at 4500~~

~~rpm (5000 g) for 20 min at RT. This is done to aid in Western Blotting sensitivity to collagen Type I.~~

- ~~**Cell Lysate** – Take entire quantitiy~~
- ~~**Cell Culture Media** – Take 1 ml~~

J. Preparing Microplate Solutions – 96 well microplate is labeled in rows (A, B, C, etc…) and columns (1, 2, 3, etc…). To speak of a specific well just put the correct number with the correct letter such as A1.

1. Locate Blanks, and Standard Protein solutions in columns 1 and 2. Standards and samples should always be done in duplicate.

    - A1, A2 – Blanks (0 mg/ml) each
    - B1, B2 - 5 µl Solution C (.01725 mg/ml) each
    - C1, C2 – 5 µl Solution B (0.345 mg/ml) each
    - D1, D2 – 5 µl Solution A (0.69 mg/ml) each
    - E1, E2 – 5 µl Solution SP (1.38 mg/ml) each

2. Locate Samples – place in subsequent columns as necessary.
3. Add 25 µl of Reagent A to each well using multichannel pipette.
4. Add 200 µl of Reagent B to each well using multichannel pipette.

K. Reading Microplate

1. Open software and default file named TotalProteinQauntDefault, and prepare it for the sample at hand.
2. Set microplate reader to read absorbance at 700 nm.
3. Rock 96 well microplate for 15 min.
4. Place in microplate reader. Shake with auto shake once before reading.
5. Depress the READ button on the SOFTmax Pro window to read the absorbance of the well mixtures.

L. Microplate Reader Software – *A basic understanding of the software is necessary before beginning. Read sections of the manual that are pertinent to the desired assay being performed, and run through the tutorial file provided electronically in the SOFTmax Pro folder.*

1. Open the shortcut on the desktop to SOFTmax Pro, the microplate reader software package.
2. Under the File menu choose Open, and select the file named Bob.
3. Choose the TotalProteinQauntDefault file. This is a default template that will need to be modified for each trial, but the basic outline of the file will be used in all cases. Modifications that may be needed:
    - Number of experimental samples and control samples in the Template window.
    - The concentration values for the standards in the template window. If a different standard is being used, this will need to be modified.

- Functions that are used to give desired output. Right now they indicate absorbance values, concentration values, and in the samples window, the number of microliters necessary to obtain 1 microgram of protein.

4. To further understand the software, open the generic tutorial file provided by the company. Then construct a new outline and template accordingly.

M. Interpreting Results – The results obtained are subject to change if you are not using the TotalProteinQauntDefault file.

Standard Curve:

3. Values for the slope and y-intercept will be given below the standard curve. These values can be used to make a function that will determine the concentration in each sample.
4. The R^2 value below the graph indicates the accuracy of the line to fit the data. A value above 0.75 is legitimate. A value above 0.9 is very good.

Sample Data:

4. The Values column indicates the absorbance at 700 nm of each well.
5. Any average column takes the average of the duplicate trials
6. The Concentration column has a function to determine the concentration of protein (μg/ml) using the standard curve data.
7. The Miclit/Micgram (microliters/microgram) column gives the number of microliters necessary to obtain 1 microgram of protein. This information is desired to perform Western Blotting.

<u>Go on to Western Blotting or other testing.</u>

WESTERN BLOTTING PROTOCOL

Time Period: Prepare to spend 2-3 days on technique with frequent breaks.

Theory behind SDS Gels – Sodium Dodecysulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is normally used to assess the purity of a protein as well as estimate a proteins molecular weight. Polyacrylamide gel is polymerized from a solution of acrylamide monomers into crosslinking chains forming a porous semisolid matrix suspended in water. Gels are rated according to (%C) the crosslinker to acrylamide monomer ratio of the monomer solution.

$$\%C = \frac{\text{g crosslinker} \times 100\%}{\text{g acrylamide} + \text{g crosslinker}}$$

This matrix is buffered to weakly basic, so most proteins will be anionic and migrate to the anode of the gel when an electric current is applied. The rate at which the proteins move in the gel is affected by the electric current, the pore size of the gel, and the size of the protein (i.e. smaller proteins move faster in the gel) (Horton et al., 2002).

Sodium dodecysulfate (SDS) is an anionic detergent with a long hydrophobic tail that binds to the hydrophobic side chains of amino acids at a constant ratio of 1.4g of SDS to 1g of polypeptide, proportional to the molecular weight of the protein (Hames, 1998). Larger proteins bind to more SDS molecules. This ratio ensures that all SDS-protein complexes have a similar mass:charge ratio, eliminating the intrinsic charge of the protein as a factor affecting migration (Rybicki and Purves, 2001). Reducing agents such as β-mercapaethonal denatures the protein and dissociates multimeric proteins into its subunits by cutting the disulfide bonds of the protein, causing extended conformations in the polypeptides and thus eliminating the effects of the shape of the protein in migration (Lodish et al., 2000). This leaves protein size (mass) as the only factor affecting the separation of proteins in electrophoresis.

Reference:
http://www.cquest.utoronto.ca/botany/bio349s/techniques/assessingcells/2SDSpage.html

Buffering Solutions:

1. 10x Running (Electrode) Buffer pH 8.3
   Making 1 Liter:
   30.3 g Tris Base
   144.0 g Glycine
   10.0 g SDS
   Dissolve with d $H2O$ to a final vol. of 1 L

   Store at 4 °C, if precipitate occurs, warm at Rm Temp before use.

   Use: Dilute to 1x concentration by taking 50 ml 10x mixture and adding

450 ml of d H_2O.

Mini-PROTEAN 3 Cell Instruction Manual – pg. 14 & Dr. Haut Donahue's Lab Manual.

2. Sample Buffer (SDS Reducing Buffer)

3.55 ml	d H_2O
1.25 ml	0.5 M Tris-HCl, ph 6.8
2.5 ml	glycerol
2.0 ml	10% (w/v) SDS
0.2 ml	0.5% (w/v) bromophenol blue
9..5ml	Total Volume

Store at Rm Temp.

Premade Lamelli Sample Buffer can also be used.

For premade or self mixed sample buffer:
Add 50 µl β-Mercaptoethanol to 10 ml sample buffer prior to use. Dilute sample 1:2 with 2X Lamelli sample buffer.

Mini-PROTEAN 3 Cell Instruction Manual – pg. 14, and Dr. Haut Donahue's Lab Manual and Santa Cruz Research Brochure pg. 8.

3. Transfer Buffer - Tris Glycine Saline (TGS) pH 8.3

Making 1 Liter:

25 mM Tris Ultrapure	3.03 g Tris
192 mM glycine	14.4 g glycine
20% v/v methanol	200 ml methanol

Add dd H_2O to reach 1 Liter.

Mini Trans Blot Transfer Cell Instruction Manual – Configuration pg. 10, and Tammy Donahue's Lab Manual.

4. Tris Buffered Saline (TBS)

10 mM Tris-HCl
150 mM NaCl
pH 8.0

Santa Cruz Research Brochure pg. 8.

5. Washing Buffer - Tris Buffered Saline Tween 20 (TBST) 0.1 (0.5)% Tween 20

1000ml TBS
1ml Tween 20

30 ml needed per /wash, when membrane placed in large Petri dish. Should be vortexed to mix.

Mini Trans Blot Transfer Cell Instruction Manual – Configuration pg. 10.

6. Membrane Blocking Buffer (Blotto) – TBST w/ 5% Non-fat Milk (Carnation instant powder milk.)
   30 ml TBST
   1.5g Nonfat Milk

30 ml needed per incubation. 10 ml TBST w/ 0.5 g Nonfat Milk used in primary and secondary antibody incubations. This reduces the amount of antibody used.

Transfer Cell capacity with cooling unit inserted – 650 ml.

Preparing Total Protein Sample for Western Blotting

1. After obtaining concentration of total protein in sample using quantification methods (i.e modified Lowry or Bradford), calculate maximum amount of total protein (μg) contained in 10 μl concentrated sample with the lowest total protein value. Then calculate the volume of sample necessary to achieve the same amount of total protein for all other samples and place in a microcentrifuge tube. It is necessary to do this for each sample because each sample will most likely have a different protein concentration.
   a. Collagen makes up 75% of the total protein concentration, and 90% of the collagen is type I.
2. Add twice the amount of 2X sample buffer to the sample.
3. Dilute this value up to 30 μl with deionized water (dd H_2O) for each sample.
1. Heat mixture at 100˚C for 3-5 min in a hot water bath.
2. Flick and centrifuge sample (≤ 10 sec) to mix solution, and bring the sample to the bottom of the tube.

Perform steps 2-5 for marker and standard protein solutions also. Standard protein concentrations should cover range of what will be found in samples. 10 μl of marker should always be used as a check for gel loading and transfer efficiency.

Gel Specifications: 7.5% Express Gels
10 sample wells (30 μl volume each)
0.1 x 10 x 8 cm
Cat # E-4325-010

A. Running Gel (1 1/2 hour @ 100 V)

1. Precast Gel Preparation
   a. Remove Ready Gel from the storage pouch.
   b. Gently remove the comb and rinse the wells thoroughly with distilled water or running buffer. To do this obtain a 1 ml pipette, fill wells with distilled water and using a dry paper towel drain water from wells by placing towel at opening of gel drawing water from wells while holding the gel angled downward (X2).
   c. Cut along the dotted line at the bottom of the ready gel cassette with a razor blade.

   OR

   c. Pull the clear tape at the bottom of the ready gel cassette to expose the bottom edge of the gel.

2. Constructing Running Cell

   a. Open cams to release existing casting plate.
   b. Place gel cassette sandwich into the slots at the bottom of each side of the electrode assembly. Be sure the short plate of the gel cassette sandwich faces inward toward the notches of the U-shaped gasket.
   c. Lift the gel cassette sandwich into place against green gasket and slide into clamping frame.
   d. Press down on the electrode assembly while closing the two cam levers of the clamping frame to format the inner chamber and to insure a proper seal of the short plate against the notch on the U-shaped gasket. Short plate must align with notch in gasket.
   e. Lower the inner chamber assembly into the mini tank. Fill the inner chamber with 125 ml of running buffer until the level reaches halfway between the top of the taller and shorter glass plates of the gel cassettes. Check for leaks. Do not overfill, may cause problem with transfer.
   f. Add 200 ml of running buffer to the mini tank, or lower chamber.

3. Gel Loading
   a. Spin down solution before beginning to get al liquid to the bottom of the tube.
   b. Be sure all bubbles are out of wells before beginning. Do this by tapping on the outside walls of the gel to get them out.
   c. Always use small pipette tips. Large ones separate gel walls decreasing volume to level below wells making it very difficult to load correct amount. Gel loading tips should be used.
   d. When obtaining sample, wipe pipette tip on inside of microcentrifuge tube to remove excess.
   e. To load gels, guide the pipette with other hand index finger near the tip of the pipette and press tip against the inside surface of the outside gel cassette plate and guide it into the well. Release all fluid without over pushing on the pipette.

1. Attach to power supply.
    a. Place cover on cell in color coded fashion.
    b. Attach leads in color coded fashion to power supply.
2. Set voltage requirement to 100 V and allow the amps to vary.
    a. Depress the run button on the power supply. Should see effervescence due to hydrolyses of solution created by electrical current.
3. Gel should be run to bottom within 1-2 hours. Need to watch gel.

B. Protein Transfer (Mini Trans Blot Transfer Cell Instruction Manual pg 5) **(Overnight – 18 hours)**

1. Make 1 L of transfer buffer (TGS)
2. Cut membrane and filter paper to dimensions of gel. **Wear gloves when handling membranes!**
3. Equilibrate gel and soak filter paper and fiber pads in transfer buffer (TGS) for 15 min. Removes contaminating electrophoresis buffer salts.
4. PVDF membranes must first be wetted in 100% MeOH, and then soaked in transfer buffer (TGS) for 15 min. Do this while running gels.
5. Prepare Gel sandwich **Order:**
    i. Gray side of cartridge down
    ii. Fiber pad
    iii. Filter paper
    iv. Gel-remove ridge and excess well strippings and cut one corner for location purposes using a piece of plastic or other sharp edge. Place face up on filter paper.
    v. Membrane
    vi. Filter paper
    vii. Fiber pad
    viii. Close it up

***Make sure there are no bubbles present at any time during creation of sandwich! Roll them out with a glass rod or put on glove and use fingertips.**

***Membrane must be on positive side of transfer case in order to allow the negatively charged proteins to relocate to the membrane from the gel.**

6. Place cassette in module.
7. Add cooling unit with ice shavings to opposite side. If ice shavings not available, fill cooling unit with water and place in freezer until frozen(Do this immediately after Transfer is complete to have ready for next time).
8. Fill with Transfer Buffer (TGS). Transfer cell capacity – 650 ml with cooling unit inserted.
9. Add stir bar to help maintain temp and ion distribution. Set speed to max without having stir bar jump around.
10. Put on lid and connect to power supply in color coded fashion. Set the amperage to be constant and allow the voltage to vary.

Transfer Conditions (pg 8) – Overnight (18 hours); 30 V; 90 mA @ 4˚C
1 hour; 100 V; 350 mA @ Rm Temp

11. Upon completion, disassemble sandwich and remove membrane for development.
12. Clean cell, fiber pads, and cassettes with lab detergent and rinse well with deionized water.
13. Filter pads and gel can be pitched if transfer was successful indicated by the existence of rainbow marker.

C. Membrane Blocking

1. Block membrane with TBST containing 5% Nonfat Milk at Rm Temp on rocker for 1 hour.
2. Primary Antibody (°1) mouse anit-Collagen type I Incubation.
   a. Dump out TBST with 5% nonfat milk
   b. Place ˚1 1:20 in blotto for 1 hr at Rm Temp in rocker or overnight (18 hr) at 4°C. (10 ml blotto w/ 500 µl °1)
3. Wash membrane with 30 ml TBST.
   a. 1 x 15min
4. Secondary Antibody Incubation (°2) Rabbit anti-Mouse.
   a. Dump out TBST wash
   b. Place ˚2 1:2500 in blotto for 1 hr at Rm Temp in rocker, or overnight at 4°C. (10 ml blotto w/ 4 µl °2).
5. Wash membrane with 30 ml TBST.
   a. 1 x 15min
   b. 4 x 5min

D. Color Development (ECL kit detection reagents instruction manual pg. 14) (1 hour)

Equipment to bring with: Timer, Scissors, X -Ray Cassette, Saran Wrap.

1. Mix equal vol. of solution 1 & 2 for total vol of 2 ml. Final vol req. is 0.125 ml/cm^2 membrane.
2. Drain excess wash buffer off membrane and dab edges off in paper towel.
3. Cover necessary surface of membrane with ECL reagent mixture and incubate for 1 min at Rm Temp.
4. Place sheet of plastic or saran wrap on flat plate (surface).
5. Drain excess detection reagent by holding membrane tightly with forceps at one corner.
6. Place membrane protein side down on Saran Wrap and wrap up gently smoothing out air bubbles.
7. Place wrapped membrane in X-ray film cassette face up. Make sure there is no water on outside surface of saran wrap. **Film cannot get wet!**
8. Cut piece of film to dimensions of membrane and place over membrane in cassette.
9. Expose film to membrane for various times depending on best results (2.5-10min) by clamping cassette shut.

10. Place film in developer for 5 min. See instructions on making developer below.
11. Place film in Fixer for 3-5 min. See instructions on making fixer below.
12. Wash film in Water and view under light.

Steps 8 – 12 should be done in dark room with safety lights on.

Repeat process from steps 7-11 until satisfied with results, and to have multiple records.

Making Fixer and Developer

- **Fixer** – 500 ml volume. Take 124.5 ml Sol A, add 13.7 ml Sol B, bring final volume to 500 ml with dH_2O. To make 3.8 L (use entire contents of both solutions) 946 ml Sol A, add 1.4 ml Sol B, bring final volume to 3.8 L with dH_2O.
- **Developer** – 500 ml volume. Take 52.1 g developer and bring volume to 500 ml with dH_2O. To make 1.9 L (entire contents of developer) take 198 g developer and bring up to final volume with dH_2O.