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hPSC Passaging and Propagation on laminin521 V.1

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We use this protocol and it's working

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Abstract

hPSC Passaging and propagation using Laminin521 and EDTA. Laminin actively supports survival, prevents spontaneous differentiation, and increases plating efficiency, thus net expansion, and enables 100% confluent single cell layer epithelial monolayer culture. EDTA-based (Gentle Cell Dissociation Reagent) minimizes stress and enhances viability. Cell lines require little or no adaptation.

Attachments

PDF

EDTA (Gentle Cell) P...

95KB

Guidelines

Survival in as single cells is supported by ROCKi at 10uM, which blocks apoptosis downstream of loss of cell-to-cell E-cadherin signalling. We routinely use 20uM at plating to ensure maximal survival. Thiazovivin 1uM is equivalent to ROCKi 10uM. CEPT cocktail is superior to either. CloneR (StemCell Tech) is best for single cell cloning survival but ingredients and mechanism is not published. Standard spit is 1:10, i.e. **50K/cm2**, e.g. ~0.5M cells/6wp well). ROCKi or equivalent lasts for 2 days. On day 2 after passage if cells have not reached self supporting quorums (>5 cells/colony) re-feed with ROCKi supplemented medium

Seeding density: split cells at 1:10 or 1:20 bby area to a new well or count and seed at 0.05M cells/cm2. Unlike other substrates cells can be seeded at >0.05M/cm2 without ROCK inhibitor Y-27632 (ROCKi) since they will migrate to form colonies before they die. Lower seeding density does requires ROCKi even on laminin.

IMPORTANT: to ensure **normalized cell seeding density**, it is important to mix cell suspension immediately (<30sec) before removing aliquot for counting, and again immediately before seeding. Single cells soft-pellet quickly by gravity. If seeding takes too long, periodically mix full volume of cell suspension. If seeding multiple plate types, adjust **master seeding stock** to a fixed concentration and calculate the volume to add per well type well.

Confluent density:
Cells on laminin 521--unlike other substrates--can be brought to 100% confluency without differentiation. They will pack in at 0.5M cells/cm2 in a completely flat epithelial sheet with apical pole up, continue dividing, but not differentiate, resembling a homogeneous honeycomb appearance. We typically passage at 100% confluence or just before, but can passage up to 4 days post-confluence as long as no differentiiaton is observed (it should not be).

Feeding volume and frequency: Seed cells in at least 2.5 ml/6wp well. during the first half week, if using StemFlex, days may be skipped. As cells proliferate, increase feeding volume and feed daily. After 1 week, feed volume is 7-8ml/6wp well and 8-9ml if 100% confluent.

Passage interval: generally ~1/week. see confluent density note above.

Other medias:
This protocol is compatible with any common hPSC medium, conditioned medium, MTsr1 (plus), conditioned medium, etc. Increase feed frequency and do not skip-days feeding if media is not additionally pH buffered and has stabilized FGF (like StemFlex)

Other substrates
We have not tested other suppliers laminin521 substrates. Passaging protocol works well for substrates other than laminin521 (geltrex or vitronectin). ROCKi or equivalent is required for single cell passage on these substrates. Passage before colonies are too large (crowding in the center, or vertical growth) or begin to touch (can spur differentiation).

Materials

	MATERIAL	SUPPLIER	CATALOG NUMBER	USE
	Gentle Cell Dissociation Reagent	STEMCELL Technologies	100-0485	Cell dissociation
	Human Recombinant Laminin 521	Biolamina	LN521-05	Plate Coating
	Y-27632 dihydrochloride	R&D Systems	1254/50	ROCK inhibition
	StemFlex	Thermo	A33493	PSC media
	CEPT cocktail kit	Tocris	7991	PSC survival

Troubleshooting

Plate Coating

1 **PREPARE LAMININ WORKING SOLUTION:** Dilute laminin521 stock (100ug/ml) to 5 ug/mL in PBS with Mg2+ and Ca2+, (1:20 dilution). IMPORTANT: Mg and Ca cations in PBS required for laminin function, do not use PBS without Mg2+ and Ca2+

1.1 VOLUMES OF WORKING SOLUTIONS REQUIRED FOR DIFFERENT PLATES

	REAGENT	4wp (0.5 mL/well)	6wp (1.5 mL/well)	24wp (0.25 mL/well)
	Laminin521	0.11 mL = 110 uL	0.5 mL = 500 uL	0.4 mL = 400 uL
	PBS(Final Volume for 1 plate with spare)	2.2 mL	10 mL	8 mL

2 Coat laminin521: minimum 2 hrs at 37 degrees. Plates may also be coated at 4 degrees sealed 1-2 days, but warm in the incubator 1 h before use.

*** Used laminin521 may be stored at 4 degrees for 1 month and reused 3 x.
IMPORTANT: Re-supplement with 1:40 (2.5 ug/mL) fresh laminin at each re-use.

Cell Passaging

3 Aspirate medium

4 Wash with PBS -/- (with 25mM glucose, optional)

5 Add **500**-1000 uL (per 24wp well) / 1.5 - 2.0 mL (per 6wp well) of Gentle Cell Dissociation Reagent (GCDR) or Accutase

IMPORTANT: Accutase should NEVER be warmed in the water bath. It will activate the enzymes and the whole flask will be useless. Warm at room temperature. Activate at 37 degrees inside the incubator only

6 Incubate at 37 degrees till cells slough with gentle touch, 10-30 min for GCDR, usually 5-10 min for accutase. (check every 10 min by moving the plate gently)

7 Pipet with p1000 to remove cells (>90%), and transfer to a 15 mL tube containing DMEM

8 Gently triturate to single cells (and homogenate for cell counting/seeding)

9 Rinse well 1x with fresh 1mL GCDR and pool to 15ml (repeat if necessary)

10 Spin 300 ×g for 5 minutes

11 Aspirate and resuspend in 1 ml PBS -/- (+glucose if available) or GCDR (**steps 8-10 are optional**)

12 Top up to appropriate counting volume (5 mL, ~1M cells/mL), homogenize and remove 20 uL for count - prepare counting sample in an eppendorf tube before applying to the slide.

13 Spin 300×g for 5 min

14 Count while spinning

15 Resuspend at appropriate [ ]** in StemFlex + Suppl

** standard split ration for weekly passage is 1:10-1:20 =0.025-0.05M cells/cm2

16 If using laminin521, collect/save coating solution right before seeding and store at 4 degrees. Do not let substrate dry.***

Protocol references

Chromosomal instability during neurogenesis in Huntington's disease. Albert Ruzo [1], Gist F Croft [1], Jakob J Metzger [1 2], Szilvia Galgoczi [1], Lauren J Gerber [1], Cecilia Pellegrini [1], Hanbin Wang Jr [1], Maria Fenner [1], Stephanie Tse [1], Adam Marks [1], Corbyn Nchako [1], Ali H Brivanlou
Development. 2018 Jan 29;145(2):dev156844. doi: 10.1242/dev.156844.

CEPT cocktail
Chen Y., Tristan C.A., Chen L., Jovanovic V.M., Malley C., Chu P.H., Ryu S., Deng T., Ormanoglu P., Tao D., et al. A versatile polypharmacology platform promotes cytoprotection and viability of human pluripotent and differentiated cells. Nat. Methods. 2021;18:528–541. doi: 10.1038/s41592-021-01126-2.