# Passaging hES/iPS cells using Versene EDTA

**Purpose:** Versene EDTA is a gentle non-enzymatic cell dissociation reagent suitable for routine passaging of human pluripotent stem cells (hPSCs). EDTA acts as a chelating agent that sequesters calcium from the culture system. As calcium is required by cell adhesions proteins, specifically cadherins, cell attachment can be disrupted when calcium is removed. This enzyme-free protocol does not require centrifugation or scraping (as required for dispase, collagenase, and common single celling enzymes), thereby reducing cell stress and promoting maximum cell survival. Due to the increased survival rate, more aggressive passaging ratios can be obtained. EDTA dissociates the colonies into consistently sized and small aggregates, which helps achieve even distribution of colonies and predictable expansion after passaging. This protocol also improves the efficiency of the passaging process as there are no washing or centrifugation steps.

**Materials:**

- Human pluripotent stem cells (70-80% confluent)
- TeSR media
- Versene EDTA 0.02%
- PBS
- 5mL pipettes
- Matrigel coated 6 well plate

**Procedure**:
*Reagent volumes are for 1 well of a 6-well plate*

1. Mark areas of differentiation using the objective marker or pen.
2. Aspirate off the spent media and aspirate marked areas of differentiation in the well using the aspirating tip. Remember to change the tip between different cell lines to avoid cross-contamination.
3. Wash each well with 1mL PBS. Gently add the PBS and other liquid reagents by pipetting against the edge of the well.
4. Add 11mL of room temperature Versene EDTA to each well and incubate at 37°C for 6-9 minutes. Incubation times will vary based on cell density.
5. During the incubation step, aspirate the DMEM-F12/Matrigel solution from the previously coated Matrigel plate and add 2mL of TeSR media per well.
6. After incubation, check the cells under a microscope. Cells should appear shiny and separated, with small gaps or holes appearing inside colonies. Cells should still be attached to the surface. In some cases, colonies will have slightly curled edges, and minimal amounts of floating cells/debris will be visible.
7. When cells appear as mentioned in step 6, gently aspirate Versene EDTA from the well without disturbing the loosely attached cells.
8. The split ratio for EDTA passaging is usually between 1:8 and 1:20. Determine the amount of TeSR media required to achieve your desired ratio. Do not add more than 5-6mL per well, as overfilling may lead to cross-contamination.
9. Use a 5 mL pipette to add TeSR to the well you are passaging. Hold the pipette perpendicular to the growth surface and dispense media to remove the loosely attached cells. The pipetting action should not be overly aggressive. If cells do not come off the surface easily, a gentle scraping action can be used. If scraping is routinely needed, your incubation time should be increased.

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10. Add the determined volume of cell suspension into the new plate containing TeSR to achieve the desired passaging ratios.
11. Before returning the plate to the incubator, observe passaged cells at the microscope (5x) to confirm plating densities are consistent. Small consistently sized aggregates should be should also be observed. Transfer the plate to the incubator shelf and shake the plate in a cross-like pattern to ensure even plating (repeat and up-down/left-right motion 3-4 times).
12. Observe passaged cells the following day. Small aggregates should be firmly attached with typical hPSC morphologies. Small colonies often have slightly irregular (spiky) edges. Small amounts of cell debris will be visible. Feed daily with 2mL of TeSR until passaging is needed again.

**Troubleshooting/ Tips & Tricks:**

- Avoid aggressive pipetting as it will result in very small colony fragments and may lead to survival issues.
- If the cells do not come off easily after gentle pipetting, gently scrape the cells off the plate with a glass 5mL pipette. If scraping is routinely needed, you should increase the EDTA incubation time.
- If the cells are at higher densities (over 80% confluency), 8-9 minutes of incubation is needed in most cases.
- Once the passaged plate is placed in the incubator, incubator doors should be closed gently. Slamming the door will result in uneven plating with aggregates/colonies concentrated towards the center of the well.
- When passaging multiple lines at the same time, be cautious with inadvertent increases to incubation times. Over-incubation may limit survival and generate smaller aggregates than desired. Additionally, when seeding cells after passaging be careful to work somewhat quickly. Aggregates can loosely attach within a few minutes. If seeded plates sit around for too long prior to dispersing colonies with a shaking action uneven distribution of colonies will result.
- While other passaging methods are available for routine maintenance of hPSCs (dispase and collagenase), we find EDTA passaging to be the most efficient, reliable and reproducible. Single-cell passaging methods (TrypLE and Accutase) should only be used in niche circumstances and not for routine maintenance.
- Several manufactures produce non-enzymatic passaging solutions similar to EDTA that work well for routine hPSC maintenance needs. We find little to no discrepancies between these products and EDTA to be the most economical option.

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