Homogenisation Protocol

Materials

- Tissue lysis buffer
- Precellys 2 ml ceramic bead (1.4 mm) containing homogenisation tubes (Stretton Scientific, #03961CK14)
- Precellys24 automated homogenisation machine (Stretton Scientific, #03119.200)

1. Remove tissues from freezer and store on dry ice.
2. Cut a piece from the first sample using a scalpel and weigh aiming for a weight of approximately 100 mg.
3. Place in a homogenisation tubes, labelled on both the cap and the sides, and add 1ml of ice-cold tissue lysis buffer. Place on ice.
4. Repeat for each sample, using the original sample as a size guide.

   NB. Hearts should be cut to ensure the ventricular tip is included. When very small tissues are used (from dwarf animals) and 100 mg of tissue is not available, the volume of tissue lysis buffer should be reduced proportionally to the quantity of tissue used. For hypothalami, use hypothalamus tissue lysis buffer.
5. Place tubes in Precellys machine maintained in a room kept at 4°C and homogenise three times for 20 seconds on each occasion, with a 15 second gap between each occasion. Leave to settle for five minutes.
6. If any tissue mass is still visible, repeat homogenisation at the same settings.
7. Centrifuge tubes for 30 minutes at 13 000 g, at 4°C.
8. Carefully remove supernatant using a 1ml pipette and transfer to a labelled 1.5 ml Eppendorf tube.
9. Place on ice to proceed to protein content determination or store at minus 80°C.

BCA Assay

Materials

- BCA kit (Pierce Perbio Science, #23227)
- Tissue lysis buffer

Part A: Preparation of the BSA (bovine serum albumin) for calibration curve:

1. Label 8 × 1.5 ml eppendorf tubes from A to H.
2. Use the table below as a guide to serially dilute the BSA to prepare a set of standard proteins. Use tissue lysis buffer as the diluent.
3. Prepare a standard curve on the first column of the 96 well BCA plate. Pipette 25 µl of Tube A into each of wells A1 and B1. Repeat with Tube B into A2 and B2 and continue up to (and including) Tube H. (Rows=Letters, Columns=Numbers)

Tube	Volume of diluent			Final concentration
A	0 µl	+	1 ml from stock at 4ºC	2 mg/ml
B	500 µl	+	500 µl of BSA	1 mg/ml
C	500 µl	+	500 µl from vial B	0.5 mg/ml
D	500 µl	+	500 µl from vial C	0.25 mg/ml
E	500 µl	+	500 µl from vial D	0.125 mg/ml
F	500 µl	+	500 µl from vial E	0.0625 mg/ml
G	500 µl	+	500 µl from vial F	0.03125 mg/ml
H	500 µl		-	0mg/ml
NB. Vortex well before transferring from one tube to the another				

Part B: Preparation of the BCA working reagent:

In a 50 ml falcon tube, prepare a sufficient volume of BCA working reagent based on the number of samples to be assayed. 200 µl of the BCA solution is required for each sample/well in the assay, made up of BCA reagent A and BCA reagent B in a ratio of 50:1 (196 µl of A and 4 µl of B).

A = (196 x [No samples]) x 2(for duplicates) + 980 (quick test) x 1.6* µl

B = (4 x [No samples]) x 2(for duplicates) + 20 (quick test) x 1.6* µl

* extra for pipetting errors

To improve accuracy round up volume required of sample A and pipette into tube, then deduct the extra with a smaller volume pipette.

Part C: Protein samples:

Once that the protein samples have thawed mix them thoroughly using the vortex and keep them on ice throughout the procedure.

Protein dilution 'quick test': to determine necessary dilution

The quick test involves serially diluting a protein sample and comparing the resulting colour after incubation with BCA working reagent (by eye) to the 2 mg/ml bovine serum albumin (BSA) standard. Use the sample from the largest piece of tissue, or the one with the darkest colour.

- Label 5 × 1.5 eppendorf tubes with STD, 1:5, 1:10, 1:20 and 1:40 dilutions.
- Put 25 µl of BSA 2 mg/ml into tube STD.
- Serially dilute the randomly selected protein sample into 1:5, 1:10, 1:20 and 1:40 dilutions as follows

Dilution	*Diluent*		*Final volume*
STD		25 µl BSA	25 µl
1:5	40 µl +	10 µl of Protein	25 µl
1:10	25 µl +	25 µl of 1:5	25 µl
1:20	25 µl +	25 µl of 1:10	25 µl
1:40	25 µl +	25 µl of 1:20	25 µl (discard 25 µl)

- Incubate all 5 tubes with 200 µl of BCA working reagent for 15 minutes at 37ºC.
- Compare the colour of each sample tube with the BSA (usually dark violet)
- Choose the "best" dilution factor that falls within the colour range of the assay (i.e. choose the sample that is lighter than the BSA, but not too light).
- Prepare the appropriate dilution of each protein in a separate eppendorf tubes, using tissue lysis buffer as diluent aiming for a total volume of 100 µl.

4. Pipette 25 µl of each unknown (diluted) sample on to the BCA plate in duplicate (or triplicate). Place sample one in wells C1 and D1, then sample two in C2 and D2

continuing down column. Label plate or draw diagram marking which samples are in which wells.

5. Transfer the BCA working reagent into a square box and use multi-channel pipette to transfer 200 µl from the box to the BCA assay plate.
6. Put the lid on the plate and incubate at 37ºC for 30 minutes.
7. Leave the plate for 2-3 minutes at room temperature and then proceed to reading.

Part D: Procedure for reading the plate using the Wallac plate reader:

1. Put the plate in the reader and remove the lid.
2. Press the 'define the platemap' button and select 'photometry' and then '*Bradfrod assay - absorbance 562'*.
3. Press 'next': the plate will appear as selected blue wells.
4. Press 'next' again: enter a title for the assay and add a comment if required.
5. Press 'finish': the count will begin.
6. Immediately after the beginning of the count select menu 'instrument' and 'end after plate'.
7. Once complete, open 'latest results'
8. Using Microsoft Excel, construct a calibration curve and use this to calculate the protein concentration of each sample.

Part E: Make up aliquots

- Make up aliquots of appropriate quantities of protein for desired experiments (e.g 300 µg) based on the calculations from the BCA curve.
- Freeze in Eppendorf tubes at minus 80ºC
- Aliquotting is important as protein will degrade with each thaw-refreeze cycle

Preparation of Antibody-coated Beads

Materials

- Protein G sepharose beads in 20% ethanol (Amersham, #17-0618-01)
- α1 and α2 antibodies (Prof G Hardie, University of Dundee). **NB.** Each batch of antibodies is a different concentration and quantity needed must be calculated
- Immunoprecipiation (IP) buffer

1. Calculate quantity of beads required:
   a. Protein G sepharose beads: 10 µl/sample taking into account the 20 % ethanol
   b. α1 antibody: 2.5 µg/sample
   c. α2 antibody: 2.5 µg/sample

Example: 300 samples, α1 Ab conc. 2 mg/ml, α2 Ab conc. 2.3 mg/ml

PG volume for 300 samples	= 300 × 10 µl = 3000 µl
+PG vol/4 to account for 20 % eth	= **3750 µl**
α1 volume/sample	= 2.5 µg/2 mg/ml = 1.25µl
α1 volume for 300 samples	= 300 × 1.25 µl = **375µl**
α2 volume/sample	= 2.5 µg/2.3 mg/ml = 1.087 µl
α2 volume for 300 samples	= 300 × 1.087 µl = **326.1 µl**

2. Add required volume of Protein G sepharose beads to a labelled 15ml falcon tube. Make sure beads are well suspended prior to aspiration – 100 % ethanol or IP buffer may be added to the beads bottle to dilute the beads.
3. Wash the beads five times with 5 ml of ice cold IP buffer as follows:
   a. Use an automatic pipette to add 5 ml of IP buffer to the falcon tube. Vortex well.
   b. Centrifuge for 1 minute at 3000 g 4ºC.
   c. Aspirate the supernatant (IP buffer) and discard.
4. Re-suspend final pellet in 5 ml of IP buffer.
5. Add required amount of α1 and α2 antibody and mix for 45 minutes on a roller at 4ºC (in the cold room).
6. Centrifuge for 2 minutes at 3000 g, at 4ºC.
7. Wash the beads five times with 5 ml of ice-cold IP buffer (as per step 3).

8. At enough IP buffer to make the final volume into 1ml aliquots, each enough for 20 samples. Pipette into labelled tubes and store at 4ºC. E.g. for 300 samples

    Number of samples per 1ml aliquot = 20

    Total volume of beads + Ab = number of samples/20 = 15 ml

    Final volume of mixture required = **15 ml**

α1 and α2 AMPK Immunoprecipitaton

Materials

- High sodium immunoprecipitation (NaIP) buffer
- Herpes-Brij (HB) buffer

1. Add 50µl of antibody-coated beads to protein samples (Use 200 µg for samples to run in duplicate on the AMPK assay; use 300 µg for samples to run in triplicate).
2. Mix for 2 hours on roller at 4ºC (cold room).
3. During this time, label the required number of 1.5 ml eppendorf tubes as 1A, 1B, 1C, 2A, 2B, 2C etc.
4. Once the samples have mixed, centrifuge for 5 minutes at 3000 g 4˚C.
5. Wash mixture once with 3 ml of ice-cold NaIP buffer as follows:
   a. With an automatic pipette add 3 ml of NaIP buffer to tube. Vortex well.
   b. Centrifuge for 2 minutes at 3000 g, at 4ºC.
   c. Aspirate the supernatant (buffer) and discard.
6. Wash the mixture once with 2 ml of ice-cold HB buffer (as in step 5).
7. Re-suspend final pellet in 300 µl of ice-cold HB buffer (200 µl if duplicates only).
8. Mix well using a 100 µl pipette and divide into the labelled tubes 1A, 1B, 1C etc.
9. Centrifuge for 2 minutes at 13000 g, at 4ºC.
10. Remove 80µl of the supernatant and use the remaining 20 µl of sample for AMPK assay (or freeze at -20˚C for up to two weeks).

Master Mixes

Materials

- γ^{32}P ATP (Perkin-Elmer, # NEG002A250UC)
- cold ATP 100 mM
- $MgCl_2$ 1 M
- AMP 1 mM (10^{-3})
- SAMS peptide 100 µM (10^{-4}) (Upstate, #12-355)
- Hepes-Brij (HB) buffer

Use HB buffer as diluent for all solutions

Working solution (1ml)

10 µl γ^{32}P ATP 10 mCi/ml (specific activity 250-500 cpm/pmol)

10 µl cold ATP 100 mM

25 µl $MgCl_2$ 1 M

955 µl HB buffer

Positive Master Mix

10 µl/sample Working solution (containing γ^{32}P ATP)

10 µl/sample AMP 1mM

10 µl/sample SAMS peptide 100µM

Negative Master Mix

10 µl/sample Working solution (containing γ^{32}P ATP)

10 µl/sample AMP 1mM

10 µl/sample 10 µl HB buffer

1. Calculate the total volume of each master mix required, at 30 µl/sample. Make excess to allow for pipetting error.
2. Prepare Master Mixes in labelled 15 ml falcon tubes, vortex at each stage of mixing. Aliquot into 1.5 ml eppendorf tubes (label with start date of γ^{32}P ATP) and store at -20ºC in the radioactive freezer.
3. Change gloves and dispose.

AMPK Assay

Materials

- Positive and negative working solutions
- P81 phosphocellulose paper squares (Millipore, #20-134)
- 1% (v/v) orthophosphoric acid (11.76 ml of 85% orthophosphoric acid in 1L water)
- Optiscint Hisafe scintillation cocktail (Perkin-Elmer, #1200-434)
- 8ml Midi-vial for scintillation counter (Perkin-Elmer, #6000288)

1. Turn on 30˚C shaker and radioactive room oven. Defrost and vortex Master Mixes.
2. Remove 20 µl immunoprecipitate samples from freezer at the last minute. Keep samples on ice at all times.
3. Ensure sample order is varied between treatment groups and controls. E.g. for an experiment with controls numbered 1, 2, and 3 and treatment samples numbered 4, 5, and 6, run: 1, 4, 2, 5, 3, 6 etc.
4. Add 30µl Positive Master Mix to the 20 µl frozen samples A and B. This initiates the reaction. Add 30 µl of Negative Master Mix to negative controls, labelled C
5. When Master mix added to all samples, transfer into 30ºC shaker. Secure rack to shaker and incubate for 20 minutes at 350 rpm. Change gloves.
6. Lay out P81 phosphocellulose paper squares on paper labelled with sample order, two or three squares per sample depending on whether control included. Cut corners to identify 6 and 9, 68 and 89, 66 and 99 or 69 and 96.
7. Put 5 µl Positive Master Mix and 5 µl Negative Master Mix onto the final 2 paper squares respectively (for the total count) and place on foil square. Keep at room temperature behind a radioactive screen – do not wash with acid or water.
8. Prepare 1% othophosphoric acid.
9. Remove the samples from shaker and put on ice. Take first sample and mix the contents with a pipette 4-8 times. Spot 35µl of reaction fluid onto the appropriate paper square. After liquid has soaked in (1-2 seconds), drop square into a plastic box containing orthophosphoric acid to stop the reaction.
10. Repeat for all the samples, maintaining correct order.
11. Stir squares gently with a magnetic stirrer for 5 minutes. Drain solution into a designated radioactive sink (pour into a beaker to avoid losing squares).
12. Perform a second wash with 1% acid and a third wash with distilled water.

13. Using forceps, transfer the squares onto paper behind a radioactive screen. Place in the oven at 70ºC and warm for 15 minutes until dry. Change gloves.
14. Immerse dry squares in 5ml of Optiscint Hisafe scintillation fluid in 8ml plastic vials. Place the two Positive and Negative Master Mix squares in two additional tubes and mark with a star (do not reuse). Place tubes into racks and proceed to counting. *Scintillation tubes can be reused if CPM<80.*
15. Procedure for counting using the Wallac scintillation counter:
    - To start counting: Click Protocol >> Easy count >> Start
    - To view latest results: Click Results >> Latest results
    - Once complete, to export results file: Click File >> Export (to floppy disk).
16. Clean work area with 'Decon' and distilled water. Monitor using the Geiger counter and record in monitoring sheets in records folder.
17. Record ATP use and disposal on Isostock.

Calculations:

1. AMPK activity is expressed as nanomoles of phosphate incorporated into substrate peptide per minute per milligram protein.
2. Calculate the **actual CPM** for each sample by subtracting from each sample count its negative control, e.g. 1A – 1C, 1B – 1C, etc. In assays where only 3 negative controls are used, these are averaged and their mean is used for all samples.
3. Calculate **nmol of ATP incorporated per min,** knowing that the CPM obtained by 5µl of Positive Master Mix = 1.66 µl of ATP, i.e. **Actual CPM** × 1.66/CPM Positive Master Mix.
4. Adjust for the quantity of protein used: the **nmol of ATP incorporated per min** are divided by (the quantity of protein used at the initial step of immunoprecipitation / 2 or 3, depending on whether duplicates or triplicate were used), then multiplied by 0.7, as only 35 µl of the 50 µl reaction fluid is spotted onto the square (35/50 = 0.7). i.e. **nmol of ATP incorporated per min** / (300/3) x 0.7
5. Multiply the result by 1000 to reflect per **mg** of protein.
6. Calculate the mean of the two positive aliquots of each sample.

Western Blotting Protocol

Materials

- NuPage gradient (4-12%) bis-tris gel (Invitrogen,12 well = #NP0322 BOX)
- Laemmli loading buffer (Sigma, #S3401-1VL)
- Hi-mark, pre-stained, high molecular weight ladder (Invitrogen, #LC5699)
- MES running buffer (Invitrogen, NP0002-02)
- Primary antibody of interest
- Appropriately raised secondary antibody 800CW (Li-Cor, #926-32211)

Transfer buffer

- Tris-base 30.4 g + glycine 14.14 g, disolve in 800ml off ddH_2O
- Add methanol 200 ml (replace with ddH_2O for large proteins)
- Stir well with magnetic stirrer

Phosphate buffered saline (PBS)

- Dissolve 5 tablets (Sigma P4417) in IL ddH_20

Blocking buffer

- 5% milk = 2.5 g of non-fat milk powder (Tesco) in 50 ml 1x PBS-Tween

Western Blotting Protocol for tAMPK and pAMPK

1. Prepare 15-40 μg of protein solution. Run two equally loaded gels, one for tAMPK and one for pAMPK
2. Pipette protein into 0.5 ml Eppendorfs with Laemmli loading buffer (Sigma, S3401-1VL). Max loading in a 10 lane gel is 25 μl/well but try not to go much over 20 μl. Therefore, use the weakest of samples to calculate the maximum quantity of loading buffer that can be used. Load at least 5 μl but try for 10 μl. Then add that amount of loading buffer to each sample. Consider putting loading buffer in empty lanes to keep gel running straight. Centrifuge 10 seconds if solution not at bottom.
3. Pipette 10 μl prestained broad range marker (New England Biolabs, P7708) into each of two 0.5 ml Eppendorfs. These do not require loading buffer
4. Denature Eppendorf contents at 95°C for 5-6 minutes, then put on ice. Centrifuge 10 seconds
5. Invitrogen NuPage 10% bis-tris gel (10 well = NP0306BOX); remove white strip and place in tank with highest bit of plastic on the outer side. Gently remove the comb. Fill tank centre until it overflows to halfway up the outer tank with MES running buffer (Stored as x 20, dilute 25 μl in 500ml ddH_20) (Invitrogen, NP0002-02).
6. Pipette in protein and marker. Marker on left.
7. Run at 95v until marker begins to separate (~15 mins); then run at 125v until protein crosses bottom of gel (check at 1hr, and then every 15 mins). Appx 1 hr 20 mins.
8. Cut 1 piece of nitrocellulose membrane (Whatman protran, 0.45 μm, 10-401-196) and 2 pieces of blotting paper (BioRad fiber pads 9.5 x 15.2x6mm, 1704086) per gel to size. Soak Membrane in water and blotting papers in transfer buffer (stored as x5 – 20ml transfer buffer [Glycine 70 g + Tris base 15 g in 1L ddH_20], 20 ml methanol, 60 ml water) for the final 10 minutes of running.
9. Open transfer machine and lay one piece of blotting paper down and place a membrane on top.
10. Remove line of gel from bottom with spatula and then open gel with spatula, keeping larger plastic side on bottom. Trim gel top.
11. Pick up blotting paper and membrane and invert onto gel. Flip other way up and using scalpel, gradually lever corner away from plastic so that gel lies on membrane.
12. Use scalpel to lift edges gently to remove air bubbles
13. Trim excess gel from bottom then place on transfer machine with marker on left.

14. Cover with second dampened blotting paper, roll.
15. Transfer at 0.4 A for one gel and 0.8 A for two gels for 40 mins – 1hr
16. Block with 40 ml 5% milk (2.5 g of non-fat milk powder in 50 ml 1xPBS Tween) for 90 mins at room temperature on a roller.
17. Wash 4 times with washing buffer (PBS + Tween), twice fast, twice 10 mins.
18. Add primary antibody to 5 ml 5% milk: 5 μl (1:1000) tAMPK (Cell Signaling, 2603) or 5 μl (1:1000) pAMPK (Cell Signaling, 4188).
19. Add GAPDH antibody to each of these tubes (5 μl in 10ml 5% milk = 1:2000) (Santa Cruz, FL-335). Roll in cold room overnight
20. Wash 4 times with washing buffer, twice fast, twice 10 mins.
21. Add secondary antibody: 0.5 μl anti-rabbit (800) in 5 ml 5% milk = 1:10,000 to each tube. Cover with foil. Roll for 90 mins at room temperature.
22. Wash 4 times with washing buffer, twice fast, twice 10mins.
23. Read on LiCOR system.
24. Store in 1x PBS without Tween in fridge.
25. Correct each protein for GAPDH (i.e. pAMPK/GAPDH) then present as pAMPK/tAMPK

Western modification for large proteins (pACC)

1. Consider running two separate gels to measure pACC on one and GAPDH on the other
2. Run at 100v until marker begins to separate (~15 mins); then run at 150v until protein crosses bottom of gel, and then 15 mins more. If this is taking some time, consider placing in ice box to prevent gel from overheating.
3. Primary antibody: 10ul pACC (Upstate 1 μg/ul polyclonal anti-rabbit, 07-303) in 5 ml 5% milk.
4. If results still not clear, consider using a looser gel

Isolation of rat neonatal cardiomyocytes

Courtesy of S Brouliette, Department of Translational Medicine and Therapeutics

Equipment

ADS buffer x10

- NaCl 68.0 g
- HEPES 47.6 g
- NaH2PO4 1.38 g
- Glucose 6.0 g
- KCl 2.05 g
- If red ADS needed – phenol red 0.02%
- Make up to 1L
- Adjust pH to 7.4
- To make up x1, dilute in ddH_2O and recheck pH is 7.4

Complete Neonatal Rat Medium (NRM)

- Take fresh bottle of DMEM (high glucose)
- Remove 100ml
- Add 100ml of 199 medium (HEPES modification)
- Add 60ml Horse Serum (heat inactivated)
- Add 27.5ml FBS (heat inactivated)
- 13.5ml 1M HEPES
- 6ml PS
- 6ml L-glutamine

Enzyme Solution

- Dissolve 24mg type II collagenase, CLS2 (Worthington) in 49ml ADS. Add 1ml of 30mg/ml pancreatin (Sigma 4x NF). Filter and place on ice.

Ice box, petri dish, 50ml bottle,

Procedure

- Anaesthetise neonates by inhalation of isoflourance (Vedco, St Joseph, MO 64507)
- Kill neonates with cervical dislocation and spray with 70% ethanol.
- Transfer to flow cabinet, remove hearts and place in petri dish of ADS on ice.
- Trim away atria, put hearts into a second petri dish, then cut heart 3-4 times to introduce ADS.
- Place hearts in 50ml tube.
- Wash hearts twice with cold ADS buffer,
- Transfer to petri dish and cut into small cubes using micro-scissors and forceps
- Transfer tissue pieces to 75cm^2 flask, incline flask and aspirate ADS buffer with wet 25 ml pipette
- Add 10 ml enzyme solution. Place flask in shaking water bath at 37C at 75 rpm for 5min
- Triturate the solution by 25 ml pipette - avoid bubbles
- Take off and discard supernatant (blood and debris)
- Add 10ml fresh enzyme, return to shaking water bath for 20 minutes (see table below)
- Meanwhile, aliquot 5 x 2 ml of FBS into 50ml tubes and keep at 37°C
- Remove flask, transfer supernatant using a 10 ml pipette to a 50 ml tube containing 2 ml of FBS – this is collection 1
- Add 8ml enzyme and return flask to shaking water bath (Step2)
- Spin collection 1 at 800rpm for 5 minutes, remove supernatant to waste, re-suspend cells in 4ml warm FBS and store in incubator with lid open to allow gas exchange - label this tube “Cells”
- Repeat for each collection – adding each batch of cells to the tube labelled “Cells” in 4ml FBS each time.

	Step1	*Step2*	*Step3*	*Step4*	*Step5*
Enzyme	10ml	8ml	8ml	6ml	6ml
Time	20mins	25mins	25mins	15mins	20mins

Pre-plating

- Spin tube of cells, 800 rpm x 5minutes. Aspirate serum. Re-suspend cells in 25 ml NRM.
- Dispense cells through 100 μm sieve into 5 Primaria (BD Biosciences, 353803) petri dishes (good for fibroblast attachment). Rock dishes side to side to distribute the cells

evenly.

- Culture for 45 - 60 minutes.
- Remove plates from incubator. Bang once to dislodge cardiac myocytes.
- With medium, rinse the dishes and collect the cells to 50ml tube.
- Repeat if needed.
- Count and seed.

Adjust cell density

96 well plate	100 μl	4×10^4 cells/well
6 well plate	1.5 ml	6×10^5 cells/well
24 well plate	400 μl	1.6×10^5 cells/well
2 well chamber	1 ml	4×10^5 cells/well
8 well chamber	250-300 μl	$1\text{-}1.2 \times 10^5$ cells/well
4 well chamber	300 μl	1.2×10^5 cells/well

Neonatal rat cardiomyocytes treatment

1. Prep and plate neonatal cardiomyocytes in 6 well plates (density 6 x 10^5 cells/well). Thirty pups provide enough cardiomyocytes for 10 x 6 well plates
2. Re-feed day 2; treatment day 3
3. Do not starve cells as this activates AMPK
4. Remove media and wash with 1 ml warmed PBS
5. Dilute drug in warmed NRM so that 2 ml contains appropriate concentration of drug
6. Incubate for desired time
7. Lyse cells with 450 μl *cell lysis buffer* (and protease inhibitor tablet) for 30 minutes in cold room (4°C).
8. Proceed to BCA assay

Drug calculations:

AICAR (MW 258.2)

Drug in vial 50mg

258.2mg/ml = 1M

50/258.2 in 194μl = 1M

50mg in 1.94ml = 0.1M stock solution (x100 Rx dose)

Add 20μl of 0.1M stock solution to each well containing 2ml = **1mM conc**

hGH

1.33mg in 4 IU vial

Dissolve in 1.33ml water = 1mg/ml

Store as 8 x 100μl aliquots + 10 x 50μl aliquots of stock solution

Dilute one aliquot 1/10 with media (to buffer any pH change) = 0.1μg/μl

Add 20μl of 0.1μg/μl solution to each well containing 2ml = **1μg/ml**

hIGF-I

Drug in vial 50μg

Stock solution: 0.1μg/μl, therefore add 500μl diluent

Store as 10 x 50μl aliquots each containing 5μg drug

Dilute 1 aliquot in 450μl = 0.01μg/μl = 10ng/μl

Add 15μl of 10ng/μl to each well containing 2 ml = 150ng in 2ml = **75ng/ml**