UNIVERSITY OF ULSTER

Human Pancreatic Beta Cell Lines Culture Protocols

Thawing Human Cell Lines for culture:

- Locate vials in liquid nitrogen and quickly remove with forceps.
- Take vial to sterile flow hood, gently screw top off slightly to release any pressure. Close top again tightly.
- Quickly transfer to water bath at 37^{o}C, and gently agitate vial to thaw cells making sure no water gets into the lid.
- When thawed transfer vial back to sterile flow hood and empty content into 10mls of pre-warmed RPMI-1640 medium with L-Glutamine (Gibco, cat no. 21875) supplemented with 10% foetal bovine serum (v/v) (Gibco, cat no. 10106-151) and 100U/ml penicillin and 0.1g/l streptomycin antibiotics (Gibco, cat no.15070063), swirl gently to mix.
- Centrifuge vial at 900rpm for 5min to pellet cells. Pour off supernatant (to wash off DMSO from the freezing medium prior to culture).
- Resuspend cells in 10mls of RPMI 1640 (with P/S and FBS as above) by gentle agitation prior to transfer to small vented tissue culture flask (approx. 25cm^2).
- Return flask to the incubator at 37^{o}C for overnight culture.
- Replace media the following day.
- Once small flask is confluent (2-4 days after thawing dependent on the human cell line) transfer cells to a medium tissue culture flask (approx. 75cm^2)
- 1.1B4, 1.1E7 and 1.4E7 all have different growth rates on initial culture from frozen stocks and may take up to a further 7 days to become confluent in a medium flask. Once confluent cells are transferred to large tissue culture flask for routine culture (approx.175cm^2, we use Cellstar flasks from Greiner Bio-One GmBH but have found no differences in culture in any standard non-coated flasks)

Routine culture and passage of Human Cell Lines:

- All HCL's are routinely cultured in RPMI 1640 culture medium with L-Glutamine (Gibco, cat no. 21875) supplemented with 10% foetal bovine serum (v/v) (Gibco, cat no. 10106-151) and 100U/ml penicillin and 0.1g/l streptomycin antibiotics (Gibco, cat no.15070063).
- Culture medium is made under sterile conditions in a tissue culture hood and stored at $4^{o}C$.
- To passage confluent cells in sterile flow hood, open tissue culture flask and tip off culture medium into a beaker.
- Wash cells twice with 10ml of pre-warmed Hanks Balanced Saline Solution, HBSS (Gibco, cat no. 14185045. Bought in as concentrated stock and diluted and autoclaved as per suppliers instructions. Once autoclaved diluted HBSS stocks are kept at room temperature).
- Pour off any excess HBSS and add 10ml trypsin (Gibco, cat no. 15400054. Bought in as x10 stock and diluted with autoclaved HBSS. Once diluted trypsin can be stored at $4^{o}C$).
- Gently swirl trypsin around flask and pour off excess. Return to $37^{o}C$ and monitor detachment of cells at intervals by an inverted microscope, usually takes approx. 10min for full detachment.
- Return flask to sterile hood and add 10ml of RPMI 1640 medium, pipetting up and down to dislodge cells.
- Place supernatant with dislodged cells into sterile universal tube and spin at 900rpm for 5 min.
- Return to hood, pour off supernatant and resuspend cells in 10ml RPMI 1640. Transfer appropriate amount of resuspended cells to a new tissue culture flask (routinely use cell star flasks from Greiner bio-one, Germany) and add fresh pre-warmed medium.
- Return to incubator at $37^{o}C$ (5% CO_2, 95% air).

Freezing down Human Cell Lines

Freezing Media

10% DMSO (Dimethylsulphoxide) (Sigma Chemicals Ltd)
40% Foetal Calf Serum (FBS – Gibco, as previous)
50% RPMI 1640 with L-Gluatmine (Gibco, as previous) – supplemented with Pen/Strep and FBS (as is used for routine culture)

- make under aseptic conditions in sterile universal tubes
- store at –20^{o}C until use (thaw in water bath to 37^{o}C before use)

To freeze down cells:

- Tip out media
- Wash with HBSS, trypsinise as described previously.
- Resuspend in RPMI 1640 culture medium (with P/S and FBS), spin down and resuspend again in 10ml of culture medium, perform cell count.
- Spin down and resuspend in appropriate amount of freezing medium. (As a guide 1 large confluent flask of the human cell lines can usually be distributed amongst 7-10 vials usually at a concentration of 1.5 – 2 x 10^{6} cells per vial).
- Use routine cryo vials – should be sterile and taken out of packaging in the hood. Write the cell line, date, cell concentration and initials clearly with permanent pen on each vial.
- Place approx. 1ml of cell suspension in freezing medium into each vial.
- Close lid (usually screw close) and place vials at –20^{o}C for 6-8 hours and then transfer to –70^{o}C or –80^{o}C overnight. Cells can then be placed in liquid nitrogen the following day and their location noted until needed.

Acute Insulin Secretion Test Protocol

- Approximately 24 hours prior to performing the insulin release studies, harvest cells from routine culture
- Seed 250,000 cells per well/ml media into 24-well plates (Iwaki, non-coated)
- Return the 24 well plates to the incubator for overnight culture
- Prior to the acute tests (insulin release studies) remove media by inverting the 24 well plate onto a tray, then gently press inverted plate onto clean tissue to remove any excess liquid and incubate cells in each well with 1ml of KRBB containing 1.1mM glucose for 40 minutes at 37^{o}C.
- Remove preincubation reagent by inverting plate as before and add pre-warmed acute test samples to each well, made up KRBB, for 20 minutes at 37^{o}C (1ml/well).
- After 20 minutes take plates from the incubator and carefully remove the 1ml of supernatant from each well and place into pre-labelled LP3 tubes (Sarstedt). Samples can then be stored immediately at -20^{o}C until further analysis.
- Note – the human cell lines secrete small amounts of insulin as compared to primary islets or even clonal rodent cell lines; thus very sensitive assay technique is required to measure insulin release.

(Presently we are using D+ Glucose from BDH (Product Code: 101174Y) for our acute test studies on the Human Cell Lines).

Human Insulin Standard Preparation

- Preparation of the insulin standard is performed on the day of the RIA, and standards made up in triplicate
- Human Insulin (Sigma – I2643) is made up as a 1mg/ml stock in 2mM HCL.
- The stock buffer is then diluted 1:100 in KRBB buffer to give a final concentration of 10ng/ml
- A range of standards are then made from this starting concentration – 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039, 0.019, 0.009 ng/ml, all dilutions are made in KRBB buffer (as in acute test).

- 200µl aliquots of the standards are processed for Insulin radioimmunoassay as for experimental samples and a standard curve generated after measurement on the 1261 Multigamma Counter (Wallac, Finland).
- Unknown samples for the Human Cell Line glucose-stimulated insulin release studies tend to fall between 0.02ng/ml (0mM) and 0.07ng/ml (16.7mM) on the standard curve (with higher values seen when in combination with other secretagogues).
- It is important that human insulin standards are made up in same type of buffer as the unknown samples and that the volume of each added in the assay is the same.

RIA

- On the day of the Radioimmunoassay, thaw the LP3 tubes containing acute test samples on a cold tray and withdraw 200µl aliquots from each in duplicate. Antibody, I^{125} label and dextran-coated charcoal are all subsequently added and samples processed for Insulin immunoassay over the following 3 days. Sensitivity of RIA can be enhanced by 24h delay in adding tracer followed by 48h to reach equilibrium.

Insulin content

- Samples for insulin content are taken from wells of confluent cells plated at the same time as for the acute test (250,000 cells per well in a 24 well plate). After overnight incubation cells are lysed in each well in 1ml of acid-ethanol solution with vigorous pipetting. Acid ethanol samples containing lysed cells and cellular insulin are centrifuged and the supernatant then stored at -20^{o}C. Samples are processed for RIA in the same way as the acute test samples but aliquots are diluted 1:10 in RIA working buffer (see attached appendix) – i.e. 20µl of each sample is added to 180µl of RIA working buffer, to give a 200µl sample. Again these are processed in duplicate.

Appendix

Recipe for KRBB, used in insulin release assay for HCLs

A.

KRB salts mix

	Final Conc (in 100ml)	MW	mg/100ml media	For x10 conc in 100ml
NaCl	115mM	58.44	672.1mg	6.721g
KCl	4.7mM	74.56	35.04mg	350.4mg
$CaCl.6H_2O$	1.28mM	219.08	28.042mg	280.42mg
KH_2PO_4	1.2mM	136.09	16.33mg	163.3mg
$MgSO_4.7H_2O$	1.2mM	246.48	29.578mg	295.78mg

Prepare 100ml of above and store on 10ml aliquots at -20^{o}C until use – keeps for approximately 4 weeks.

B.

$NaHCO_3$ (MW 84.01), final concentration in KRBB is 24mM. Add 201mg /100ml buffer on day of KRBB preparation.

C.

Hepes (MW 238.3) – make up 23.83% w/v, store 1ml aliquots at -20^{o}C until use. To make 25 ml of stock Hepes weigh 5.957g and add 25ml distilled water to give 25 x 1ml aliquots.

To make up KRBB:

- Thaw 10ml stock tube KRB salts mix, 1 tube Hepes.
- Add distilled water to 95ml and add 201mg of $NaHCO_3$, place on stirrer to mix well
- Add bovine serum albumin (BSA) to give final concentration of 0.1%; this prevents loss of insulin secreted by adsorption onto plastics
- pH with 1M NaOH to 7.4
- Make up to 100ml with distilled water

Working RIA Buffer

- Prepare working RIA buffer by dissolving bovine serum albumin (BSA) 0.5% (w/v) in 40mM sodium phosphate buffer pH 7.4.

*** If RIA is not available, detection has been obtained using the Ultra Sensitive Kit by Mercodia and by extending the assay range with lower concentrations to obtain optimal insulin secretion and detection.

*** Configuration as pseudoislets has also been shown to enhance functionality (Guo-Park et.al., 2012)