

Fetal Liver Cultures

Set Up:

3 X 10cm dishes filled with 1X sterile DPBS

2X 6well TC dish filled with 1X sterile DPBS (per pregnant female): to wash embryos.

2X 6well TC dish with 5mls complete supplemented RPMI, with a cell strainer

placed in each well (per pregnant female): to break up fetal liver and resuspend in media.

3 X 50ml glass jars full of 100% EtOH, DPBS or H2O

Dissection table

Dissection tubes

1.5 ml eppendorf tubes filled with 500 μ l tail lysis buffer and 10 μ l proteanase K. (2 tubes per embryo = ~16 tubes per pregnant female... assuming 8 pups)

3 X 12 well TC plates seeded with T220 cells (NIH3T3 cells that express IL-7) (per pregnant female. You will want 2-4 wells per embryo, depending on assays to be done.)

** Be sure to be trained by Kevin Mills on how to properly follow this protocol. There are techniques that cannot be fully explained in this protocol.

Done on Benchtop

Harvest Embryos from E13 to E14 pregnant females.

Remove embryos and keep them in embryonic sac, still attached to each other.

Place in 15 mls of room temp sterile 1X PBS in a 10cm TC dish, transfer to hood.

Using sterile techniques from this point on:

Transfer embryos (still together) into a fresh 15 mls of room temp sterile 1X PBS in a 10cm TC dish

Remove embryos from each other, but do your best to keep them in their embryonic sacs and place each embryo into 5mls of 1X PBS (in one well of a 6 well TC dish)

Break embryos from sac and drop into a fresh well of DPBS in 1 well of a 6well TC dish. Rinse by swishing gently with tweezers

Remove from DPBS and place on a clean area of the lid of the 6well dish used for washing.

Remove red clump of cells on the chest region of the embryo (this is the fetal liver)

****do not take any of the white tissue as these are embryonic fibroblasts and will contaminate your fetal liver culture.**

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Place this clump of fetal liver cells into the cell strainer that has been placed into one well of a 6well TC dish containing 5mls of RPMI. Be sure that the cell clump is submerged in media while in the cell strainer.

Using a sterilized (EtOH'd, flamed and cooled, then rinsed in sterile DPBS) glass rod, gently massage the cell clump through the filter and into the media. Discard the cell strainer. Now you have fetal liver lymphocytes resuspended in ~5mls of RPMI.

Remove two one mm size pieces of tissue from the remaining embryo and place onto two tubes of tail lysis buffer + PK. Now you have two separate samples for DNA prep and genotyping from each embryo. This is very useful in determining and confirming genotyping...you'll see.

Perform a cell count (#cells/ml) on the resuspended cells in each well (each embryo) using a hemocytometer.

Calculate volume needed from each embryonic cell sample well in order to obtain 2×10^6 cells per ml and transfer this volume to one well of a 12 well TC dish plated with 1ml T220 cells. If necessary (if you have very few cells to work with) you can use as little as 1×10^6 cells per well of the T220 seeded plate.

****When adding fetal liver cells to T220 cell cultures, DO NOT remove the media from the T220 culture. This media will supplement the fetal liver media with constituents needed for development and survival.**

Add 1×10^6 to 2×10^6 cells per well of a 12 well TC dish plated with T220 cells. Use desired number of wells per sample. Usually 3-4 wells are used per sample.

Bring the volume in each well up to 4mls total (remember to consider the 1ml already in each well from the T220 cultures).

The cells should be fine for up to 4 days before they need to be fed.

To feed cells use a media derived on 50% Fresh supplemented RPMI plus 50% T220 conditioned media.

T220 conditioned media is normal Supplemented RPMI that was used to grow T220 cultures, and then removed after 1 to 2 days of T220 culture. Thus, you have RPMI with all of the proteins and cytokines (including IL-7) expressed by the T220 cells.

To feed the cells you gently, using a disposable glass Pasteur pipette, pull off the old media by gently aspirating the top layer of media until the volume is reduced to ~500 μ l.

Add 3mls of 50%RPMI/50%T220Conditioned media to each well to feed.

This will need to be done as the culture loses its pink color and begins to turn orange. The cultures should be fed often enough so that they won't turn yellow. Yellow (acidic) media will kill the cells. Thus this will need to be done every day following 6 days of

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culture. The cells begin expanding rapidly and thus require continuously fresh media to support their growth. You will see that it can turn yellow very quickly!

Collect cells from well as necessary depending on assays performed.

To confirm that the cells grew properly, and BEFORE you can use them for data collection, you must be sure that the wild types developed to B cells.

To test for this you must utilize FACS Calibur.

Remove approximately 2 aliquots of 1×10^6 cells from each sample and place into two facs tubes. Spin this volume down to $\sim 50\mu\text{l}$. Will Schott or Ted Duffy can teach you this technique.

Stain one of these $50\mu\text{l}$ aliquots with $10\mu\text{l}$ of a B cell cocktail containing antibody-fluorophore ; B220-PE-Cy7 (1:20), CD43-PE (1:60), CD19-FITC(1:80), and IgM- APC (1:20).

Stain the second aliquot with $10\mu\text{l}$ of a T cell cocktail containing antibody-fluorophore; CD8-FITC (1:100), CD3-PE (1:20), and CD4-APC (1:100). See Will Schott to help with antibody cocktail preparations.

Keep the cells in the dark and incubate with antibody 30min at 4°C .

Wash with $200\mu\text{l}$ RPMI (or any other appropriate Facs buffer) and spin for 5 min at 1000rpm

Pour out supernatant firmly and resuspend the remaining $\sim 50\mu\text{l}$.

The cells are now ready to be analyzed by flow cytometry. See Kevin Mills or Will Shott.

Will Schott will usually be able to do the staining for you if you bring him the tubes containing the cells and give him plenty of notice.