SOLUTIONS AND REAGENTS

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)

STOCK SOLUTION

MTT	500 mg (Sigma M2128)
PBS	100 ml

Mix with vortex for 20 minutes, then sterile filter. Wrap bottle in aluminum foil or paper to block out light as MTT is light sensitive. Store at 4°C. Prepare fresh stock each month.

WORKING SOLUTION

MTT stock solution	10 ml
RPMI1640 + 20% FBS	40 ml

Prepare just prior to use and pre-heat to 37°C before use. To use add 1 ml of working solution to 2 ml of RPMI 1640/20% FBS containing the hollow fiber samples.

2.5% PROTAMINE SULFATE (P/S)

Protamine sulfate	2.5 gm (Sigma P4380)
normal saline (0.9%)	100 ml

Add P/S slowly to saline while stirring vigorously. Stir for 20 minutes or longer if particles are still present. Sterile filter and store at room temperature. Prepare fresh stock monthly.

NORMAL SALINE (Quality Biological #14-106-5)
STERILE DISTILLED WATER (Quality Biological #14-219-5)
RPMI 1640 w/o L-GLUTAMINE (Quality Biological #12-113-5)
L-GLUTAMINE 200 mM (Life Technologies/Gibco #320-5030)
FETAL BOVINE SERUM (Hyclone Laboratories #A-1111-L)
TRYPSIN/EDTA (Life Technologies/Gibco #610-5300)
PHOSPHATE BUFFERED SALINE (Quality Biological)
70% ETHANOL (NON-DENATURED)
0.4% TRYPAN BLUE

TISSUE CULTURE

GENERAL

Cell lines (adherent and suspension) are split once weekly on Thursday or Friday. Cells are fed once weekly in addition to splits, generally on Monday or Tuesday. All cell lines are generally passed in T75 flasks.

PROCEDURE: Rinse flask with normal saline, aspirate and add 3-5 ml of trypsin/EDTA. After cells loosen, add 10-15 ml of medium. Split new passage from this suspension without centrifugation. Cells are grown in RPMI 1640 with 10% FBS and 2mM glutamine.

Adherent lines: 1:40 and 1:80 (backup) in 20 ml of medium*
Suspension lines: 1:40 and 1:80 in 30 ml of medium*

passage dilutions depend upon cell line and number of flasks needed for experiments.

CELLS ARE CARRIED ONLY 20 PASSAGES.

EXPERIMENT PREP

Cells are split the previous week into T150 flasks at densities that will result in 70-90% confluency on the day of experiment. Cells are fed the day before the experiment by removing half of the existing volume of medium and replacing with an equal volume of fresh medium.

DAY OF EXPERIMENT

1. Remove medium from cell flask(s) and place into a sterile tube on ice. This is the **conditioned medium** which is used to resuspend the cells.
2. Wash cells once with saline.
3. Remove the saline and add 3-4 ml of trypsin solution. *Incubate at 37°C to loosen cells as needed *
4. Add 10 ml of fresh medium to the flask, rinse thoroughly and transfer to an appropriate size centrifuge tube. Pool contents of flasks if more than 1 flask of a cell line is used.
5. Centrifuge 10 minutes at 1000 rpm at 4°C.
6. Pour off medium and resuspend cells in the residual volume by flicking the tube. DO NOT VORTEX.
7. Resuspend the cells in 5 ml of the conditioned medium and place on ice.
8. Do viability and density counts using trypan blue dye exclusion assay.
9. Add the appropriate volume of RPMI 1640/20% FBS to adjust the cells to the desired density.
10. Store the cells on ice until used.

HOLLOW FIBER PREP

MATERIALS NEEDED

- hollow fibers (Spectrum Medical)
- sterile filtered deionized/distilled water
- chromatography columns
- large volume syringe with 20 gauge needle
- 70% EtOH (non-denatured)
- autoclavable pipette pan with cover (Nalgene)
- scissors
- RPMI 1640 with 20% FBS
- autoclave bags
- gloves (non-sterile)
- forceps

PROCEDURE

Wear gloves during all fiber manipulations to reduce transfer of body oils to fibers.

1. Using gloves, remove fibers from storage container and place them on work surface (previously wiped with EtOH to clean).
2. Position fibers so that badly crimped ends can be cut off and remaining lengths of fibers are even.
3. Cut the fibers into 12-15 inch lengths so they will fit into the pipette trays.
4. Pop open the ends of the tubing by gently squeezing across the crimp.
5. Flush the fibers with 70% EtOH individually using a syringe and 20 gauge needle and place them into a pipette tray containing 70% EtOH.
6. Store fibers in EtOH at room temperature with fibers being allowed to soak a minimum of 72 hours (can be stored for weeks if necessary).

→FROM THIS POINT ON DO NOT ALLOW THE FIBERS TO DRY OUT OR THE PROCESS HAS TO BE STARTED OVER AGAIN←

7. Fill a second pipette tray with 300-500 ml of filtered water. Individually rinse the fibers with water and transfer into the water-filled pan.
8. Cover the pan and place into the autoclave. Autoclave at STP for 40 minutes (or whatever is required to change the sterility control pellet).

****FROM THIS POINT ON, ALL WORK WITH THE FIBERS MUST BE CONDUCTED UNDER STERILE CONDITIONS USING STERILE TECHNIQUE****

9. Place autoclave pan containing sterile fibers into a plastic bag to prevent evaporation/contamination and place the fibers in the refrigerator until used.

FIBER IMPLANT PREPARATION

MATERIALS NEEDED

- rocker plate
- ice pack shaped around 15cc tube
- sterile drapes (2)
- 6-well plastic plates
- stainless steel sterile work surface
- sterile forceps (2 pair)
- smooth-jawed needle holders
- 15 ml sterile conical centrifuge tube
- bacteriological incinerator
- 10 cc syringe with 20ga needle (chilled)
- 5 cc syringe with 20ga needle (chilled)
- sterile gloves
- sterile scissors
- conditioned hollow fibers

→Petri dishes should be labeled, filled with 2 ml of RPMI 1640/20% FBS and stored at 4°C until needed.
→Work surface should have 2 cm demarcations so that hollow fibers can be prepared at the proper length.

PROCEDURE

→CREATE AND MAINTAIN A STERILE FIELD THROUGHOUT THE PROCESS←

1. Empty the BSC of all materials and wipe down with ethanol (including the interior glass). Put on a cuffed long sleeve gown/lab jacket.
2. Place the bacteriological incinerator, rocker plate and pipette tray containing the conditioned hollow fibers into the hood. Spray the external surface of the hollow fiber pan with 70% EtOH. Turn on the bacteriological incinerator so it can come to temperature.
3. Secure the “sculptured” ice pack to the rocker plate in the BSC.
4. Insert a sterile 15cc conical tip centrifuge tube into the ice pack. Remove the cap from the tube - do not touch the threads or opening of the tube as this tube will be used to hold the cell syringe so the “mouth” of the tube needs to be sterile to not contaminate the syringe.
5. Place the wrapped autoclaved work surface into the hood and unwrap being careful to not touch the tray or inside of the paper. Slide the tray to one end of the hood by grabbing the non-sterile end of the wrapping paper and pulling on it.
6. Unwrap all sterile instruments and drop them onto the work surface being careful to maintain sterility. Add the 10 cc and 5 cc syringes along with two 20 gauge needles.
7. Sterily open the sterile drapes.
8. Don sterile gloves - **DO NOT TOUCH ANYTHING NONSTERILE FROM THIS POINT ON.**
9. Have gloved hands sprayed with 70% ethanol by assistant to remove/restrict glove powder.
10. Sit down and pull up to the hood.
11. Unfold 1 sterile drape and spread along the width of the hood surface being careful not to touch the upper working surface side to anything nonsterile.
12. Unfold second drape and spread parallel to and slightly overlapping the first drape.
13. Place stainless steel work surface onto the sterile field drapes. Assistant discards the paper wrapping from the sterile work surface.
14. Gloved (nonsterile) assistant removes lid from the pan of hollow fibers maintaining sterility of the inner lid surface.
15. Assistant opens tube of cold media under the hood and holds while the 10 cc syringe is filled. Media filled syringe is placed to one side while assistant removes the tube.

FIBER IMPLANT PREPARATION cont=d

16. Assistant holds tube containing the cell suspension in the hood while the 5 cc syringe is filled with cell suspension. A 5 cc syringe is used for the cell suspension because it is the size syringe that will fit nicely into the 15 cc conical tube which is located in the ice pack.
17. Place the cell syringe into the 15 cc conical tip tube located in the ice pack so the cells remain cold and mixed. DO NOT TOUCH THE TUBE EXTERIOR or THE ICE PACK- unsterile!!! It is important to keep the cells cold to reduce agglutination and to increase viability.
18. Using sterile forceps remove 1-15 fibers from the sterile pan and place them onto the work surface where they are flushed with fresh medium.
19. Remove cell syringe from holder and aspirate a small volume of air.
20. Slide the hollow fiber over the needle until the bevel is covered. Inject a small volume of air through the fiber to remove any residual medium then fill with the cell suspension being careful to exclude air bubbles.
21. Place the needleholder into the incinerator and heat for a few seconds (generally 3-5 seconds is adequate). Heat seal the loose end of the hollow fiber by briefly clamping the end with the needleholder. Heat seal the end of the fiber attached to the needle and lay the fiber down.
22. Recap the cell syringe needle (CAREFULLY) and return it to the ice holder.
23. Using the medium filled syringe cover the exterior of the fibers with medium to prevent them from drying out . 3-6 fibers can be processed at a time but care must be taken to prevent the fibers from drying out or the medium osmolality from increasing to a deleterious level.
24. Using the 2 cm marks on the work surface to indicate distance, heat seal every 2 cm. Cut the individual samples apart in the center of the heat seal. The heat seals should be clear, not white, in appearance. The desired length of a heat seal is 3-4 mm so that when the samples are cut apart each sample has a 1.5 - 2 mm long sealed “flap”. Generally, the first heating marks the point that should be sealed and a second heating is done while exerting a slight pull across the seal to produce the actual heat seal. If necessary, the seal can be reheated until the desired effect is achieved. PVDF heat seals well when wet or when dry. For the purpose of cell samples it is important that the fiber not be allowed to dry.
25. Assistant places 6 well plates into the BSC and removes the lid so that 6 fibers can be transferred into each well for storage. Transfer the 6-well plates to the incubator after fibers are added.
26. Repeat steps 18-25 until the proper number of samples have been generated.
27. Incubate at 37°C in an humidified 5% CO_2 environment.
28. Implant fibers into host mice (athymic nudes generally) after overnight (or longer depending upon cell line) incubation. The overnight incubation allows the cells to stabilize following trypsinization and allows for a sterility check prior to in vivo implantation.

****When using more than one cell line for an experiment, implants must be color-coded to differentiate cell lines following in vivo implantation. The fibers are available from the manufacturer in blue, yellow, green and white.****

NOTES ON FIBER IMPLANT PREPARATION

1. VISUALLY CHECK THE CELLS IN THE SYRINGE TO DETERMINE WHETHER THEY HAVE AGGLUTINATED OR AGGREGATED WHILE SITTING. IF THEY HAVE THEN ATTEMPT TO DISAGGREGATE BY GENTLY MIXING. IF THEY DISAGGREGATE USE THEM. IF THEY REMAIN AGGLUTINATED THEN NEW CELLS SHOULD BE GOTTEN. FOR SOME CELL LINES IT IS VERY PROBABLE THAT THEY WILL AGGLUTINATE/AGGREGATE - FOR SOME OF THESE LINES IT IS BEST THAT THEY BE HELD IN A CENTRIFUGE TUBE IN ICE RATHER THAN IN THE SYRINGE AS THEY STAY COLDER AND THUS LESS LIKELY TO AGGREGATE.

2. SERUM PROTEINS BEGIN TO COOK ONTO THE NEEDLEHOLDERS AS THEY ARE USED. THIS INTERFERES WITH EFFICIENT HEAT SEALING. THE FORCEPS CAN BE CLEANED BY SCRAPING WITH THE BLADE OF THE SCISSORS OR BY WIPING WITH AN ETHANOL IMPREGNATED SWAB. IF ETHANOL IS USED THE FORCEPS SHOULD BE HEATED TO REMOVE THE ALCOHOL PRIOR TO COMING IN CONTACT WITH CELL SAMPLES.

3. DO NOT SQUEEZE FIBERS EXCESSIVELY, ESPECIALLY WITH THE FORCEPS DURING THE PROCESSING. THE SITES WHERE SQUEEZING OCCURRED ARE MORE OFTEN THAN NOT DEVOID OF ANY CELL GROWTH.

4. FIBERS WHICH DEHYDRATE (TURN WHITE) SHOULD NOT BE USED. IF THEY HAVE NOT YET BEEN EXPOSED TO CELL SUSPENSION THEN THEY CAN POSSIBLY BE REPROCESSED.

5. IF FIBERS CONTAIN A FLATTENED OR BENT PLACE THEN HEAT SEALS SHOULD BE PLACED SO THE DAMAGED AREA CAN BE REMOVED.

6. IF FIBER SAMPLES TURN WHITE THEN THEY ARE UNACCEPTABLE FOR USE.

7. TEMPERATURE FLUCTUATIONS SHOULD BE AVOIDED AS MUCH AS POSSIBLE AS THEY ARE VERY DELETERIOUS TO THE CELLS.

ASSAYING IMPLANTS

COLLECTION

1. Fill 6 well plates with 2 ml of RPMI 1640/20% FBS and place into incubator. Transfer plates to *in vivo* laboratory for sample collection.
2. Sacrifice mice (3 maximum at any given time). Collect subcutaneous fibers into SC labeled wells. Collect intraperitoneal fibers into IP labeled wells. At retrieval wipe the fibers **GENTLY** with gauze to remove mouse cells/debris adhering to the exterior of the fiber.
3. Return plates containing fibers to incubator as soon as possible.

**** Maintaining temperatures at or near 37°C is very important to retaining cell viability.**

4. Transfer dishes from *in vivo* laboratory to *in vitro* laboratory. Minimize temperature fluctuations as much as possible.

ASSAY

1. Incubate plates from *in vivo* laboratory for a minimum of 30 minutes at 37°C in 5% CO_2 to allow temperature and sample condition to stabilize.
2. Add 1 ml of MTT working solution (pre-warmed to 37°C) to each 2 ml well of fibers.
3. Incubate plates for 4 hours at 37°C in a 5% CO_2 atmosphere.
4. Aspirate MTT - dispose of as chemical waste as this is a possible mutagen.
5. Add 2 ml of protamine sulfate solution to each well.
6. Store at 4°C for 24 hours (can go over a weekend without causing a problem).
7. Aspirate protamine sulfate.
8. Add 2 ml of protamine sulfate for a second wash.
9. Store at 4°C for at least 2-4 hours (can be stored for at least 2 weeks).
10. Wipe implants with gauze to remove any stained debris from the exterior of the fiber and transfer to 24 well plates with 1 fiber per well for drying and extraction. Cut the fibers in half so they will lie flat in the bottom of the wells.
11. Allow to dry overnight in a BSC.
12. Add 250 µl of DMSO (100%) to each well making sure that the fibers are lying flat in the bottom of the wells for proper extraction to occur.
13. Place the plates on an orbital shaker and cover to protect from light (MTT is light sensitive).
14. Extract for 4 hours at room temperature.
15. Transfer 150 µl of sample from each extraction well to the appropriate well on a 96-well plate. When transferring triplicate samples, the same pipette tip can be used for all 3 samples provided the sample with the least color is transferred first to prevent color carry-over.
16. Read plates at 540 nm in a standard microtiter plate reader using water as the blanking well.

NOTE: It is possible to skip the second protamine sulfate wash provided the volume of the first wash is at least 50% greater than the volume of the MTT solution used to stain the cells.

FIBER IMPLANTATION, TREATMENT & COLLECTION

MATERIALS NEEDED

- fiber samples
- metofane anesthetic and anesthesia jar
- 2x2 or 4x4 gauze squares
- surgical stapler
- surgical staples
- Nolvasan disinfectant
- scissors
- forceps
- CO_2 gas

PROCEDURE

1. Anesthetize mice with methofane gas.
2. Wipe skin over abdomen with disinfectant.
3. Make a small dorsolateral incision into the abdomenal cavity (about at the location of the kidney).
4. Insert the desired intraperitoneal hollow fiber implants.
5. Close the incision with a surgical staple.
6. Lay the mouse in ventral recumbency and make a small nick incision at the nape of the neck.
7. Place 2 hollow fiber samples into an 11 ga. tumor implant trocar and slide it through the skin incision and down the back of the mouse.
8. Deposit the fiber samples subcutaneously over the pelvic region of the mouse.
9. Repeat the subcutaneous implant process to deposit a third fiber.
10. Close the skin incision with a surgical staple.
11. Place mice into cage for recovery.
12. Begin treatment with anticancer compounds 1-4 days following implantation.
13. Treat animals with desired compound on desired schedule (generally qd X 4).
14. On day of fiber collection, sacrifice mouse with CO_2 gas.
15. Open the peritoneal cavity and remove the ip fibers (generally found in the dorsal area of the peritoneum.
16. Wipe the fibers gently to remove mouse tissue.
17. Transfer the fibers into pre-warmed media in 6 well plates.
18. Incise the skin adjacent to the subcutaneous fibers and reflect the skin to expose the fibers.
19. Remove the fibers and gently wipe them to remove mouse tissue.
20. Transfer the fibers into pre-warmed media in 6 well plates.
21. Transfer the samples to the in vitro laboratory for analysis.

Fiber Source

The fibers are PVDF hollow fibers with a 500kDa MWCO, a 1.0 mm ID and available in 36@ lengths

They are available in 4 colors as follows:

S9320101 Biopore white hollow fibers
S9320202 Biopore blue hollow fibers
S9320103 Biopore green hollow fibers
S9320104 Biopore yellow hollow fibers

The supplier is

The Spectrum Companies
23022 La Cadena Drive
Laguna Hills, CA 92653
Telephone: (714) 581-3880
FAX: (714) 855-6120

Our contact person is F. Jesus Martinez