IMMUNOLOGY-RELATED PROTOCOLS

I1: Guidelines for the Culture of T Cell Lines and Clones

We work with two types of antigen specific T cell: either T cell hybrids, which are created by fusion of lymph node cells from antigen immunized mice to the thymoma BW5147, or T cell lines and clones which are established from the lymph node cells of antigen immunized mice. Whereas T cell hybrids proliferate continuously, and are thus available on a daily basis, T cell lines and clones are propagated by stimulation every 14 days with antigen, APC and IL-2 and are only available at the end of the 14-day cycle. Each has its advantages, but only T cell lines and clones can be used in adoptive transfer experiments.

The key to efficient cell culture is to plan ahead and prepare all of the reagents and components needed ahead of time. This can be done the afternoon before in the case of inert material such as 96 well plates that need to be labeled and formatted for proliferation assays and flasks that need to be labeled for restimulations. The labile components such as media and antigens should be prepared the morning the experiment is to be performed.

Minimize the length of time T cells are out of the incubator. It is best to avoid prolonged exposure to room temperature and levels of CO2 that are inadequate to maintain the pH of the DMEM. I suggest you follow the recommendations below and break the restimulation and testing into separate tasks. Do not take a significant break between the time you remove the old flasks from the incubator until you have placed the new flasks with the appropriate number of T cells (generally 5.0×10^5) for restimulation back into an isolator box and in the incubator and the cells for the proliferation assay on ice.

Prepare all of the BSS, DMEM/FBS/EL-4SN and other media you will need, such as non EL-4 supplemented DMEM prior to removing the T cells from the incubator. Prepare the flasks needed for restimulation by writing the T cell designation, date, antigen and concentration on the new flasks. Tape these flasks together in the order that the old flasks have been set up. In addition, label a 15 ml centrifuge tube for each T cell line or clone for transfer of the cells required for a proliferation assay Another thing to consider is the stability of peptide antigens it is best to minimize their contact with warm temperatures.

Thaw peptides solutions in a beaker of warm water as soon as you take it out of the freezer. Prepare the antigens you need for the experiments prior to removing the cells from the incubator.

Once you have removed the T cells from the incubator work quickly, resuspend the T cells and take an aliquot for counting and count the samples immediately. After finishing the counts, determine the volume you will need for testing and for restimulation. Unless you require more than 2 ml of cell suspension to get enough T cells for restimulation, there is no need to pellet the cells. Therefore you can add the required volume of cell suspension to the pre-labeled flask for that T cell line or clone. Use the same pipette to transfer the number of cells you need for the proliferation assay to the pre-labeled 15 ml centrifuge tube.

When you have transferred the T cells to the new flasks, place both the new flasks and the old flasks back in the isolator box, gas, and place back into the incubator.

The tubes with cells to be used for the proliferation assay should be pelleted, the medium aspirated and placed on ice until you are ready for them. NOW YOU MAY TAKE A BREAK IF YOU WANT.

It is now time to prepare the spleen cells. I recommend that everyone wait until all T cell lines and clones to be restimulated are in the flasks. That way the spleen cells are added when they are as fresh as possible. Estimate the number of spleen cells of each strain required that day and sacrifice the mice and take the spleens. I recommend that if more than one strain of mouse is needed, each strain be done by a different worker. Take both strains at the same time and process the spleens and irradiate the cells promptly. As soon as they are back from the irradiator they should be added to the waiting flasks into which the T cells have been previously placed. The cells required for the proliferation assays should be placed on ice until use.

I2: Lymph Node Proliferation Assays and Establishment of Antigen Specific T Cell Lines

Immunization

Materials

CFA (Freunds Complete Adjuvant)
0.14 M NaCl
1M HCl
1M NaOH
2 x 1.0 ml Hamilton syringes
26 - 30 Ga. ½-inch needle

Antigens to be emulsified in CFA should be dissolved or dialyzed against 0.14 M NaCl and the pH adjusted to neutrality by either HCl of NaOH as required. Antigens are emulsified by drawing equal volumes of the antigen and CFA into 2 1.0 ml Hamilton syringes and mixing by pushing back and forth through a connecting needle. The antigen is properly emulsified when it becomes more viscous and when a drop placed on the surface of a beaker of cold water does not leave an oil slick. Immunization doses vary but typically range from 10 - 100 µg in 50µl CFA. Mice are immunized on the underside of the tail by inserting a 26 - 30 Ga. 1/2 inch needle approximately 1/2 in from the base of the tail and insetting it full length.

Harvest of lymph nodes

The inguinal and peri-aortic lymph nodes are primed by this route and are harvested into a tube of sterile BSS. It is best to work quickly and sacrifice only 2 mice at a time. Once the lymph nodes are collected they are disrupted by use of a ground glass homogenizer and processed as follows:

Materials

15 ml tubes
Clicks' medium
BSS

Procedure

1. Spin at low speed to pellet debris by bring rotor up to 500 rpm and turning off the centrifuge

2. Transfer cell suspension to a fresh 15 ml tube, leaving pelleted debris behind

3. Spin cell suspension 1000 rpm 5 min. and wash by resuspension in 5 ml Clicks' medium and pelleting @ 1000 rpm for 5 min. Repeat wash and resuspend in 5 ml Clicks

4. Take a 0.1 ml aliquot and dilute with 0.9 ml BSS and count on hemocytometer.

Proliferation assays

Materials

Clicks' medium
0.5% NMS (normal mouse serum)
flat bottom 96 well plates
saline (0.14M NaCl)
BSS
1.0 µCi ^{3}H thymidine

Procedure

1. Prepare Clicks' with 0.5% NMS in advance (100 mls)

2. We generally use 1.0 X 10^{6} cells per well in flat bottom 96 well plates in 0.2 ml Clicks and the larger cultures are set up with 1.0 X 10^{7} cells in 2.0 mls, therefore either dilute or pellet and resuspend lymph node cells to 5.0 X 10^{6} cells /ml.

3. The number of replicates is usually 2 - 4 depending on cell number and other desired use of cells.

4. Antigens must be sterile and are added in 10 µl saline, the dose varies with the purpose of the experiment and availability of antigen but typically ranges from 0.05 - 5.0 µM.

5. It is a good idea to perform serial dilutions of antigens, a convenient dilution factor is 1:3.16 (31.6 µl into 68.4 µl diluent) because every 2nd dilution gives 1:10 overall, we generally set up 6 dilutions of antigen.

6. Use only the inner 60 wells of a 96 well plate and add 0.2 ml BSS to the outer wells to prevent evaporation.

7. Plates are harvested from 3 - 6 days after the cultures are set up, but usually the plates are pulsed with 1.0 µCi ^{3}H thymidine in the afternoon of day 4 and harvested the next morning.

Larger cultures for the establishment of antigen specific lines

To obtain larger cell numbers for fusions and establishment of antigen specific T cell lines 1.0 X 10^{7} cells are cultured in 2.0 ml Clicks. The appropriate concentration of antigen to be used should be established from the results of the proliferation assays. The cultures are incubated for 4 - 6 days, collected, pelleted and transferred into DMEM/10% FBS supplemented with 50 U/ml mouse IL-2 at a cell density of 2.5 - 5.0 X 10^{4} /ml. These cells are culture for a further 8 - 9 days to give a total time of 14 days at which time they can be tested by proliferation assays and used to establish line as described.

I3: Density Gradient Separation of Antigen Activated T Cells from Lymph Node Populations

Materials

BSS
FBS
0.4µ syringe filter
sterile 15 ml centrifuge tube
sterile 15 ml polystyrene centrifuge
DMEM/10% FBS/ 50u/ml IL-2
sterile plugged pasteur pipettes
5.0 ml pipettes
10 ml pipettes
75 cm flasks

Procedure

Before taking the cells out of the incubator:

1. Prepare 10.0 ml BSS/15% FBS by adding 1.5 ml FBS to 8.5 ml BSS and filtering through a 0.4µ syringe filter into a sterile 15 ml centrifuge tube;

2. Filter approx. 4.0 ml Lympholyte M into a 15 ml sterile centrifuge tube;

3. Coat a sterile 15 ml polystyrene centrifuge tube with FBS by filtering approx. 2.0 mls of FBS through a 0.4µ syringe filter and after replacing the cap rolling the tube to coat it evenly on the inside surface, taking care not to get FBS on the cap, aspirate the FBS, spin briefly in the centrifuge (bring up to 1500 rpm and shut off), aspirate the FBS that collected at the bottom of the tube;

4. Place 3.0 ml of the filtered Lympholyte into the FBS coated tube;

5. Prepare approx. 200 ml of DMEM/10% FBS/ 50u/ml. IL-2 and filter sterilize;

6. Freshly filter or prepare 200 ml of DMEM/10% FBS.

After the above steps are completed:

7. Harvest the cells from 24 well plates into a sterile 15 ml centrifuge tube by using a sterile, plugged pasteur pipette, titurating the cell suspension to resuspend as many cells as possible;

8. Pellet the cells by centrifugation for 5 min. @ 1500 rpm, aspirate off the medium and resuspend in 5.0 ml sterile BSS/15% FBS using a 5.0 ml pipette, add the remaining 5.0 ml BSS/15% FBS and mix by pipetting up and down, taking care not to draw the cells suspension into the cotton plug at the top of the pipette;

9. Draw the cell suspension into a 10 ml pipette tilt the tube with the lympholyte cushion and the pipette to approximately 45 and touch the tip of the pipette to the side of the tube approximately 1 cm above the Lympholyte, carefully layer the cell suspension over the 3.0 ml cushion of Lympholyte taking care to disturb the interface as little as possible, when all of the cell suspension has been added place the tube upright and centrifuge at 1500 rpm for 15 min. with the brake off;

10. When the centrifuge has stopped carefully remove the tube and transfer to the hood upright and place into a rack, you should see a distinct tight band of cells at the interface of the lympholyte with the BSS/FBS, aspirate off approximately 7 - 8 ml of the BSS/FBS taking care not to disturb the band of cells, collect the cells in a plugged pasteur pipette by first squeezing the bulb and placing the tip of the pipette to the level of the band of cells and slowly releasing the bulb while sweeping the interface area, it does not matter if you draw up some of the Lympholyte;

11. Transfer the cells to a fresh 15 ml centrifuge tube and fill with DMEM/10% FBS pellet by centrifugation @ 1500 rpm for 5 min. and wash twice more;

12. Resuspend the pellet in 5.0 ml DMEM/10% FBS/ 50u/ml. IL-2 and add 2.5 ml to each of two 75 cm flasks and divide the remainder of the DMEM/10% FBS/ IL-2 between the two flasks, gas and seal and place in the incubator;

Check cells daily for growth and if necessary, add additional DMEM/10% FBS/IL-2 to flasks, generally 100 ml at a time, if a flask with 200 ml of medium needs to be fed, split it into two separate flasks and bring each to a total of 200 ml.

I4: Polyethylene Glycol (PEG) Mediated T Cell Fusion

The fusion parent of choice is the TCR $\tilde{a}\tilde{b}$variant of BW5147, to this are fused T cells from a line or clone reactive to the antigen of interest. Typically the T cells are obtained by harvesting the inguinal and periaortic lymph nodes of mice primed to the antigen of interest with CFA/antigen. The lymph nodes are disrupted into cell suspensions and cultured for 4 days in Clicks medium with 0.5% NMS and then placed in IL-2 for 3 days to expand. T cell clones and lines can be successfully fused following an analogous schedule, in this case the clone or line is set up in a standard re-stimulation flask with 5.0×10^5 T cells, 2.0×10^7 irradiated NOD spleen cells in 20 ml DMEM/10% FBS with 50U/ml IL-2. The cultures are

incubated for four days at which point they are subjected to Lympholyte M separation and culture for 3 days in 50 U/ml IL-2, the cells are then counted and washed as below.

Hybrid cells are selected by culturing the fused cells in hypoxanthine/aminopterin/thymidine (HAT). The aminopterin component of HAT inhibits a key enzyme in purine and thymine biosynthesis, cells in culture can survive this inhibition due to the presence of an alternate pathway and the exogenously added hypoxanthine and thymidine, a key enzyme in this pathway is not functional in BW5147 and this cell line dies in the presence of HAT. The presence of a normal enzyme encoded in chromosomes of the normal T cell component of the hybrids allows them to survive in HAT.

Materials

200 ml of serum free DMEM @ 37
200 ml of BSS @ 37
200 ml DMEM/10% FBS @ 37
3 1 ml aliquots of 50% PEG
50 ml polypropylene tube
0.4 µ syringe filter
15 ml tube
50 ml tubes
sterile plugged pasteur pipettes
25 ml pipette
10 ml syringe

Prepare all in advance and filter

Procedure

Preparation of 50% PEG solution

1. Place PEG (MW=1500) (usually in a 50 ml polypropylene tube) in boiling water,
2. Once the PEG has melted weigh out 2 - 3 grams noting weight to second decimal,
3. Quickly add 1.0 ml / gram of serum free DMEM, mix and sterilize by filtration through a 0.4 µ syringe filter into a 15 ml tube,
4. Remove approximately 3 ml and place approximately 1 ml in each of 3 sterile 15 ml tubes.

T cell fusion

1. Count BW5147s and remove 3.0 X 10^7 viable cells, pellet in 50 ml tubes and pool into one tube, pellet and leave at room temperature

2. Count T cells to be fused and remove 2.5 X 10^7 to 5.0 X 10^7 cells, pool pellets and add to pellet of BW5147 cells, bring volume to 50 ml with DMEM/HEPES and wash, resuspend cells and repeat wash two more times

3. After the final pelleting aspirate off the DMEM and spin tube again for 5 min to get last traces of medium, aspirate carefully and get the pellet as dry as possible, during this last spin add about 1600 ml 39 – 40^{o}C tap water to a clean 2 L beaker

and tape one of the aliquots of PEG to the beaker with the PEG solution below the surface of the water

4. Hold the tube with the cells approximately horizontal and tap firmly against the lip of the hood and rotate the tube to distribute the pellet of cells over as much surface area of the conical bottom as possible. Once the pellet has been distributed, loosen the cap and rest the tube in the water. Draw the PEG solution into a sterile plugged pasteur pipette, remove the cap from the tube of cells and place it in a water filled beaker in the back of the hood. Dribble the PEG over the sheet of cells from a height of the rim of the tube over a period of approximately 45 seconds shaking the tube and gently tapping against the side of the beaker while keeping the lower part of the tube in the water, after all of the PEG has been added replace the cap and place the tube in the water bath while a 10 ml syringe is filled to the limit (approx. 15 ml) with 37 ^{o}C DMEM/HEPES;

5. 45 seconds after the PEG addition was completed begin to dilute out the PEG by adding the DMEM drop-wise and gently swirling to mix the PEG with the DMEM, after all 15 ml have been added, gently fill the tube with DMEM using a 25 ml pipette, use care to minimize the shear forces due the fragile nature of the fusion products at this time, incubate the tube at 37 ^{o}C for 10 min and pellet the cells (10min @1000 rpm), aspirate off the DMEM and loosen pellet by gently tapping on the hood surface, gently add 25 ml DMEM/HEPES, pellet, loosen pellet by tapping and resuspend in approximately 40 mls of DMEM/10% FBS

6. To minimize shear forces that might disrupt hybrids it is essential that all pipetting be done gently without trapping the tip of the pipette on the bottom of the tube. The cells should be distributed into 4 96 well plates @ 0.1 ml of cell suspension per well

7. Approximately 24 hours after the fusion add the HAT supplement to the plates by preparing 40 - 50 ml of DMEM/10% FBS with 2X the final concentration of HAT

8. If the fusion is a success, hybrid growth will be apparent at day 4 - 5 by examination with an inverted 'scope, the hybrids will be ready for transfer to flasks approximately 10 - 14 day after the fusion.

I5: Transfer, Expansion, Testing and Cloning of T Cell Hybrids

If the fusion is successful, hybrid growth will be visible by examination of the wells of the 96 well plates using the inverted microscope. If the fusion is a success, the hybrids will overgrow the wells within 10 - 14 days. The hybrids should be transferred when approximately 50% of the bottom of the well is covered with cells or when the medium begins to turn orange, whichever occurs first. In order to keep track of individual T cell lines and clones it is necessary to have a concise, consistent nomenclature system. In this laboratory T cell hybrids are designated by an alpha numerical system in which block capital letters abbreviate the strain and antigen, and numbers designate the order in which T cell hybrids were transferred to flasks from the 96 well plates. For example the current fusion used NOD mice and peptide B:9-23 of insulin so the designation NIB-1, NIB-2, NIB-3 etc., will be used. In order to ensure stability and monoclonality all T cell hybrids that are

investigated in detail are cloned by limiting dilution as described below. The system described below has been developed over a period of years to minimize the amount of labor and the potential for cross contamination microbial contamination.

Key features of T cell hybrids

1. At this stage, we have a good deal of labor, reagents and supplies invested in the cells that were fused, we want to protect our investment. Therefore, filter all media to be used for the propagation of the T cell hybrids the day they are to be used, even if you filtered it the day before.

2. One key to successful and efficient cell culture in general is to have all media and other required components ready so that you can minimize the time the cells spend at ambient temperature. Proceed briskly while the culture plates and flasks are out of the incubator and plan your work to take breaks after the manipulations are performed and the cells are back in the incubator.

3. All hybrids are initially grown in DMEM/10% FBS/HAT, they grow with a doubling time of approximately 16 - 24 hours and have to be diluted in fresh medium approximately 1:20 every 2 days.

4. When we are in the process of transferring and feeding T cell hybrids, the first thing that should be done upon arriving at the lab each day is to carefully examine the plates and flasks of hybrids to ensure that they are not overgrown or too alkaline. In addition, it is best not to transfer hybrids into cold medium therefore a bottle of DMEM/10% FBS should be removed from the refrigerator and placed in the incubator.

5. When they are in HAT medium all tests and media replenishment must be done with either HAT (for the first 3 weeks to ensure the complete death of BW5147) or HT (from 3 weeks after fusion).

6. The HAT must be diluted out by serial feeding with HT at least 1:1000 before non-supplemented DMEM can be used.

7. The most efficient way to deal with large numbers of small flasks it to tape them together, groups of 5 are convenient because the culture boxes will accommodate 10 flasks in each 3 rows.

Transfer of T cell hybrids from 96 well plates to flasks

<u>Materials</u>

DMEM/10% FBS/ HAT *prepare in advance*
sterile plugged pasteur pipettes

<u>Procedure</u>

1. Remove the plates from the box and inspect them for growth either by use of the inverted ‘scope or by holding above and looking at the bottom of the plate. Go

through all four plates and circle the wells that are ready for transfer with a lab marker.

2. After all four plates have been marked starting at plate 1 and going from left to right, top to bottom number each well that has been circled and make a note of the number of wells to be transferred, place plates back in the box and in the incubator while the flasks are prepared.

3. Take out as many 25 cm flasks as necessary to have one flask for each well to be transferred, tape the flasks together in groups of 5 label each flask with the appropriate cell line number and add 2.0 ml of freshly filtered DMEM/10% FBS/HAT to each flask, close the cap snugly.

4. Place the flasks in numerical order at the back of he hood.

5. Take the fusion plates out of the incubator and starting with plate #1, use a sterile plugged pasteur pipette to transfer the contents of each well into the appropriate flask, using a new pipette for each well.

6. When all of the wells have been transferred, place the plates back in the incubator and place the flasks into a culture box with the cap loose enough to allow gas exchange, gas the box and place in the incubator.

Testing T cell hybrids for antigen specificity

Conventional T cell lines are tested for antigen responsiveness by proliferation assays, due to the fact that T cell hybrids are essentially transformed tumor cell lines that proliferate continuously, it is not possible to use proliferation. Another consequence of stimulation of T cells is the elaboration of cytokines, among which is IL-2, one of the easiest to measure. There are two steps involved in use of cytokine production as a measure of T cell activation; first, the hybrids are cultured with antigen and antigen presenting cells (APC) overnight to allow for the stimulation of IL-2 production and the release of this into the medium; second, the culture supernate (medium) is tested for the presence of IL-2. We use a bioassay to quantitate IL-2 that is based on the availability of a cell line, CTLL that is strictly dependent on IL-2 for viability. This is performed by setting up the test wells in the top row of a 96 well flat bottom plate and culturing overnight, the next day 0.1 ml of DMEM/10% FBS/Hat or HT is added to the lower 6 rows, leaving the second row empty. Aliquots of the culture are transferred to the second and third rows and serial dilutions are performed to the bottom of the plate. Meanwhile the CTLL cells are prepared and added to the dilutions of the culture SN

> *Please note: Although any manipulation involved in the propagation of T cell hybrids must adhere to the strictest standards of cell culture to prevent microbial contamination as well as cross contamination.*

<u>Standard assays conditions</u>

2 wells for each hybrid, one with APC + antigen the other with APC only
1.0 X 10^5 T cells from the hybrid
1.0 X 10^5 lymphoma APC OR 1.0 X 10^6 spleen APCin 0.3 ml of DMEM/10% FBS/ HAT or HT culture 18 - 20 hours

Materials

15 ml centrifuge tube
96 well flat-bottomed plates (one for each 6 hybrids to be tested)
0.1 ml DMEM/ 10% FBS/ HAT or HT
multichannel pipettor
DMEM/10% FBS

Procedure

1. Keep the cells in numerical order and count the T cells (or estimate number when you have become proficient) and remove enough for the test (2.0×10^5) and place in 15 ml centrifuge tube (do not forget to label each tube with the cell line # and make absolutely certain that you record this information in your lab notebook as you do the counts), pellet the cells and aspirating off the medium, place the tubes in numerical order after pelleting.

2. Once you have determined the number of lines that need to be tested count the lymphoma line or spleen cells and take enough cells to give 2.0×10^5 lymphoma cells per line to be tested or 2.0×10^6 spleen cells for each line to be tested and pellet.

3. Open the required number of 96 well flat bottomed plates (one for each 6 hybrids to be tested), mark the plates to be used for the assay with the number of the line tested over the two test wells required for each hybrid.

4. The total volume of medium is 0.3 mls therefore time is saved if the T cells are resuspended in 0.5 ml and 0.2 ml added to each of the 2 wells.

5. The APC are resuspended to either 1.0×10^7 cells per ml for the lymphoma or 1.0×10^6 cells per ml for spleen cells and 0.1 ml of this cells suspension is added to each well.

6. The desired antigen is added to the well in 10 - 20 µl and the plates are cultured overnight.

7. After 18 - 20 hours of culture remove the plates from the incubator and add 0.1 ml DMEM/ 10% FBS/ HAT or HT to each well in the lower 6 rows.

8. Using a multi channel pipettor remove an aliquot of 50µl of medium from the culture well and add it to the second row of wells, take another 50µl aliquot and add it to the third row, which also contains 0.1 ml medium, mix the culture SN with the medium by pipetting up and down several times minimizing the formation of bubbles transfer 50 µl of this to the next row down and mix and repeat until you have diluted down to the bottom of the plate.

9. A total of 5.0×10^3 of the indicator cell CTLL are added to every well in the lower 7 rows of the plate, there are a total of 84 wells therefore 4.2×10^5 CTLL cells are needed for each plate.

10. Count the CTLLs and take out enough for all of the plates to be tested, pellet and wash 4 X with 5.0 mls of DMEM/10% FBS, it is necessary to wash extensively to remove traces of the IL-2 that the CTLL are grown in order to get a satisfactorily low background.

11. The CTLL cells are added to the plates in 20 µl of DMEM/10% FBS and 5.0 X 10^3 are added per well therefore resuspend the CTLLs to 2.5 X 10^5 cells per ml and add.

12. Incubate overnight and assess CTLL viability by visual inspection using the inverted 'scope.

Freezing T cell hybrids of the desired antigen specificity

It cannot be emphasized enough that as soon as a T cell hybrid is determined to be sufficiently reactive to the antigen to be of interest, at least three vials of this line should be frozen. The freezing of cells is described in another protocol

Cloning T cell hybrids

For most purposes it is essential that T cell hybrids be clonal, that is that the population arose from a single cell, in order to accomplish this the T cell hybrids are cloned. The goal of cloning is to have individual wells in 96 well plates that have a single viable hybrid cell. There are two general methods for cloning, first is micromanipulation in which single cells are transferred from a cells suspension into a well of a 96 well plate, the second method achieves a single cell per well by limiting dilution. The method of limiting dilution cloning used in this laboratory is described below.

Please note:
DMEM/ 10% FBS is used for all cultures
Cloning of T cell hybrids is done in 96 well flat bottom plates

Materials

96 well plates
DMEM/ 10% FBS
15 ml centrifuge tubes

1. For each line to be cloned four 96 well plates, one each with an average of 10, 3, 1, and 0.3 cells per well are set up.

2. The cells are added to the cloning plates in 0.1 ml of medium, therefore to get an average of 10 cells per well a cell suspension @ 100 cells / ml is required.

3. Count the T cell hybrid line or lines to be cloned and determine the total dilution required to give 15 ml @ 100 cells / ml.

4. Perform the serial dilutions in 15 ml centrifuge tubes and transfer 1500 cells to a 15 ml tube and fill to 15 ml with medium.

5. Distribute the cell suspension into the plate labeled 10 cells / well, this will require approx. 9.6 ml, dilute the remaining 5 ml of cells up to 15 ml by adding 9.6 ml medium, this dilution will give approx. 3 cells per well when 0.1 ml is added to each well, distribute the cells in the 3 cells/well plate and repeat this dilution and distribution of cells for a 1 cell per well plate and a 0.3 cell per well plate.

6. When all of the plates have been prepared place them in a culture box, gas and place in the incubator.

7. Clone growth is apparent after 7 - 10 days and when the clones have expanded to cover approx. half of the area of the well transfer to a flask with 2.0 ml medium, we generally transfer 20 wells per cloning

8. Always transfer clones from the lowest dilution possible, therefore use the 0.3, and 1 cell per well plates to obtain as many clones as possible.

9. The clones are expanded, tested and frozen in a manner identical to that used for the uncloned lines, in general 3 or 4 of the most strongly reactive clones are selected for freezing and approx. 5 vials of each clone should be frozen.

I6: Freezing BW5147 and Hybrid T Cells for LN2 Storage

Materials

sterile glass media bottle
0.45µ filters
sterile 15 ml polypropylene centrifuge tubes
Freezing medium (FM) *(vol:vol:vol)*:
    10% DMSO
    20% FBS
    70% DMEM

To make: mix the components for 200 ml of FM in a sterile glass media bottle (DMSO can dissolve the nitrocellulose membranes used in filters unless it is thoroughly mixed with the other components prior to filtration) and then filter through a 0.45µ filter. Aliquot at 10 ml/ tube in sterile 15 ml polypropylene centrifuge tubes and store @ -20 until needed.

Please Note: The 10% DMSO in FM is necessary for cryoprotection but is also toxic to cells at room temperature, the best results are obtained if the freezing medium and freezing vials are kept very cold, the work is completed quickly and the cells are transferred to the freezer quickly.

Procedure

1. Count the cells that are to be frozen and make sure that they are not above 1.0 X 10^6 per ml and that you have enough for at least one vial, 5.0 x 10^6 cells.

Standard freezing conditions are 5.0 X 10^6 cells per vial in 1.0 ml FM, for most purposes the minimum number of vials of each cell line to be frozen (not necessarily on the same day) is 3 - 4.

2. Make a chart of the cells to be frozen on a given day in your notebook with the cell counts, volume taken for freezing, total cell number frozen and number of vials to be frozen.

3. If you are freezing more that 2 cell lines list them on the chart in alpha numerical order and arrange the flasks in the same order to avoid confusion and mixing of cells.

   NEVER HAVE MORE THAN ONE FLASK OF CELLS OPEN IN THE HOOD AT ANY TIME.

4. Take the required amount of freezing medium from the -20 freezer, thaw in warn tap water and after thawing place on ice to chill thoroughly.

5. Label each vial on the white label area with the name of the cell line, the date and your initials, place them in the freezing block in the order that the lines are listed in your notebook.

6. Transfer the cell suspension to either 15 ml or 50 ml centrifuge tubes, depending on the volume and pellet by centrifugation for 5 min @ 1500 rpm, aspirate off the medium using a new pasteur pipette for each tube and place the tubes on ice for 5 - 10 min to chill.

7. Working quickly, resuspend the first tube of cells in the appropriate volume of freezing medium and place 1.0 ml per vial in the appropriate vial and screw the top on firmly, proceed with the remainder of the cells to be frozen in the same manner.

8. After all of the cells have dispensed, seal each vial as tightly as possible using your fingers and place in a styrofoam rack, cover with another styrofoam rack and place in the -70 freezer.

9. Keep a written record of the number of vials of cells and dates of freezing in your notebook, devote a page or two to this at the back of the notebook and reference the page in your notebook where the counts are recorded, in addition keep a record in an Excel document on the computer.

   PLEASE NOTE: -70 is not low enough for long term storage and it is essential that cells be transferred to liquid nitrogen and that stocks be split between at least two LN2 storage units asinsurance in case of a "melt down". Cells can be transferred the next day or up to a few weeks later, do not leave at -70 for extended periods of time. The date of transfer to LN2 should also be recorded in the notebook and in the Excel document.