www.lonza.com
U.S. Scientific Support: 800-521-0390
scientific.support@lonza.com
EU/ROW Scientific Support: +49-221-99199-400
scientific.support.eu@lonza.com
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Clonetics™ B-ALI™ air-liquid interface medium

Instructions for use

Safety statements

THESE PRODUCTS ARE FOR RESEARCH USE ONLY. Not approved for human or veterinary use, for application to humans or animals, or for use in clinical or *in vitro* procedures.

WARNING: CLONETICS™ AND POIETICS™ PRODUCTS CONTAIN HUMAN SOURCE MATERIAL, TREAT AS POTENTIALLY INFECTIOUS. Each donor is tested and found non-reactive by an FDA approved method for the presence of HIV-I, hepatitis B virus and hepatitis C virus. Where donor testing is not possible, cell products are tested for the presence of viral nucleic acid from HIV, hepatitis B virus, and hepatitis C virus. Testing can not offer complete assurance that HIV-1, hepatitis B virus, and hepatitis C virus are absent. All human sourced products should be handled at the biological safety level 2 to minimize exposure of potentially infectious products, as recommended in the CDC-NIH manual, Biosafety in Microbiological and Biomedical Laboratories, 5th edition. If you require further information, please contact your site safety officer or Scientific Support.

NOTE: Before starting the cell culture process make sure parts not included in this kit have been purchased: Lonza ALI guaranteed NHBE cells (CC-2540S); rat tail collagen type 1, BD Biosciences No. 35-4236; and Costar PET transwell plates, Corning No. 3470. Lonza recommends using a 24-well plate format.

Unpacking and storage instructions

1. Check all containers for leakage or breakage.
2. For cryopreserved cells, remove cryovials from the dry ice packaging and immediately place into liquid nitrogen storage. Alternatively, thaw and use the cells immediately. If no dry ice remains, please contact customer service.
3. BulletKit™ instructions: upon arrival, store B- ALI™ growth basal medium and B-ALI™ differentiation basal medium at 4-8°C; store SingleQuots™ at -20°C in a freezer that is not self-defrosting.
4. ReagentPack™ subculture reagents are sterile-filtered and then stored at -20°C until shipment. Subculture reagents may thaw during transport. They may be refrozen once. If you plan to use within 3 days, store at 4°C. Trypsin/EDTA solution has a limited shelf life or activation at 4°C. If, upon arrival, trypsin/EDTA is thawed, immediately aliquot and refreeze at -20°C. We recommend that the HEPES-BSS and the trypsin neutralizing solution be stored at 4°C for no more than one month.

NOTE: To keep trypsin/EDTA fresh and active after thawing, you may aliquot it into sterile centrifuge tubes and re-freeze at –20°C. Using media or reagents other than what's recommended will void the cell warranty. Please contact Scientific Support if you need help selecting media and/or reagents.

Preparation of B-ALI™ growth and B-ALI™ differentiation media

All work should be done in a laminar flow hood. Decontaminate the external surfaces of all supplement vials and the medium bottles with ethanol or isopropanol. Complete the following 3 steps in order:

1. **Realiquoting the inducer**
   BEFORE reconstituting the medium, realiquot the B-ALI™ inducer SingleQuots™ (No. 00556823) which contains a 1.0 ml stock solution. DO NOT add the 1.0 ml inducer aliquot to the 500 ml B-ALI™ differentiation medium bottle. **The inducer must be re-aliquoted and frozen for future use.** Thaw the 1.0 ml vial and re-aliquot into approximately thirty 33 µl samples in sterile amber tubes or vials or store clear vials in a dark place like an enclosed cardboard box. Immediately transfer the re-aliquoted inducer vials to -20°C.
2. **B-ALI™ growth medium**
   Thaw and add recommended volumes of each SingleQuots™ to the 250 ml bottle of B-ALI™ growth medium using the volumes in table 1. Keep reconstituted media at 4°C. When changing the medium, do not warm up the entire bottle (i.e. for a 24-well plate with 24 inserts, warm up 15 ml of media). After SingleQuots™ are added to basal medium, use within one month.
3. **B-ALI™ differentiation medium**
   From the same thawed SingleQuots™, add each growth factor to the 500 ml bottle of B-ALI™ differentiation media using the volumes in table 1. Store reconstituted media at 4°C. **Do not complete the B-ALI™ differentiation medium with**

inducer until ready to start the airlift and feed cultures (the inducer must be used fresh).

a. The first batch of "complete" B-ALI™ differentiation medium will be prepared the day of the airlift (day 0 of the differentiation process). Use fresh aliquots of inducer for each subsequent media change in the basal chamber.
b. Thaw and mix very well a realiquoted inducer vial and add 10 µl of inducer to every 5 ml B-ALI™ differentiation media or 30 µl for every 15 ml media.
c. When changing media do not warm up the entire bottle (i.e. for a 24-well plate with 24 inserts, warm up 15 ml of media).

SingleQuots™	Volume to add to 250 ml B-ALI™ growth medium	Volume to add to 500 ml B-ALI™ differentiation medium
BPE	1.0 ml	2.0 ml
Insulin	0.25 ml	0.5 ml
Hydrocortisone	0.25 ml	0.5 ml
GA-1000	0.25 ml	0.5 ml
Retinoic Acid	0.25 ml	0.5 ml
Transferrin	0.25 ml	0.5 ml
Triiodothyronine	0.25 ml	0.5 ml
Epinephrine	0.25 ml	0.5 ml
hEGF	0.25 ml	0.5 ml

Table 1. Preparation of the B-ALI™ growth and differentiation media

Thawing of cells / initiation of culture process

Handling of the NHBE is comprised of two steps: short expansion of the guaranteed NHBE cells and differentiation of the expanded NHBE cells.

Expansion of B-ALI™ guaranteed NHBE cells in T-75 flask

1. Do not seed cells directly into inserts. Expand one B-ALI™ guaranteed NHBE cell cryovial into a single T-75 flask in B-ALI™ growth medium.
2. Add 25 ml of B-ALI™ growth medium to the T-75 flask and allow the vessel to equilibrate in a 37℃, 5% CO_2, humidified incubator for at least 30 minutes.
3. Wipe cryovial with ethanol or isopropanol before opening. In a sterile field, briefly twist the cap a quarter turn to relieve pressure, and then retighten. Quickly thaw the cryovial in a 37℃ water bath being careful not to submerge the entire vial. Watch your cryovial closely; when the last sliver of ice melts remove it. Thawing the cells for longer than 2 minutes results in less than optimal results.
4. Resuspend the cells in the cryovial and using a micropipette, dispense **all** cell suspension into the T-75 flask set up earlier. Gently rock the culture vessel to evenly distribute the cells and return to the incubator.
5. Centrifugation should not be performed to remove cells from cryoprotectant cocktail. This action is more damaging than the effects of DMSO residue in the culture.
6. Day 1 after seeding: refeed the T-75 flask with 25 ml pre-warmed B-ALI™ growth medium.
7. Day 2 after seeding: if by day 2 in the morning confluence is greater then 50%, then the cells need to be seeded onto inserts before the end of the day.
8. Day 3 after seeding: the cells should be sufficiently confluent to be harvested for seeding onto the transwell inserts (see figure 1).

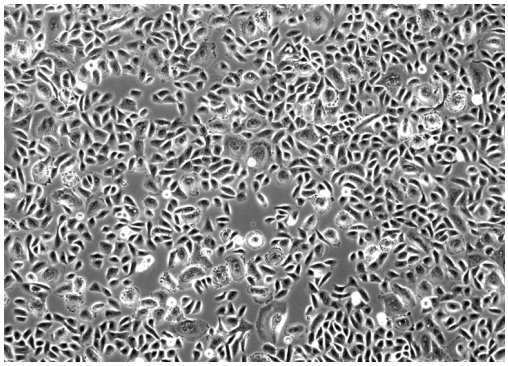

Figure 1. NHBE morphology (10X) in the expansion mode; ~80% confluence in B-ALI™ growth medium

Seeding inserts

The following instructions are for seeding the collagen coated 24-well transwell plate; at this time, we do not recommend using any other size plate.

Collagen coating of the inserts

NOTE: All work is to be performed in a laminar flow hood.

1. By day 3 prepare the filter plate by coating inserts with collagen.
2. Make a 0.03 mg/ml collagen solution. Divide the concentration of the collagen stock solution by the desired working concentration (in this case 0.03 mg/ml).

Example:
4.7 mg/ml (stock concentration) = 157 (dilution factor)
0.03 mg/ml (working concentration)

3. In this example, for each microliter of stock collagen, add 157 µl of PBS in a sterile tube. For 6 inserts a volume of 1 ml of 0.03 mg/ml collagen solution is sufficient.
4. Coat inserts with 100 µl/well from the 30 µg/ml collagen solution. Leave plate in the incubator for 45 min.
5. Aspirate the collagen solution from each well; Add 150 µl PBS per well, gently remove PBS and leave plate in the hood.

Harvesting cells for insert seeding

NOTE: Lonza warrants its Clonetics™ cells only if Lonza subculturing reagents are used. The recommended subculturing reagents for these cells are trypsin/EDTA (CC-5012), trypsin neutralizing solution (CC-5002), and HEPES buffered saline solution (CC-5022). These reagents can be purchased individually or together as part of the Reagent Pack™ subculture reagents (CC-5034).

Trypsinize the T-75 flask, count and plate 50,000 cells per insert in 100 µl B-ALI™ growth medium in the apical chamber, and 500 µl B-ALI™ growth medium in the basal chamber. Follow the protocol below:

1. Warm 5 ml of trypsin/EDTA to 37℃ in a water bath.
2. Allow 10 ml of HEPES buffered saline solution (HEPES-BSS) to come to room temperature.
3. Allow 5 ml of trypsin neutralizing solution (TNS) to come to room temperature.
4. Warm up 15 ml of B-ALI™ growth medium at 37℃ in a water bath. Do not warm up the entire bottle (i.e. for a 24-well plate with 24 inserts requires 15 ml of media).

In a laminar flow hood:

1. Aspirate the medium from the T-75 flask.
2. Rinse the cells with 10 ml of room temperature HEPES-BSS. DO NOT forget this step. The medium contains complex proteins and calcium that neutralize the trypsin.
3. Aspirate the HEPES-BSS from the flask.
4. Cover the cells with 5 ml of trypsin/EDTA solution.
5. Examine the cell layer microscopically.
6. Allow the trypsinization to continue until approximately 90% of the cells are rounded up. This entire process takes about 2-6 minutes.
7. At this point, rap the flask against the palm of your hand to release the majority of cells from the culture surface. If only a few cells detach, you may not have let them trypsinize long enough. Wait 30 seconds and rap again. If cells still do not detach, wait and rap every 30 seconds thereafter.
8. After cells are released, neutralize the trypsin in the flask with 5 ml of room temperature trypsin neutralizing solution. If the majority of cells do not detach within 6 minutes, the trypsin is either not warm enough or not active enough to release the cells. Harvest the culture vessel as described above, and either re-trypsinize with fresh, warm trypsin/EDTA solution or rinse with trypsin neutralizing solution and then add fresh, warm medium to the culture vessel and return to an incubator until fresh trypsinization reagents are available.
9. Quickly transfer the detached cells to a sterile 15 ml centrifuge tube.
10. Rinse the flask with a final 2 ml of HEPES-BSS to collect residual cells, and add this rinse to the centrifuge tube.
11. Examine the harvested flask under the microscope to make sure the harvest was successful by looking at the number of cells left behind. This should be less than 5%.
12. Centrifuge the harvested cells at 1500 rpm for 5 minutes to pellet the cells.
    a. Aspirate most of the supernatant, except for 100-200 µl.
    b. Flick the 15 ml tube with your finger to loosen the pellet.
13. Count viable cells and seed into the apical chamber 50,000 cells per insert in 100 µl B-ALI™ growth medium.
14. Add 500 µl of B- ALI™ growth medium to the basal chamber in all wells containing the inserts.
15. Next day remove media and refeed cultures with prewarmed B-ALI™ growth medium as follows: 100 µl in the apical chamber and 500 µl in the basal chamber. Do not warm up the whole B-ALI™ growth medium bottle but only enough for a media change for the number of inserts utilized.

NOTE: Check morphology and confluence daily. Allow the cells to maintain confluence until day 3 at which time the monolayer should have the appearance from figure 2.

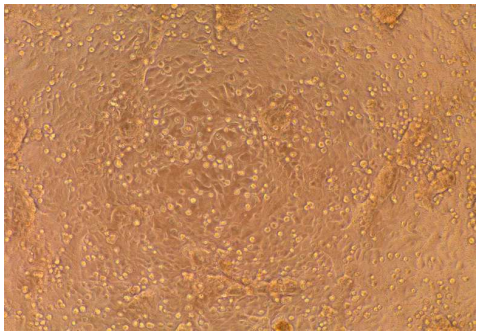
Figure 2. NHBE morphology before airlift

Air lift procedure

1. On day 3 after seeding, warm up sufficient volume of B-ALI™ differentiation medium (as prepared in the media preparation step; see table 1). Thaw and **mix well** one inducer aliquot. Add 10 µl of the inducer for every 5 ml of media. For each media change prepare FRESH complete (including inducer) B-ALI™ differentiation medum by thawing each time a realiquoted inducer vial.
2. Remove B-ALI™ growth medium from the apical and basal chambers and add 500 µl B-ALI™ differentiation medium to the basal chamber ONLY. This is the "airlift" step. DO NOT add medium to the apical chamber - this should remain "dry" until the end of your culture period. Feed every second day by adding 500 µl of pre-warmed B-ALI™ "complete" differentiation medium (including B-ALI™ inducer) to the basal chambers. For weekend schedule, feed Friday at the end of the day and next Monday first thing in the morning.

Ordering information

CC-2540S	B-ALI™ guaranteed NHBE cells	≥500,000 cells
00193514	B-ALI™ BulletKit™	Contains B-ALI™ growth basal medium, B-ALI™ differentiation basal medium, and B-ALI™ SingleQuots™ kit
CC-5034	ReagentPack™	Contains 100 ml each of trypsin/EDTA, HEPES, and TNS

Product warranty

Lonza warrants its cells only if Lonza media, reagents, and protocols are followed.

Quality control

The B-ALI™ BulletKit™ is tested on B-ALI™ guaranteed NHBE cells for TEER, mucin secretion and cilia formation during 20 days in culture. The media kit contains sufficient volumes for 30 days of post airlift culture in a complete 24 well transwell format. HIV-1, hepatitis B and hepatitis C are not detected for B-ALI™ guaranteed NHBE cells. For detailed information concerning QC testing, please refer to the certificate of analysis (COA).