

PROTOCOL

# EDTA-based Passaging of ESI Human Embryonic Stem Cells

## INTRODUCTION

The protocol below outlines passaging of 1 well of a 6-well plate containing ESI human embryonic stem (hES) cells cultured on Matrigel® hESC-qualified Matrix at a 1:6 split ratio using an EDTA-based dissociation solution.

Passaging using an EDTA-based dissociation solution can minimize cell death and allows for rapid cell attachment upon re-plating and resumption of the cell cycle<sup>1</sup>. Over time, this methodology enables rapid expansion of cell lines with minimal introduction of environmental and handling stresses.

hES cell cultures should be passaged when the colonies have reached their ideal colony size without becoming overgrown, or when colonies have reached approximately 60 to 75% confluency across the well. Allowing the colonies to grow too large or at a higher confluency can adversely affect viability after dissociation and induce selective pressure on the cell cultures, even if the cells appear healthy prior to passaging. ESI hES cells cultured following this protocol are generally passaged between a 1:6 and 1:12 split ratio.

## WHEN TO PASSAGE

- Approximately every 3 to 4 days in culture.
- Cultures are beginning to differentiate.
- Colonies have become large but not yet overgrown.
- Cultures have reached 60 to 75% confluency across the well.

## ! IMPORTANT TIPS

### Timing is critical.

Cells can easily over-dissociate if left in EDTA solution too long or if incubated at elevated temperatures. Unlike enzymatic dissociation, human ES cells in EDTA solution must be incubated at room temperature for best results. Passage only 1 to 2 wells at a time, and monitor the progress of the cell dissociation under a microscope once the EDTA solution is added.

### Gentle passaging and pipetting.

Keep small colony aggregates. Do not dissociate into single cells, as this often leads to poor survival and abnormal karyotypes<sup>2,3</sup>. When passaging, aim to plate cell aggregates of approximately 20 cells each.

### Split ratios are variable.

The split ratio will change for each passage based on cell number, culture health, technique, and other variables. Adjust split ratios based on experience for each cell line and user. If unsure of what split ratio to use, plate the collected cells into multiple wells in a range of seeding densities. (Plate 3 wells with 1:6, 1:12, and 1:18 split ratios, for example).

## REQUIRED MATERIALS

### Equipment

- 37°C humidified incubator (5% CO<sub>2</sub>)
- 15 mL conical tube
- 5 mL pipets
- 6-well tissue culture plate

### Reagents

- ESI hES cells
  - hES cell culture media
  - Corning® Matrigel® hESC-qualified Matrix
  - Dulbecco's PBS, without Ca<sup>2+</sup> and Mg<sup>2+</sup>
  - 0.5 mM EDTA solution (see Appendix for recipe)
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## EXPERIMENTAL PROTOCOL

### PREPARE PLATES & MEDIA:

1. Coat 6 wells of a 6-well tissue culture plate with Matrigel hESC-qualified Matrix as directed by manufacturer.
2. Prior to use, allow the Matrigel-coated plate to equilibrate to room temperature for at least 1 hour.
3. Just before dissociating cells for passaging, aspirate the liquid Matrigel solution from the wells and replace with 2 mL of hES cell culture media per well. Set aside.
4. Place 6 mL of hES cell culture media in a 15 mL conical tube. Allow this media aliquot to warm to room temperature before use.



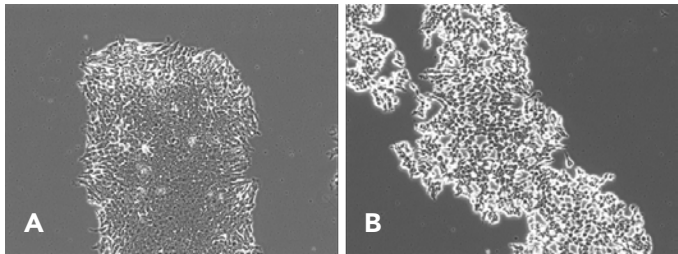
Sensitive components in cell culture media can degrade rapidly at 37°C. Warming small aliquots daily as needed will maintain the shelf life and activity of the cell culture media.

### DISSOCIATE CELLS:

5. Aspirate the media from 1 well of cells to be passaged.
6. Wash the well briefly with 2 mL of PBS (without Ca<sup>2+</sup> and Mg<sup>2+</sup>). Aspirate the PBS wash.
7. Add 1 mL of room temperature 0.5 mM EDTA solution to the cells.
8. Incubate the culture at room temperature for 3 to 5 minutes, or until cells begin to separate uniformly throughout the entire colony. Do not allow the cultures to over-incubate with the EDTA solution.



After 1 to 2 minutes of incubation, observe the colonies under a phase-contrast microscope to closely monitor the progress of the EDTA incubation. Proceed to steps 9 and 10 immediately once the cells within colonies appear rounded, and begin to separate from each other uniformly throughout the colony. Refer to images on page 3.



**Figure 1. ESI-017 hES cells treated with 0.5 mM EDTA solution, 10X magnification.** **A.** This colony has been treated with 0.5 mM EDTA for approx. 2 minutes at room temperature. The cells are beginning to separate along the edges, but this colony needs more time to incubate before it is ready to dissociate from the plate. **B.** This colony has been treated with 0.5 mM EDTA for approx. 4 minutes at room temperature. The cells are rounded and uniform separation is observed throughout the entire colony. This colony is ready to passage. Note that the cells within this colony are found in clusters and not as single cells. If this colony was left in the EDTA solution any longer, the cell clusters would dissociate to single cells, negatively affecting cell viability.

### COLLECT CELL AGGREGATES:

9. As soon as the cells appear rounded and uniform separation is seen throughout the colonies, carefully aspirate the EDTA solution from the well. Do not rinse.
10. Immediately add 3 mL of the pre-warmed hES cell culture media (prepared in step 4) to the well. There should be 3 mL of media left in the tube.

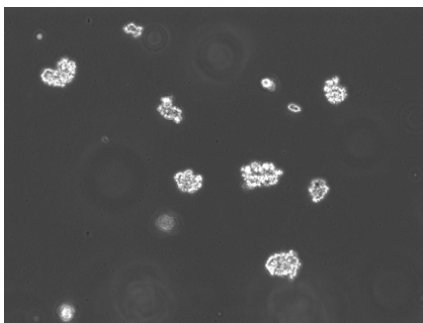


The  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in the culture media will neutralize the action of the EDTA. At this point, healthy cells will begin to reattach. Detach and collect cell aggregates quickly but gently.

11. With a 5 mL pipet, take up the 3 mL of media from the well, and very gently dispense it against the culture surface to dissociate the cells from the dish. Repeat 1 to 2 more times, if needed. Be careful not to over-pipet the cell suspension.
12. Dispense the cells gently into the 15 mL conical tube containing an additional 3 mL of pre-warmed media.



The colonies will break apart into smaller cell aggregates from the action of the EDTA solution and the force of the media while rinsing off of the plate. No further pipetting is needed at this step. Too much pipetting can lead to single cells or very small cell aggregates, and an overall decrease in cell survival after passaging. See Figure 2.



**Figure 2. ESI-017 hES cell aggregates after dissociation and collection, 10X magnification.** This image shows the optimal size of colony pieces after dissociation during passaging.

**PLATE CELLS:**

13. Pipet the solution very gently 1 time to mix, and dispense 1 mL of the cell suspension drop-wise into each of the 6 new Matrigel-coated wells.
14. Place new cell culture plate in 37°C incubator at 5% CO<sub>2</sub>.
15. Rock plate back-and-forth and side-to-side to evenly distribute the colony pieces across the well.
16. Incubate undisturbed at 37°C and 5% CO<sub>2</sub> overnight.

**CARE AND MAINTENANCE:**

17. Replace cell culture media every day with 2.5 mL of fresh hES cell culture media, warmed to room temperature.
18. Monitor cells daily and passage as needed.

**APPENDIX****RECIPE:****0.5 mM EDTA Dissociation Solution**

- 50 µL EDTA (@ 0.5 M)
- 50 mL PBS (without Ca<sup>2+</sup> and Mg<sup>2+</sup>)

**COMMERCIAL SOURCES:**

REAGENT	SOURCE	CAT. NO.
ESI hES Cell Lines	ESI BIO	ES-706
Matrigel hESC-qualified Matrix	Corning	354277
TeSR-E8 Medium	Stem Cell Technologies	05940
PBS/EDTA Cell Release Buffer (pre-made dissociation reagent; optional)	Primorigen	S2702-100ml
Dulbecco's PBS, without Ca <sup>2+</sup> and Mg <sup>2+</sup>	Invitrogen	14190-250
EDTA, 0.5 M, pH 8.8	K&D Medical	RGF-3130

**REFERENCES**

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2. Thomson A, et al. (2008) Human embryonic stem cells passaged using enzymatic methods retain a normal karyotype and express CD30. *Cloning Stem Cells.* 10:89–106.
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4. Garitaonandia I, et al. (2015) Increased Risk of Genetic and Epigenetic Instability in Human Embryonic Stem Cells Associated with Specific Culture Conditions. *PLOS One.* 0118307.

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