ES culture medium

DMEM-F12(invitrogen 21331-020)	500 ml
NON-ESSENTIAL AMINO ACID (x100) (invitrogen 11140)	6.3 ml
200 mM L-GULUTAMINE(x100) (invitrogen 25030)	6.3 ml
KSR（final 20%）(invitrogen 10828)	125 ml
2-MERCAPTOETHANOL(x1000stock)（final 0.1 mM）	630uL

bFGF (Peprotech 315-09final 10ng/ml)

penicillin/streptomysin

Dissociation buffer

2.5% Trypsin	1 ml	
10mg/ml Collagenase IV	10 ml	(Invitrogen17104-019)
KSR（final 20%）	20 ml	
100 mM $CaCl_2$（final 1 mM）	1 ml	
1×PBS	68 ml	

(Filtration is needed for Collagenase IV and $CaCl_2$.)

Freezing buffer

2 M DMSO,

1 M Acetamide,

3 M Propylene glycol (1,2-Propanediol)

/human ES medium

Dissolve 0.59g Acetamide in 6ml of ES medium

Filtrate 0.22um

Add 1.42ml of DMSO and 2.2ml of Propylene glycol

Add ES medium (final 10ml) and mix

Make aliquot and stock in -80C

Cells were cultured on a feeder layer of inactivated MEF cells that were seeded at 1x 10^6 per 10cm plate) in hES medium.

For passaging, hES cell colonies were detached and recovered as small clumps from the feeder layer by treating them with dissociation buffer, followed by sucking the dissociation buffer, tapping the cultures and flushing them with a pipette*. Two volumes of culture medium were added, and the detached ES cell clumps were broken into smaller pieces (10–20 cells) by gently pipetting them several times. The passages were done at a 1:2 to 3 split ratio**.

For storage, the ES cell colonies were recovered as small clumps, suspended in 200ul of ice-cold Freezing buffer, and quickly frozen in a 2-ml cryogenic tube by directly submerging the tube in liquid N2.
About thawing cell lines, the mixture of ES media which is pre-warmed in 37C, is directly added to the frozen tube and is thawed by pipetting as quickly as possible. Frozen tubes should be in liquid N2 just before thawing and should not use water bath. After centrifugation, cells are resuspended and pipetted, and cells are plated onto a feeder layer.

*These cells can be mechanically passaged.
**If we use ROCK inhibitor(Y-27632), the survival and cell growth are improved.

Y-27632 (Calbiochem; water soluble) was added to culture medium at 10 mM 1 h before detaching the cells from the feeder layer and also upon seeding the cells onto a new MEF layer. A single half day treatment of Y-27632 was sufficient for enhanced survival of dissociated hES cells in low-density adhesion culture on MEF cells.

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