Scope

This culture guide describes the \textit{in vitro} culture of normal human prenatal esophagus epithelial cells using DV Biologics' cells, media, and solutions. These cells are comprised of multicellular aggregates rather than a single cell suspension. Any changes in the experimental conditions may have negative effects on cell survival and may yield abnormal cell growth.

Materials and Equipment

\begin{itemize}
  \item Esophagus Epithelial Cells (PD016-F)
  \item Cell Dissociation Solution (CCS101)
  \item Culture Vessel Coating Solution (CCS102)
  \item Pro-Conditioned Epithelial Cell Medium (D-PRO)
  \item 1× PBS (Ca$^{2+}$/Mg$^{2+}$-free)
  \item 15ml & 50ml tubes
  \item Dimethyl Sulfoxide (DMSO)
  \item Dulbecco’s modified Eagle medium (DMEM)
  \item Fetal Bovine Serum (FBS)
  \item TrypLE Express (Invitrogen)
  \item Ice
\end{itemize}

\begin{itemize}
  \item Cell culture vessels
  \item Centrifuge
  \item Cryovials
  \item Incubator, 37°C, 5% CO$_2$
  \item Pipette
  \item Water Bath, 37°C
\end{itemize}

Procedure

1. Upon receipt
   \begin{itemize}
     \item Remove vial from shipping container to check that it is still frozen—if thawed, contact DV Biologics.
     \item Transfer frozen vial to liquid nitrogen until you are ready to thaw and begin cell culture.
   \end{itemize}

2. Coating—prior to thawing cells
   \begin{itemize}
     \item Thaw CCS102 at 4°C for 2-3 hours.
     \item Warm CCS102 solution to room temperature prior to use.
     \item Add CCS102 to the desired cell culture vessel as suggested in the table below.
     \begin{table}[h]
       \centering
       \begin{tabular}{|c|c|}
         \hline
         Cell Culture Vessel & Medium Volume (ml) \\
         \hline
         12-well & 0.4 \\
         6-well & 1.0 \\
         T25 & 2.5 \\
         T75 & 7.5 \\
         \hline
       \end{tabular}
     \end{table}
     \begin{itemize}
       \item Incubate at room temperature, 1h.
       \item Remove coating solution by aspiration and discard.
       \item Wash culture vessel with PBS.
     \end{itemize}

3. Thawing Cells (For details see \textit{How to Thaw Cells})
   \begin{itemize}
     \item Thaw cryovial in 37°C water bath with agitation.
     \item Transfer contents to 50ml tube.
     \item Slowly and drop-wise add 9ml of FBS to the cells with agitation.
     \item Centrifuge to pellet at 400g at 4°C for 5min.
     \item Remove supernatant and resuspend cell pellet in warm D-PRO; determine cell count (see \textit{How to Count Cells}).
     \begin{itemize}
       \item NOTE: Cells are provided as multi-cellular aggregates, thus the conventional method of cell counting often underestimates the actual cell count.
       \item Keep cells on ice
     \end{itemize}
   \end{itemize}
4. Cultivation
- Calculate the number of cells needed to obtain a $5 \times 10^4$ cells/cm$^2$ seeding density; prepare a corresponding cell suspension (see table below), using D-PRO medium.

<table>
<thead>
<tr>
<th>Cell Culture Vessel</th>
<th>Medium Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-well</td>
<td>1.0</td>
</tr>
<tr>
<td>6-well</td>
<td>2.0</td>
</tr>
<tr>
<td>T25</td>
<td>5.0</td>
</tr>
<tr>
<td>T75</td>
<td>15.0</td>
</tr>
</tbody>
</table>
- Add cell suspension to the cell culture vessel.
- Incubate at 37°C, 5% CO$_2$.
  NOTE: Cell clusters take at least 24 hours to fully adhere to the growth surface.
- Replace medium after 24 hours in order to remove cell debris, then every 2 or 3 days.
  NOTE: To maximize the number of serial passages detach cells (Section 5) at 80% confluency.

5. Detaching Cells
- Thaw CCS101 at 37°C with agitation (avoid passive thaw).
- Remove medium; wash cells with PBS.
- Add CCS101 (use same volume as suggested for medium (Section 4); incubate for 20 min at 37°C.
- Agitate culture vessel and transfer detached cell suspension into a 50ml conical tube containing two volumes of cold DMEM + 10% FBS; set tube on ice.
- Add another volume of CCS101 to the vessel and incubate 37°C, 20min; agitate culture vessel and pool detached cell suspension with the previously collected fraction.
- Wash culture vessel with one volume of PBS and pool with the previously collected fraction.
  NOTE: Detachment procedure yields multi-cellular aggregates, which is normal.
- Centrifuge the collected cell suspension 400g at 4°C for 5 min; aspirate supernatant
- Proceed with sub-cultivation (Section 6) or freezing (Section 7).

6. Sub-cultivation
  NOTE: The morphology of intestinal cells may change after its passage
- Warm CCS101 and D-PRO medium to room temperature.
- Select a culture vessel; coat the vessel with Culture Vessel Coating Matrix solution (Section 4).
- Resuspend cell pellet (from section 3.4) in D-PRO medium.
- Count Cells (see How to Count Cells).
- Refer to Section 3.3 from seeding density and prepare cell suspension using D-PRO; transfer the cell suspension to the vessel.
- Incubate culture vessel 37°C, 5% CO$_2$, replacing medium every 2 to 3 days.

7. Freezing (See How to Freeze Cells)
8. Selective Detachment (optional)—for selective isolation of epithelial cells.

   NOTE: This step should not be performed within the first 48 hours of culture.
   NOTE: the following steps assume use of a T25 flask.
   - Aspirate medium from flask.
   - Wash cell culture vessels twice with 10 ml of cold PBS (for T25 flask).
   - Using an inverted microscope, visualize an area with a mix of epithelial cells and fibroblast; note or mark the area on the flask.
   - Add 2ml of cold TrypLE solution to the culture flask.
   - Incubate for 2 min at RT; continuously observe under inverted microscope.
   - Observe until fibroblasts (and not epithelial cells) begin to detach.
   - Immediately at 5 ml of FBS to halt the reaction.
   - Aspirate solution and wash with PBS.
   - Add fresh D-Pro and incubate @ 37°C, 5% CO2.
   - Observe after 90 minute to see if desired results have been achieved.

9. Cell Characterization

   **Figure 1.** Primary culture of normal EECs: 
   A: EECs 24 hours after seeding under a phase contrast microscope at 10X. 
   B: Formation of EECs colony 72 hours post seeding. 
   C: Primary culture of normal human EECs after 5 days of culture. 
   D: Primary culture of normal human EECs after 8 days. Phase contrast image showing epithelial cells overlaying small cuboidal cells. 
   E: Single sheet of esophageal epithelial layer spread across the tissue culture plates. 
   F: CK-14 positive expression in normal human EECs visualized (10X magnification) by immunofluorescent staining after 14 days of in vitro culture. 
   G: CK-14 and GAPDH RT-PCR performed on mRNA derived from EECs, Human Umbilical Vein Endothelial Cells (HUVEC), and whole skin tissue cDNA (WSC).