

Human Alveolosphere Maintenance and Passaging Protocol – Kotton Laboratory

INTRO

This protocol describes an approach to maintain three-dimensional (3D) culture of purified PSC-derived lung SFTPC+ alveolar cells as published in: Jacob et al. Cell Stem Cell. 2017.

REAGENTS

A) cSFDM (complete serum free differentiation media):

Volume for 500 ml	Final concentration	Reference
375 ml IMDM	75%	ThermoFisher 12440053
125 ml Ham's F12	25%	Cellgro 10-080-CV
5 ml B-27 (with RA) supplement	1%	Invitrogen 17504-44
2.5 ml N-2 supplement	0.5%	Invitrogen 17502-048
3.3 ml BSA (7.5% stock)	0.05%	Invitrogen 15260-037
1 ml Primocin (100 µg/ml stock)	200 ng/ml	Invivogen NC9141851
5 ml Glutamax 100X	1X	ThermoFisher 35050-061
500 µl Ascorbic Acid (50 mg/ml stock)	50 µg/ml	Sigma A4544
1.5 ml MTG (from 26 µl in 2 ml IMDM)	4.5x10 ⁻⁴ M	Sigma M6145

B) CK+DCI Media

cSFDM Base
3uM CHIR99021 (Tocris 4423)
10ng/ml rhKGF (R&D 251-KG-010)
50nM Dexamethasone (Sigma D4902)
0.1mM 8BrCAMP (Sigma B7880)
0.1 mM IBMX (Sigma I5879)

C) Other Reagents

Rho-associated kinase inhibitor (Y-27632, Tocris 1254)
Growth Factor-Reduced Matrigel (Corning 356231)
Dispase (ThermoFisher 354235)
0.05% Trypsin-EDTA (1X, phenol red) (Life Technologies, 25300-120)

PROTOCOL

Alveolosphere dissociation and passaging method

1. Thaw 3D matrigel on ice and prepare CK+DCI media beforehand.
2. Aspirate media from matrigel drop.
3. Add 1 ml dispase (2mg/ml) to break apart matrigel drop. Leave at 37C for 1 hour, pipetting up and down once after ~30 mins.
4. Transfer 1ml dissociated organoids in dispase into a 15ml conical, add 10ml IMDM or empty media to wash.
5. Centrifuge 300g x 5 min, aspirate supernatant.
 - a. If a clear haze is seen above the pellet, the dispase has not totally dissolved and more dispase can be added to the pellet for another 20 minutes at 37C.
6. Aspirate leftovers with a P200 tip, leaving as little supernatant as possible.
 - a. It is important to remove all dispase since it will dissolve matrigel in culture as well.

7. Resuspend cells in 1ml trypsin/well, incubate at 37C for 10-12 minutes, until pipetting only 3-5 times results in single cell suspension.

Dissociate cells in a tissue culture plate so that you can visualize them in a microscope. Cells are sensitive to over-pipetting at this stage, so leave them in trypsin long enough for most of the dissociation to be enzymatic. If alveolospheres have not dissociated into single cells by 12 minutes, spin down, add fresh trypsin, and leave another 5 minutes

8. Stop with FBS-containing media, spin at 300g x 5min, wash once with 10ml IMDM or empty media.
9. Count and resuspend cells in 3D matrigel at desired concentration (400 cells/ul matrigel, 50-100ul matrigel drops in 12-well plate).
10. Place in 37C incubator for 20 minutes *without* adding media to allow the matrigel to solidify.
11. Add 1ml CK+DCI+ 10 μ M Y-27632 media over matrigel drop.
12. After 48-72 hours, change media to CK+DCI *without* 10 μ M Y-27632. Be careful when aspirating to avoid dislodging the matrigel drop. Aspirate out roughly half the media, and change to new media (ex. 1 ml/well new media in 12-well plate).
13. Change media every 2 days, or as necessary.

Alveolosphere freezing method

1. Dissociate alveolospheres into single cell suspension (as below):
 - a. Aspirate media from matrigel drop.
 - b. Add 1 ml dispase (2mg/ml), leave at 37C for 1 hour, pipetting up and down once after ~30 mins.
 - c. Transfer 1ml dissociated organoids in dispase into a 15ml conical, add 10ml IMDM or empty media to wash.
 - d. Centrifuge 200g x 4min, aspirate supernatant, repeat once more.
 - e. Resuspend cells in 1ml trypsin/ well, incubate at 37C for 5-10 minutes.
 - f. Pipet up and down 5-10 times, stop with FBS-containing media.
 - g. Spin at 300g x 5min, wash once with 10ml IMDM or empty media.
2. Resuspend in 1ml Freeze Media/well (60% FBS, 30% CK+DCI, 10%DMSO) and freeze in a cryovial (first at -80C overnight, then long term storage at -150C).

VERSION HISTORY

2017-07-10	Drafted by Anjali Jacob.	AJ
2017-08-09	RA concentration changed from 50nM to 100nM	LS
2018-04-03	Removed progenitor stage from protocol – see independent protocol to avoid confusion	FH
2020-03-20	Shortened to maintenance, passaging and freezing for COVID-19 collaborators	JH/OTH