Instruction Sheet

HBTEC Air-Liquid Interface Differentiation Medium

LM-0050

This product is for Research Use Only. This product is not approved for human or veterinary use or for use in in vitro diagnostics or clinical procedures.

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Quick Steps for Air-Liquid Differentiation of HBTEC

Good Laboratory Practices and Pre-Differentiation Cell Culture

1. Always wash hands before and after working with cell cultures.
2. Always wear eye protection and gloves when working with cell cultures.
3. Store cryopreserved cells in liquid nitrogen, vapor phase.
4. When working with cells or medium, always use a certified biological safety cabinet.
5. Handle cryopreserved vials with caution. Aseptically vent any nitrogen from cryopreserved vials in a biosafety cabinet prior to thawing in a water bath.
6. Feed cells using pre-warmed Lifeline® culture medium according to feeding guidelines.
7. Expand HBTEC in BronchiaLife™ B/T Medium (LL-0023) as detailed on page 2 to 3.

Differentiation

Day 0

1. When cells are 70 to 80% confluent and actively proliferating, passage cells using Lifeline subculture reagents (as detailed on page 4).
2. Resuspend pellet in BronchiaLife B/T Medium.
3. Inoculate cells into 24-well permeable insert plates at 50,000 cells/insert (~150,000 cells/cm^2); 100 µL of cell suspension (500,000 cells/mL) in the apical chamber and 0.5 mL of BronchiaLife B/T Medium in the basolateral chamber.
4. Incubate plate at 37°C, 5% CO_2.

Day 1

5. Gently aspirate the medium from each well.
6. Replace the apical and basolateral medium with BronchiaLife B/T Medium; 100 µL in the apical chamber and 0.5 mL in the basolateral chamber.
7. Incubate at 37°C, 5% CO_2.
8. Pre-differentiation measurement of TEER. (optional)

Day 4 Through Day 21

9. Aspirate the medium from the apical chamber on Day 4.
10. Gently replace the medium in the basolateral chamber every 2 to 3 days with HBTEC Air-Liquid Interface Differentiation Medium (LM-0049).
11. Incubate at 37°C, 5% CO_2.

Day 21

12. Differentiation is complete. Post-differentiation TEER measurement. (optional)

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Medium Storage

HBTEC Air-Liquid Interface Differentiation Medium should be stored at -20°C until ready to use. For long-term storage, HBTEC Air-Liquid Interface Differentiation Medium should be stored at -70°C or lower. Once thawed it may be stored at 2 to 8°C for up to four weeks. Users should take care to protect the medium from extended exposure to light. Do not use product beyond expiration date or for longer than four weeks after it has been thawed. Multiple freeze/thaw cycles are not recommended. Aliquots can be prepared and stored at -20°C.

Medium Preparation

HBTEC Air-Liquid Interface Differentiation Medium contains all the necessary growth factors to support the differentiation of Human Bronchial/Tracheal Epithelial Cells. HBTEC Air-Liquid Interface Differentiation Medium contains no antimicrobials and no phenol red. Antimicrobials are not required, but are recommended during differentiation due to the duration of culture and the frequency of handling of the cultures. A vial of Gentamicin and Amphotericin B (GA; LS-1104) is provided with the purchase of HBTEC Air-Liquid Interface Differentiation Medium (LM-0050) for your convenience. Phenol Red (LS-1009) may be purchased, but is not required.

Product	Part No.	Volume	Storage
HBTEC Air-Liquid Interface Differentiation Medium	LM-0050	500 mL	-20°C until ready to use. Thaw and store at 2 to 8°C for up to 4 weeks.

Components of HBTEC Air-Liquid Interface Differentiation Medium	Final Concentration
L-Glutamine	5 mM
rh EGF	0.5 ng/mL
Hydrocortisone Hemisuccinate	1 µg/mL
rh Insulin	5 µg/mL
(-)-Epinephrine-(+)-Bitartrate	1 µM
Transferrin PS	2.5 µg/mL
Apo-Transferrin	2.5 µg/mL
Triiodothyronine	10 nM
All-trans-Retinoic acid	30 ng/mL

Optional Supplements	Part No.	Volume	Concentrations of Supplement	Storage
Phenol Red Supplement	LS-1009	1 mL	33 mM	RT
Antimicrobial Supplement: Gentamicin and Amphotericin B *(Provided with purchase of LM-0050)*	LS-1104	0.5 mL	Gentamicin 30 mg/mL Amphotericin B 15 µg/mL	-20°C

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Other Recommended Products	Part No.	Unit	Storage
Normal Human Bronchial/Tracheal Epithelial Cells (HBTEC)	FC-0035	500,000 cells/mL	-150°C
BronchiaLife™ B/T Medium Complete Kit	LL-0023	Kit	2 to 8°C when prepared

Basic Aseptic Technique

Medium, cells, and reagents should only be used in an aseptic environment, a Class II biological safety cabinet with front access and filtered laminar airflow, or an equivalent device. Always wear gloves and eye protection when working with these materials. Wipe or spray all bottles and vials with 70% ethanol or isopropanol, especially around the area of the cap, before placing them in the biological safety cabinet. Allow these surfaces to dry completely before opening the bottle or vials. Transfer cells, medium or reagents with disposable sterile pipettes. Do not mouth pipette! Take up the volume needed into the pipette, being careful not to touch the sterile tip to the rim of the container or any other surface. Close the container and open the container into which the transfer is being made, again being careful not to touch any surfaces with the sterile tip. Transfer the material and close the container. Wash your hands before and after working with cell cultures. Do not block airflow in a laminar flow hood as this may compromise sterility. Ensure that biological safety cabinets are certified routinely and that the HEPA filters are replaced regularly.

Pre-warming Medium

If using less than 100 mL of medium, Lifeline® recommends warming only the volume needed in a sterile conical tube. Repeated warming of the entire bottle over extended periods will cause degradation and reduced shelf life of the medium. Medium will warm to 37°C in 10 to 30 minutes, depending on the volume. Do not leave medium in water bath for extended periods.

Additional Items Strongly Recommended for Culture and Differentiation of HBTEC

Product	Recommended Vendor	Part No.
BronchiaLife™ B/T Medium Complete Kit,	Lifeline Cell Technology	LL-0023
Rat Tail Collagen, Type 1	BD® Biosciences	35-4236 *or equivalent*
Costar® PET Transwell® Plates (24-well)	Corning®	3470 *or equivalent*
Trans-epithelial electrical resistance (TEER) meter	Millicell® ERS, Millipore®	MERS00002 *or equivalent (optional)*

Thawing and Plating Cryopreserved Cells

Pre-warm BronchiaLife B/T Medium (see BronchiaLife B/T Medium Instruction Sheet). Remove vial from dewar and check the cap to be sure that the vial is securely sealed. Spray the vial with 70% ethanol or isopropanol and transfer it to a biosafety cabinet. Allow it to dry thoroughly and carefully loosen the cap to vent any liquid nitrogen that may have entered the vial. Recap the vial and submerge only the bottom half of the vial in a 37°C water bath for approximately one minute or until vial is almost completely thawed—a small amount of ice should still be visible—thawing past this point may damage the cells. To avoid potential contamination, do not allow the vial cap to make contact with the water. Dry the vial, spray the exterior of the vial with 70% ethanol or isopropanol and place the vial in a biological safety cabinet and allow it to dry. Carefully remove the cap to avoid contamination or splatter. Gently resuspend the cells in the vial using a 1 or 2 mL sterile pipette. Do not centrifuge; the cells may be directly plated from the vial. Plate the cells into pre-warmed culture medium in the desired culture vessel at a density of 5,000 cells per cm^2. (Please see the Standard Calculation section on page 3.) Flasks with vented caps, commonly referred to as filter caps, are strongly recommended. Gently rock the culture vessel from side to side and front to back to evenly distribute cells within the vessel. Place inoculated culture vessel in a 37°C, 5% CO_2 incubator. Remove cryopreservation agents after the cells have attached by replacing the culture medium approximately 4 to 36 hours after inoculation.

Recommended Feeding Guidelines for HBTEC

Guidelines for a T-75 Flask. Adjust volumes according to culture surface area.
Every other day, remove medium and feed with 15 mL of fresh supplemented BronchiaLife B/T Medium.
Most cultures which are 50% confluent will be ready for passage the following day and should be fed with 15 to 20 mL of supplemented medium.
Do not use more than 10 mL of medium per 25 cm^2 of culture surface to ensure the depth of the medium is at a level where gas diffusion* will be sufficient to support the cells' requirements for oxygen.

**Gas diffusion gradients through the culture medium to the cells are affected by the depth of the medium. The volumes of medium recommended in this table result in a range of depths between 2 mm and 5 mm, which is compatible with general recommendations, 30 mL being at the maximum depth allowable (5 mm) for a T-75 flask.*

Passaging Cells

Normal HBTEC may be passaged once the culture is 70 to 80% confluent and actively proliferating. Normal HBTEC are contact inhibited, and it is essential that the cells be passaged before reaching confluence since post-confluent cells exhibit slower proliferation and morphological changes after passaging. Prior to passaging the cells, coat the permeable inserts with collagen as per the instructions on the next page.

Lifeline® recommends using the TrypKit™ Subculture Reagent Kit (LL-0013). Aspirate the medium from the culture vessel. Rinse the flasks with Lifeline's Phosphate Buffered Saline (PBS; CM-0001) by adding at least 1 mL of PBS per each 5 cm^2 and gently tilting the flask to cover the surface with PBS. Aspirate the PBS from the culture vessel, repeat the rinse if desired. Add at least 2 mL of Lifeline's 0.05% Trypsin/0.02% EDTA (CM-0017) per each 25 cm^2 to the vessel. Swirl gently to ensure all cells are coated with the Trypsin/EDTA. Observe the cells carefully under

the microscope. When the cells round up they are ready to be released. This normally takes from 2 to 3 minutes depending on the confluence of the cells. Do not over trypsinize as this may damage the cells. Detach the cells by gently striking the culture vessel against your hand several times. Observe the cells under the microscope to be sure they have become detached. Once the cells are fully detached, add Lifeline's Trypsin Neutralizing Solution (TNS; CM-0018) using a volume equal to the amount of Trypsin/EDTA that was originally used. Gently swirl to ensure all of the trypsin solution is neutralized. Using aseptic laboratory techniques, pipette the cells into a sterile centrifuge tube. Collect the remaining cells by rinsing the culture vessel with at least 1 mL of PBS per each 5 cm^2 and pipetting the cells into the sterile centrifuge tube. Check culture vessel under the microscope for cells still attached and repeat steps if necessary to retrieve all the cells from the vessel. All steps must be completed under aseptic conditions in a biological safety cabinet.

Centrifuge cells at 150 x *g** for 3 to 5 minutes. For best results, calculate speed for individual centrifuge type. Time may also be centrifuge dependent. Do not over centrifuge cells as this will cause cell damage. After centrifugation, the cells should form a clean loose pellet. Please consult Lifeline's technical service department if issues arise from trypsinization or centrifugation.

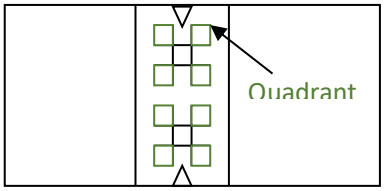
Aspirate neutralized trypsin from the centrifuge tube and resuspend the cell pellet in pre-warmed BronchiaLife™ B/T Medium by gently pipetting up and down with a 2 or 5 mL pipette. Count cells using a hemacytometer, and inoculate at 5,000 cells per cm^2 (for expansion) or 50,000 cells per insert (for differentiation) in pre-warmed BronchiaLife B/T Medium.

***To calculate RCF (x *g*):** **$RCF = 0.00001118 \times (rpm)^2 \times r$**
r = rotational radius in centimeters
rpm = rotations or revolutions per minute

Standard Calculation for Plating of Cells for Mucociliary Differentiation

Immediately prior to cell counting, gently re-suspend the cells evenly in complete BronchiaLife B/T Medium. Using a clean hemacytometer and aseptic technique, remove 25 µL of the cell suspension to a separate tube, such as a microcentrifuge tube. Add 75 µL of 0.4% Trypan Blue solution to the cell suspension in the microcentrifuge tube and allow it to sit for 1 to 5 minutes. Place 10 µL of the cell suspension into each chamber of the hemacytometer. Count a minimum of 4 quadrants on the hemacytometer (see diagram below). Dead and dying cells are permeable to Trypan Blue, viable cells will not be blue. For accurate cell counts, optimal number of cells per quadrant should be 25 to 75 cells. After counting the cells, calculate the average number of cells per quadrant. Take the cell count average and multiply by the dilution factor and by 10^4 to get the number of cells per mL. Multiply the desired inoculation density (~150,000 viable cells per cm^2) by the surface area of the vessel(s) to be inoculated. This will give you the total number of cells to inoculate one vessel. Divide the number of cells needed to inoculate the vessel(s) by the total number of cells in the cell suspension. This will give you the volume of cell suspension with which to inoculate the vessel(s). Inoculate the cells into the culture vessel(s) prepared with pre-warmed culture medium. Mix gently to evenly distribute the cells and place culture vessel(s) into the incubator at 37°C, 5% CO_2. (See TrypKit Instructions for more detailed cell counting instructions.)

Sample calculation:
Average viable cells per quadrant = 31
31 cells/quadrant x 10,000 quadrants/mL x 4 (dilution factor) = 1,240,000 cells/mL



Inoculating a 24-well permeable insert plate at 50,000 cells per insert:
Dilute cell suspension to 500,000 cells/mL:
1 mL x 1,240,000 cells/mL ÷ 500,000 cells/mL = 2.48 mL media (total)
Add 1.48 mL media to 1 mL of cell suspension.
Inoculate each insert with 0.1 mL (50,000 cells/insert)

Plating Cells for Mucociliary Differentiation

Collagen Coating of Transwells on the Day of Use

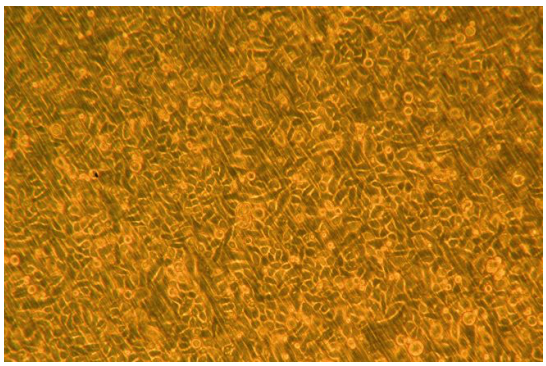
1. Dilute collagen to 0.03 mg/mL in PBS (CM-0001).
   Example: $\frac{4 \text{ mL} \times 0.03 \text{ mg/mL}}{3.68 \text{ mg/mL collagen}} = 0.033\text{mL of stock}$
2. Add 0.033 mL of collagen to 3.967 mL PBS and mix.
3. Add 100 µL of the diluted collagen solution to the culture surface of the apical chamber of each Transwell insert.
4. Incubate in a 37°C cell culture incubator 45 minutes.
5. Aspirate the collagen solution from the wells.
6. Rinse once with 150 µL of PBS and aspirate the PBS from the wells.

Inoculation of Inserts

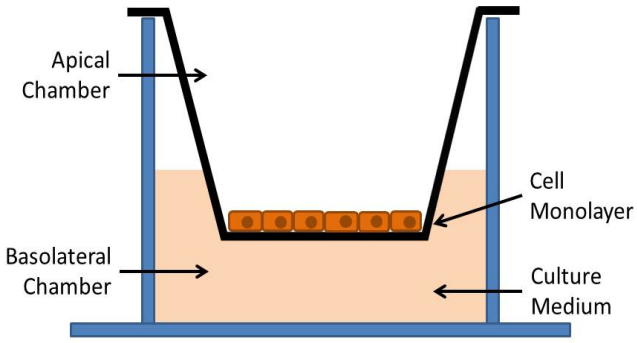
Using the passaging and cell counting instructions on page 3, inoculate HBTEC in the desired number of 24-well permeable inserts at a density of 50,000 cells/insert. The apical (upper) chamber of each well should be filled with 100 µL of HBTEC (500,000 cells per mL) to achieve 50,000 cells per insert. Add 0.5 mL of BronchiaLife™ B/T Medium to the basolateral (lower) chamber of each well. Gently rock the plate from side to side and front to back to evenly distribute cells within the insert. Never swirl the plate as this will result in uneven distribution of cells.

Place inoculated plate in a 37°C, 5% CO_2 incubator and incubate for 48 hours. Multi-well permeable insert plates (e.g. Transwells) other than 24-well plates may be used, but the volume of media and cells must be adjusted accordingly to ensure that the meniscus in the apical chamber matches the meniscus basolateral chamber.

The day after inoculation change the media using BronchiaLife B/T Medium; 100 µL in the apical chamber and 500 µL in the basolateral chamber. Allow the cells to maintain confluence until 3 days after inoculation. The monolayer should have the appearance seen in the picture below. At this time TEER (trans-epithelial electrical resistance) can be measured to record a pre-differentiation value, typically between 100 and 200 Ω per well (33 to 66 Ω•cm^2).



HBTEC, 3 days after inoculation,
prior to removal of apical medium.



Transwell insert in one well of a
multi-well plate Air-Liquid Interface Culture.

Air-Liquid Differentiation Medium Preparation

HBTEC Air-Liquid Interface Differentiation Medium contains all the growth factors necessary to support the mucociliary differentiation of HBTEC. HBTEC Air-Liquid Interface Differentiation Medium is prepared by thawing and warming to 37°C.

HBTEC Air-Liquid Interface Differentiation Medium contains no antimicrobials or phenol red. Antimicrobials are not required, but are recommended during differentiation due to the duration of culture and the frequency of handling of the cultures. A vial of Gentamicin and Amphotericin B (GA; LS-1104) is provided with the purchase of HBTEC Air-Liquid Interface Differentiation Medium for your convenience.

Initiating Differentiation

Three days after inoculating the 24-well permeable insert plate with HBTEC cells, initiate differentiation by aspirating the medium from both the apical and basolateral chambers of the inserts. Add 0.5 mL of HBTEC Air-Liquid Interface Differentiation Medium to the basolateral chamber of the wells. Return the plate to a 37°C, 5% CO_2 incubator. Do NOT add any medium to the apical chamber.

The basolateral medium should be replaced every 2 to 3 days (e.g. Mon-Wed-Fri) using pre-warmed differentiation medium. Re-feeding is performed by aspirating the medium from each insert through the open side of the insert. Using a repeating pipet or a micropipettor, dispense 0.5 mL of differentiation medium to the basolateral chamber.

If culture medium diffuses into the apical chamber of the wells it should be aspirated to remove it. Be very careful to not damage the monolayer or TEER values will be lower than expected.

After 10 to 14 days beating cilia should begin to be visible. After 21 days, differentiation should be complete. To measure TEER, add 100 µL of medium to the apical chamber of the wells. At this time TEER should be >3000 Ω per well (1000 Ω•cm^2) which is indicative of a monolayer with strong tight junctions and good integrity as an epithelial barrier.

Optional Supplements

Phenol Red:

Phenol red is a pH indicator that is not required in cell culture and may adversely influence the behavior of some cell types. Medium with phenol red will appear more yellow than red in acidic conditions and will appear more purple than red in basic conditions.

If you wish to add phenol red to Lifeline® medium, a 1 mL LifeFactor of water-soluble phenol red (LS-1009) may be purchased separately. Addition of 0.5 mL of the phenol red to 500 mL of medium will yield a 33 µM solution of phenol red and will change the medium to a strong red to reddish-purple color. The phenol red supplement may be stored at room temperature.

Gentamicin and Amphotericin B (GA):

A vial of GA (LS-1104) is provided with the purchase of HBTEC Air-Liquid Interface Differentiation Medium for your convenience. The use of GA is recommended to inhibit potential fungal or bacterial contamination of eukaryotic cell cultures. GA is best stored at -20°C; it should only be thawed once and stored at 4°C for a maximum of two weeks after thaw. Addition of 0.5 mL of GA to 500 mL of media will provide an effective concentration to inhibit bacterial and fungal cell division.

The Lifeline Guarantee

Lifeline's rigorous quality control ensures sterility and performance to standardized testing criteria. Upon request, we will provide lot-specific QC test results, material safety data sheets and certificates of analysis. See complete guarantee/warranty statement at lifelinecelltech.com or contact your technical representative for more information.

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For any question on medium preparation or cell feeding guidelines; please contact technical service at 877.845.7787. We are here to help.

Notes:

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