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702.B.3 URMC HTC Lung Tissue Digestion SOP + Worksheet 052820

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We use this protocol and it's working

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Abstract

Lung MAP HTC - BioRepository for Investigation of Neonatal Diseases of the Lung (BRINDL)

702.B.3 Combined SOP and Worksheet HTC_Lung_Tissue_Digestion_0100616rev061220

Purpose and Scope of the Procedure or Laboratory Assay

1.To dissociate and isolate single cell suspensions from human lung samples to meet the standards and needs of the Human Tissue Core.

2.This protocol covers manual gross dissociation of pieces of lung tissue (lobes or cubes) followed by enzymatic digestion and freezer storage as mixed populations of filtered and counted cells for which viability has been determined.

3.A separate protocol details the technique of freezing, thawing and further identifying and sorting these mixed cell populations.

Scientific Principles or Validation of Procedure

1.Fujino *et al.* Am J Respir Cell Mol Biol. 2012 Apr;46(4):422-30

2.Barkauskas, C. E., Cronce, M. J., Rackley, C. R., Bowie, E. J., Keene, D. R., Stripp, B. R., ... & Hogan, B. L. (2013). Type 2 alveolar cells are stem cells in adult lung. *The Journal of clinical investigation*, *123*(7), 3025.

Guidelines

Considerations:

1.This process is resource intensive. It is suggested that for a small lung donation (e.g. less than 6 months old), two people are available to complete the digestion; for a medium lung donation (e.g. six months to 18 months) that three people are available; for a large lung (above 18 months of age) that four people are present for the digestion.

2.It is suggested that the GentleMACs system be run as few times as possible for a sample. Running the GentleMacs disruption program > once has an adverse effect on cell viability.

3.When diluting the enzyme for digestion, target so that it is ready for use within 15 minutes to ensure that the integrity of the enzyme is not compromised.

4.If at all possible, one should complete the ACK *red blood cell lysis) step only once, as repeats of this step could lead to a compromise in cell viability.

5.When freezing down large numbers of cells, eg. 4000 $\times 10^6$ or greater for CBL, freeze down in 70 cryovials.

6.Certain enzymes, depending on their specific activity and/or purity, may cleave surface proteins (ex CD8).For this reason, we typically perform lung digestion with a cocktail of enzymes but also with a collagenase only +/- DNaseI cocktail depending on what the downstream use will be

Materials

Materials, Equipment & Reagents including Formulations

1.Digestion Buffer (for 1 L):

i)Divalent cation free DPBS with 10 mM HEPES-NaOH (pH 7.4)(use 10 ml of 1M stock), 150 mM NaCl (8.77g), 5 mM KCl (0.37), 1 mM $MgCl_2$ (0.1g), 1.8 mM $CaCl_2$ (0.2g) plus:

(1)Collagenase Type A from Clostridium (Roche: 11088793001 or similar); 2 mg/ml final (powder) (0.15 units/mg and 0.30 units/mL)

(a)Stored as lyophilized dry powder at 2-8°C, dry enzyme is stable for 6-12 months at 2-8°C.

(2)Dispase II (Gibco: 17105-041 or similar); 1 mg/ml final (liquid), 1:5 dilution of total digestion cocktail volume (50 units/mL stock, 10 units/mL)

(3)Elastase: Twice crystallized from Porcine Pancreas (Worthington:ESL or similar) 0.5 mg/ml final (powder) (3 units/mg and 1.5 units/mL)

(a)Elastase is unstable at pH ≤ 3.5. When stored as a dry powder the enzyme is stable for 6-12 months at 2-8°C. Elastase product codes: ES and ESL have poor solubility at neutral pH and at concentrations greater than 0.25%. It is suggested that primary solutions be made in KCl or alkaline buffers and diluted into the reaction mixtures or media, compensating for ionic strength or pH changes.

(4)DNase- Deoxyribonuclease-I (Sigma-DN-25 or similar) from Bovine pancreas; 2 mg/ml final (powder) (≥400 Kunitz units/mg of protein, ≥85% protein in prep, and 800 units/mL)

(a)10 mg/mL solution of DNAse I in 0.15 M NaCl may lose <10% of its activity when stored for a week in aliquots at −20 °C. The same solutions stored in aliquots at 2-8 °C can lose approximately 20% activity. It remains active for up to five hours at 60 °C between pH 5 and 7, and loses activity in <10 minutes at 68 °C. Activity of 1 mg/ml solution is lost at the rate of 6%/hour in acetate buffer (pH 5.0) or tris buffer (pH 7.2).

(5)Note: Trypsin digestion is not recommended as it may degrade antibody epitopes.

2.RBC lysis Buffer

i)ACK Lysing Buffer, Biowhittaker, Cat#10-548E or equivalent

3.Enzyme Neutralization Buffer

i) Divalent cation free DPBS + 10% FBS (A standard lot of FBS is reserved for LungMAP work)

4.Freezing Media 90% FBS + 10% DMSO

5.Disposables:

i)Single Edge Razor Blades and 100mm Culture Dish (for razor method)
ii)or C-tubes for GentleMACS (for GentleMACS method)
iii)100 um nylon mesh tube-top filters
iv)0.04% Trypan blue exclusion dye in saline
v)10% bleach solution
vi)Waste Pan
vii)Absorbent towels
viii)Sterile serological pipets; 50, 25,10, 5, 2, 1 mL size
ix)Sterile pipet tips; 1000, 200 and 20 µl

x)Sterile 15mL or 50mL conical polypropylene tubes
xi)Test tube racks for 15mL or 50mL conical tubes
xii)Eppendorf tubes for diluting trypan counts
xiii)Isopropyl alcohol for Mr. Frosty containers

6.Equipment:
i)Table Top Centrifuge: BD Allegra X-12 centrifuge with SX4750 swinging bucket rotor and safety inserts with seals, or equivalent
ii)Biological Safety Cabinet, Class II
iii)Hemocytometer and microscope for cell counting
iv)Pipet-men and Pipet-aid
v)Dissecting forceps and scalpel
vi)Two pairs of sharp large scissor and Two pairs or sharp small scissors
vii)Mr. Frosty, Nalgene Cryo 1°C Freezing container Cat# 5100-0001
viii)Cryogenic vials, Nalgene, Cat# 5000-1020 or equivalent
ix)Cryogenic vial racks
x)-80°C freezer and liquid nitrogen cryopreservation system for long-term storage
xi)GentleMACS Tissue Octo Dissociator with Heaters (Miltenyi Biotec Inc San Diego, CA)

Safety warnings

Safety Considerations Personnel will adhere to safe work processes outlined in U.S. Public Health Universal Precautions Guidelines for use of human blood and body fluids. PPE will be used including lab coat, closed shoes and gloves. Additional PPE is recommended for potential SARS-CoV2+ specimens. All activity will be behind shield of biosafety cabinet and/or with mask and safety glasses. Biosafety level 2 (or 2+ for SARS CoV2) practices will be followed and the work performed in the designated lab space, which is covered by annually updated IBC approved protocol. All institutional biosafety measures are followed in any manipulation of these human tissues.

Procedure

1 Record details of procedure in the Tables at end of this Worksheet. At HTC, the contents of the Worksheet needs to be transferred to BRINDL Database as soon as possible.

2 Record the time the lung tissue was received for dissociation (see Table 1)

3 Weigh the total lung tissue received for dissociation (see Table 1)

4 Record the time the spleen and thymus was received for dissociation (if applicable) (see Table 2)

5 Weigh the total tissue received of spleen and thymus for dissociation (if applicable) (see Table 2)

6 Weigh the total lung tissue to be used for dissociation (see Table 1)
i)If multiple types of tissue or protocols used, indicate on the Worksheet

7 Dissect pleura and larger airways from the lung tissue. To cut out airway +/- pulmonary arteries, follow down the major branches of the bronchi.

8 Cut the lung tissue into large pieces. Set aside the large airways (bronchi, BRR) for dissociation separate from remaining lung tissue. To ensure an accurate representation of the sample, cut remaining lung tissue into smaller pieces and combine lung tissue from multiple lobes (=CBL, combined lung lobes, if applicable) before dividing lung tissue into C-tubes.

9 Make enzyme solutions as follows, and prewarm at 37° C just a few minutes prior to use (to ensure optimal enzyme activity) 37 °C

9.1 **i)Calculating the total number of C-tubes:**
C-tubes for lung tissue (max 6 g tissue per C-tube): _____
C-tubes for lung tissue – collagenase only (col only, max 2 g tissue per C-tube): _____
C-tubes for airway (BRR, max 6 g tissue per C-tube): _____
C-tubes for spleen (around 2 g tissue per C-tube) (if applicable): _____
C-tubes for thymus (around 2 g tissue per C-tube) (if applicable): _____
C-tubes total: _____

9.2 **ii)Calculating the total volume of digestion cocktail, buffer and enzymes:**

Total Volume of Digestion Cocktail (DC) needed = Total # C-tubes _____ x 10 = _____

Digestion Buffer (DB) = Total Volume of DC needed – Vol of dispase

= ___-___ = ____ml DB needed

Enzymes Used	Weight Added to Buffer (mg)	Volume Added to Buffer (mL)	Final Conc. (mg/ml)
Collagenase	Total vol. DC x 2 =	N/A	2 mg/ml
DNase	Total vol. DC x 2 =	N/A	2 mg/ml
Elastase	Total vol. DC / 2 =	N/A	0.5 mg/ml
Dispase	N/A	Total vol. DC / 5 =	1mg/ml

10 Re-weigh the lung tissue (CBL) and the airway tissue (BRR) dissociated and record on worksheet (Table 1)

i) CBL weight: _____g

ii) CBL-COL weight: _____g

iii) BRR weight: _____g

11 Record weight of spleen dissociated, if applicable: (see Table 2)

Record weight of thymus dissociated, if applicable: (see Table 2)

12 Using GentleMACS Tissue Dissociator:

12.1 i)Add no more than 6 g of lung tissue to Miltenyi C tubes containing 10 ml of pre-warmed 1x digestion cocktail (collagenase, dispase, elastase, DNase diluted in digestion buffer). Add no more than 2 g of lung tissue to Miltenyi C tubes containing 10ml of pre-warmed 1x Col Only digestion cocktail (collagenase only). Add no more than 6 g of airway to separate Miltenyi C tube, to be digested under identical conditions as lung tissue, but kept separate from lung tissue throughout processing procedure

12.2 ii)If total weight of tissue exceeds 8 C tubes, exclude some from digest and flash freeze as CBL-BPS, and record weight of biopsy:_____g, and time biopsy was frozen: _____

12.3 iii)Cut lung tissue and airway into smaller pieces using scissors in C-tube

12.4 iv)Cut spleen and thymus tissue into smaller pieces using scissors in C tube (if applicable)

12.5 v)Dissociate lung tissue, airway, spleen, and thymus (if applicable) using the GentleMACS mouse tumor implant program 01.01

12.6 vi)Record time tissue added to enzyme and time GentleMACS run ends (See Table 1 and 2)
Enzyme_____GentleMACS _____

12.7 vii)After GentleMACS run, loosen caps and place in 37^{o}C 5% CO_2 incubator for 60 mins. Invert to mix each tube every 15 minutes (pull stuck stringy stuff off the cap and submerge in enzyme

13 Dilute the suspension with 5 ml of cold sterile DPBS containing 10% FBS and pass it through a 100 micron cell strainer into a 50 ml conical tube

14 Disrupt the residual lung fragments in the strainer using a syringe plunger and add 10 ml cold sterile DPBS containing 10% FBS to recover lung cells from the dish / C tube and through the strainer
i)Aim for at least 20ml of volume through filter into new 50ml conical. Expect filtering to take as long as 30-45 minutes per tube, depending on age of donor (older donor takes longer)

15 Wipe down caps and lips of tubes and place in centrifuge cups which have been equilibrated to 20°C in balanced positions. Attach aerosol containment lids. Pellet cells x10 mins at 1000xg, 4 ^{o}C

16 Gently decant supernatant into waste bucket containing 10% bleach and blot tube lip, watch the pellet to ensure no cell loss

17 Re-suspend cell pellet from each 50 ml conical tube in 10-30 ml of RBC lysis buffer, depending on redness of cell pellet. Combine all cell pellets, and divide evenly among twice the number of tubes to ensure equal treatment. Incubate for 5 mins at room temperature

18 Neutralize the reaction by bringing the volume up to 50 ml with cold sterile DPBS containing 10% FBS

19 Pellet cells for 10 mins at 800xg at 4 ^{O}C

20 Carefully decant supernatant into waste bucket containing 10% bleach (Pellet can still be loose)

21 Re-suspend the cells in cold DPBS containing 10% FBS (typically 40ml for CBL and 20ml for BRR, SPL, THY, use more volume if pellet is 5ml or greater) for counting cells by trypan blue exclusion method

22 Pass resuspended cells through 100 micron cell strainer to eliminate debris generated during RBC lysis

23 Count by hemocytometer:
Dilute 10µL of cells in 90µL 10% FBS in DPBS.Add 100µL 0.4% -4% Trypan Blue solution.
Both large and small cells are counted (="total). % of cells that are relatively large is calculated ("large").
Either the total or the large cell count is used for purposes of freezing cells down.
Optional: i)Take 50 million cells out for the CBL (full enzyme digestion) and pellet in 15mL conical tube.
Use this cell pellet to freeze first five vials down at 10 million cells per vial

24 Pellet cells for 10 mins at 800xg at 4 ^{O}C. Decant supernatant into waste bucket containing 10% bleach

25 Resuspend cells in appropriate volume of freezing media (90% FBS, 10% DMSO). Aliquot cells in freezing media into cryovials (1 mL per cryovial except last), using a micropipette
i)After the first 5 vials of 10×10^{6} cells/vial, the goal is 40 vials with $40 - 60\times10^{6}$ cells/vial;
if there is a large yield of cells, freeze down in up to 70 vials
ii)Measure the volume dispensed in last cryovial and record on worksheet table

26 Label cryovials with appropriate convention:
For example: D###-CBL-MIX-## for lung cells, D##-CBL-MIX-COL for lung cells digested with collagenase only, D###-BRR-MIX-## for airway cells, D###-SPL-AMIX for spleen cells, and D###-THY-AMIX for thymus cells

27 Place cryovials in Mr. Frosty, and freeze at -80 ^{O}C
i.). Record time Mr. Frosties are placed in the -80 ^{O}C (Freeze time Table 1 and 2)

28 After at least 24 hours, and as soon thereafter as possible, transfer cells to liquid nitrogen tank for long-term storage. Enter vials and location into BRINDL Database (if applicable)

Analysis of Results :

29 Use Tables below or Worksheet (702.A.X) and BRINDL Database CRF to Record the following:

a.Personnel Involved in Isolation
b.Lung Lobe or Block(s) Used for Dissociation
c.Weight of Tissue Received for Dissociation
d.Weight of Tissue Used for Dissociation
e.Method of Mechanical Dissection Used
f.Enzymes and concentrations used in digestion
g.Calculate and Record:
i)Total Cell Number Yield
ii)Total Large Cell Number Yield
iii)% Viability
iv)% of Total Large Cell Yield
v)Number Viable Cells
vi)Total Cell per gram of tissue
vii)Total Large Cell per gram of tissue
viii)Numbers of Vials Frozen
(1)# Cells per Vial
(2)Volume of Last Vial

Table 1.

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Standard Operating Procedures 702.B.3_HTC_100616rev061220

Table 1:

Specimen ID #	Start Date & Time	Weight of Tissue (g)	Live Cell Count Cell Count Done By: _____		Dead Cell Count	Enzymes used:	% Viable	# Viable Cells/gram of Tissue (x 10^6/g) Based on which cell count	# Vials Frozen	# Cells per Vial (x 10^6)
D_____-CBL	Tissue received: In enzyme: GentleMACS run ends: Frozen:	Total received: Total digested:	Hemocytometer: _____ **total** live _____x10^6/mL _____x10^6 total	Hemocytometer: _____ **large** live _____ x10^6 total large _____ % large	Hemocytometer: _____ dead Resuspended in _____ mL for count	Enzymes used:	Hemocytometer:		Vol. of last vial:	Last vial:
D_____-CBL	Tissue received: In enzyme: GentleMACS run ends: Frozen:	Total received: Total digested:	Hemocytometer: _____ **total** live _____x10^6/mL _____x10^6 total	Hemocytometer: _____ **large** live _____x10^6 total large _____ % large	Hemocytometer: _____ dead Resuspended in _____ mL for count	Enzymes used:	Hemocytometer:		Vol. of last vial:	Last vial:
D_____-BRR	Tissue received: In enzyme: GentleMACS run ends: Frozen:	Total received: Total digested:	Hemocytometer: _____ **total** live _____x10^6/mL _____x10^6 total	Hemocytometer: _____ **large** live _____x10^6 total large _____ % large	Hemocytometer: _____ dead Resuspended in _____ mL for count	Enzymes used:	Hemocytometer:		Vol. of last vial:	Last vial:

Table 2.

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Table 2:

Specimen ID #	Start Date & Time	Weight of Tissue (g)	Live Cell Count Cell Count done By _____		Dead Cell Count	Enzymes Used	% Viable	# Viable Cells/ gram of Tissue (x 10^6/g) Based on which cell count	# Vials Frozen	# Cells per Vial (x 10^6)
D_____-SPL	Tissue received: In enzyme: GentleMACS run ends: Frozen:	Total received: Total digested:	Hemocytometer: _____ **total** live _____x10^6/mL _____x10^6 total	Hemocytometer: _____ **large** live _____ x10^6 total large _____ % large	Hemocytometer: _____ dead Resuspended in _____ mL for count	Enzymes used:	Hemocy-tometer:		Vol. of last vial:	Last vial:
D_____-THY	Tissue received: In enzyme: GentleMACS run ends: Frozen:	Total received: Total digested:	Hemocytometer: _____ **total** live _____x10^6/mL _____x10^6 total	Hemocytometer: _____ **large** live _____ x10^6 total large _____ % large	Hemocytometer: _____ dead Resuspended in _____ mL for count	Enzymes used:	Hemocy-tometer:		Vol. of last vial:	Last vial:
D_____-	Tissue received: In enzyme: GentleMACS run ends: Frozen:	Total received: Total digested:	Hemocytometer: _____ **total** live _____x10^6/mL _____x10^6 total	Hemocytometer: _____ **large** live _____ x10^6 total large _____ % large	Hemocytometer: _____ dead Resuspended in _____ mL for count	Enzymes used:	Hemocy-tometer:		Vol. of last vial:	Last vial: