Protocol for Flow Cytometric of Cell Surface Antigen Expression

Purpose:
To define the procedure for the preparation of hES cells for cell surface antigen analysis using FACS.

Items required:
- Trypsin: EDTA solution
- hES growth medium – see Appendix 1
- Wash buffer – PBS (Ca²⁺, Mg²⁺ free) with 5% FCS and 0.1% sodium azide
- PBS (Ca²⁺, Mg²⁺ free)
- Primary antibodies
- Secondary antibody
- Healthy hES cell cultures
- Haemocytometer
- Centrifuge
- 15ml centrifuge tubes
- Round bottom 96 well plate
- Plate seals
- Flow cytometer

Notes:
- The cells to be assayed for these surface antigens should be from healthy stock cultures.
- High and low density 2102Ep cells should be assayed with test samples to provide a standard and a positive control for the antibodies provided.
- Feeder cells should also be assayed with test samples, provided that you have enough of the primary antibodies to do this (200µl will be needed for this).
- This protocol assumes that the procedure is carried out using a 96 well plate; however other suitable plates/tubes may be used.
- Precise protocols for immunofluorescence analyses vary between labs. The following procedure is the one recommended for use in the ISCI, to help ensure comparability. It may be that you need to change the staining or analysis protocol used in your laboratory. If you believe that the protocol used in your laboratory is significantly different from the protocol set out below, please discuss this with the co-ordinator.
- Any deviations from standard protocols should be recorded on ISCI/FRM/002 – Deviations from Standard Protocols.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Species</th>
<th>Class</th>
<th>Expected 2102Ep expression</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>High Density</td>
</tr>
<tr>
<td>Undifferentiated ES Cell Markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSEA3</td>
<td>MC631</td>
<td>Rat</td>
<td>IgM</td>
<td>Positive</td>
</tr>
<tr>
<td>-------</td>
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<td>-----</td>
<td>-----</td>
<td>----------</td>
</tr>
<tr>
<td>SSEA4</td>
<td>MC813-70</td>
<td>Mouse</td>
<td>IgG</td>
<td>Positive</td>
</tr>
<tr>
<td>TRA-1-60</td>
<td>TRA-1-60</td>
<td>Mouse</td>
<td>IgM</td>
<td>Positive</td>
</tr>
<tr>
<td>TRA-1-81</td>
<td>TRA-1-81</td>
<td>Mouse</td>
<td>IgM</td>
<td>Positive</td>
</tr>
<tr>
<td>GCTM2</td>
<td>GCTM2</td>
<td>Mouse</td>
<td>IgM</td>
<td>Positive</td>
</tr>
<tr>
<td>GCTM343</td>
<td>GCTM343</td>
<td>Mouse</td>
<td>IgM</td>
<td>Positive</td>
</tr>
<tr>
<td>L-ALP</td>
<td>TRA-2-54</td>
<td>Mouse</td>
<td>IgG</td>
<td>Positive</td>
</tr>
<tr>
<td>L-ALP</td>
<td>TRA-2-49</td>
<td>Mouse</td>
<td>IgG</td>
<td>Positive</td>
</tr>
<tr>
<td>CD90(Thy-1)</td>
<td>Anti-Thy-1</td>
<td>Mouse</td>
<td>IgG</td>
<td>Positive</td>
</tr>
<tr>
<td>CD9</td>
<td>Anti-CD9</td>
<td>Mouse</td>
<td>IgG</td>
<td>Positive</td>
</tr>
</tbody>
</table>

**Differentiation Markers**

<table>
<thead>
<tr>
<th>SSEA1</th>
<th>MC480</th>
<th>Mouse</th>
<th>IgM</th>
<th>Negative</th>
<th>Positive</th>
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</thead>
<tbody>
<tr>
<td>A2B5 (GT3)</td>
<td>A2B5</td>
<td>Mouse</td>
<td>IgM</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>CD56(NCAM)</td>
<td>B159</td>
<td>Mouse</td>
<td>IgG</td>
<td>Positive</td>
<td>Positive</td>
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<tr>
<td>GD3</td>
<td>VINIS56</td>
<td>Mouse</td>
<td>IgM</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>GD2</td>
<td>VIN2PB22</td>
<td>Mouse</td>
<td>IgM</td>
<td>Negative</td>
<td>Negative</td>
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</tbody>
</table>

**Pan human antigens**

<table>
<thead>
<tr>
<th>TRA-1-85(0K(a))</th>
<th>TRA-1-85</th>
<th>Mouse</th>
<th>IgG</th>
<th>Positive</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A,B,C</td>
<td>W6/32</td>
<td>Mouse</td>
<td>IgG</td>
<td>Positive</td>
<td>Positive</td>
</tr>
</tbody>
</table>

**Control antibody**

| P3X | Control IgG | Mouse | IgG | Negative | Negative |

All the primary antibodies are monoclonals. The secondary antibody provided is FITC tagged and recognizes mouse IgM and IgG.

**Procedure:**

1. Harvest hES cells by washing with PBS and then incubating with 1ml Trypsin: EDTA per 25cm² flask for 3 – 5 mins at 37°C.
2. When cells begin to detach, add 9ml hES growth medium per ml Trypsin: EDTA.
3. Gently pipette to form a single cell suspension, and then count the cells using a haemocytometer.
4. Transfer the cells to a centrifuge tube and centrifuge at 200 x g for 5 minutes.
5. Resuspend cells in Wash Buffer to 2 x 10⁶ cells per ml.
6. Dilute the secondary antibody 1/100 in wash buffer. **Primary antibodies are used undiluted.**
7. Distribute the antibodies at 50 l per well of a round bottom 96 well plate, two wells for each assay point. To prevent carry-over from one well to another, it is good practice to arrange to use alternate wells of the plate.
8. Add 50 l of cell suspension (i.e. 10⁵ cells) to each 50 l of antibody in the wells of the 96 well plate.
9. Seal the plate by covering with a sticky plastic cover, ensuring that each well is sealed.
10. Incubate at 4°C, ideally with gentle shaking, for 30 minutes.
11. Centrifuge the plate at 280 x g for 3 minutes, using microtitre plate carriers in a suitable centrifuge.
12. Check that the cells are pelleted, and then remove the plastic seal using a sharp motion, but holding the plate firmly, to avoid disturbing the cell pellet.
13. Discard the supernatant. The cells remain as pellets at the bottom of the wells.
14. Wash the cells by adding 100 l wash buffer to each well. Seal and agitate to re-suspend the cells. Centrifuge, as above.
15. Discard the supernatants and repeat for 2 further washes & spins.
16. After the third wash, discard the supernatants and add 50 l of the secondary antibody to each well.
17. Seal the plate, as above, and incubate with gentle shaking for 30 minutes at 4°C.
18. Centrifuge the cells and wash 3 times, as before.
19. Resuspend the cells at about 5x10^6 per ml in wash buffer and analyse using the Flow Cytometer.

Data Analysis and Reporting:

A negative control first antibody is provided and should be used for each cell line. This should be used to set a threshold for scoring the proportion of cells that are ‘positive’ for each test antigen. Typically this threshold should be set at the inflexion point on the negative control histogram, such that only 1% - 5% of the cells stained with the negative control would be scored ‘positive’. Using this threshold, the % cells scored positive with each antibody should be recorded (see Figure below).

A second region should be set on the histogram, encompassing all the cells – from this gate the ‘mean fluorescence intensity’ of the cells stained with each antibody should be recorded (see Figure below). Although this value is arbitrary, and depends on local machine settings, inclusion of the 2102Ep standard cells will permit some comparability between the results from different labs.

Figure: Immunofluorescence Histograms
Note:
In Panel A, the positive antibody stains cells substantial brighter than the negative control; probably nearly 100% of the cells would be scored as antigen positive. In Panel B, the positive histogram overlaps significantly with the negative control histogram; rather less than 100% of cells will be scored positive for this antigen. The mean fluorescence of cells stained with the test antibody in Panel B will be substantially less than the mean fluorescence of the cells stained with the test antibody in Panel A. The two numbers, mean fluorescence and % positive cells, provide two complementary numbers for comparing results between cells, antibodies and laboratories.

Please record the % cells positive and mean fluorescence of each cell line for each antibody, ensuring that data for 2102Ep cells, and for negative control antibodies for each cell line are reported.
Protocol for the Preparation of Cell Lysates for DNA and RNA Studies

Purpose:
To provide lysates from undifferentiated and differentiated hES cells. DNA extracts will be used for DNA fingerprinting and imprinted gene studies. RNA extracts will be used for gene expression analysis.

Items Required:
- Healthy hES cultures
- Trizol (Invitrogen)
- Collagenase – see Appendix 1, or suitable alternative
- hES growth medium – see Appendix 1
- Sterile pre-labelleEp Eppendorf tubes, provided by UKSCB
- Pipettes, 1ml and 5ml
- Centrifuge
- 50ml centrifuge tubes

Notes:
- Where participating laboratories are using a local protocol for growth of hES cells, in addition to the ISCI Standard protocol (ISCI/SOP/001), samples should be provided for both sets of growth conditions.
- Samples should be collected at Time Points 1 and 2.
- Samples should be collected from both undifferentiated and differentiated hES cell cultures.
- In order to minimize the risk of labelling errors, and to aid in sample processing at the UKSCB, samples should be placed only in the pre-labelled tubes provided.
- Lysates are prepared in Trizol, which will be provided by the UKSCB.
- Separate lysates are required: 2x1ml for DNA analysis and 2x1ml for RNA analysis.
- The samples will be sent from the participating laboratories to the UKSCB for DNA and RNA extraction and purification.
- Each lysate should be prepared from approximately 3 – 5 x 10^6 cells, which should be obtainable from a single 25cm^2 flask. A total of 2 x 25cm^2 flasks will therefore be required for each set of samples (RNA + DNA).

Procedure:
1. For Undifferentiated hES Cells:
   1.1. Prepare undifferentiated hES cells according to the ISCI Standard Protocol (ISCI/SOP/001) and local protocol, where appropriate.
   1.2. Remove culture medium from 2 x 25cm^2 flasks, or equivalent.
   1.3. Add 1ml of Collagenase per 25cm^2 flask, and incubate at 37°C for 8-10min, until the edges of the hES colonies start to curl.
   1.4. Gently scrape with glass beads or rubber policeman. Add 1ml hES growth medium per flask and gently resuspend cells.
   1.5. Divide into 4 x 1ml aliquots and transfer to each of four sterile, pre-labelled Eppendorf tubes (2 for RNA, 2 for DNA). Complete tubes labels.
   1.6. Centrifuge at 50 x g for 3min at 4°C. Discard supernatant, leaving hES cell pellet. Add 1ml of Trizol to each tube.
   1.7. Freeze tubes and store frozen below -70°C, until dispatched to UKSCB.
2. *For Differentiated hES Cells:*
   2.1. Prepare differentiated hES cells according to the ISCI protocol (ISCI/SOP/007)
   2.2. Gently transfer 10 day old embryoid bodies (EBs) and culture medium from two
       10cm Petri dishes to a 50ml centrifuge tube.
   2.3. Centrifuge at 50 x g for 3min at 4°C.
   2.4. Remove all but 4ml of the supernatant. Resuspend cell pellet.
   2.5. Divide into 4 x 1 ml aliquots and transfer to each of four sterile, pre-labelled
       Eppendorf tubes (2 for RNA, 2 for DNA). Complete tubes labels.
   2.6. Centrifuge at 50 x g for 3min at 4°C. Discard supernatant, leaving cell pellet. Add
       1 ml of Trizol to each tube. *The sample volume should NOT exceed 10% of the
       volume of TRIZOL.*
   2.7. Freeze tubes and store frozen below -70°C, until dispatched to UKSCB

3. *Notification to UKSCB:*
   3.1. Notify the UKSCB once all samples have been prepared.
   3.2. Package samples according to packaging instructions issued by the UKSCB.
   3.3. The UKSCB will arrange courier services and advise when samples are to be
        collected.
**Protocol for the Preparation of differentiated hES cells.**

**Purpose:**
To induce the formation of embryoid bodies from hES cells, for use in ISCI characterisation studies.

**Items Required:**
- Healthy hES cultures
- hES growth medium – see Appendix 1
- Collagenase – see Appendix 1, or suitable alternative
- Pipettes, 10ml
- Centrifuge
- 15ml centrifuge tubes
- CO₂ incubator
- Petri dishes

**Notes:**
A wide variety of techniques can be used to induce differentiation of hES cells. To identify the range of differentiation of hES cells, we propose to use this simple protocol, which generally allows extensive differentiation. This will permit comparison of the range of cell lineages that different hES lines may generate under these conditions. Any deviation from this protocol should be recorded on ISCI/FRM/002 – Deviations from Standard Protocols.

**Procedure:**

1. Aspirate medium from stock culture of hES cells and harvest clumps of cells by adding 2ml Collagenase per 25cm². Incubate at 37°C for 20-30 minutes. Undifferentiated human ES colonies should detach, leaving behind feeders and any spontaneously differentiated cells which may be present.
2. Transfer the detached colonies to a 15ml centrifuge tube and spin down at 50 x g for 3 minutes.
3. Aspirate the supernatant and gently resuspend hES colonies in 10ml of hES medium. Transfer to a sterile 10cm bacterial Petri dish (the surface of bacterial Petri dishes do not typically facilitate the attachment of embryoid bodies). Use the equivalent of 3.5 x 10⁶ cells per 10 cm dish. Prepare two 10 cm dishes for each cell line.
4. Culture under 5% CO₂ in air at 37°C, to allow embryoid bodies to form.
5. Embryoid bodies (EBs) should be fed every 3-5 days, depending upon density and size. The medium can be changed by gently transferring the EBs and old medium to a clear centrifuge tube and allowing them to settle under gravity (takes 5-10 minutes). The old medium can be aspirated and new medium gently added, taking care not to break up the EBs. Transfer to a fresh bacterial Petri dish.
6. Culture for 10 days, and then prepare samples for microbiology, RNA and DNA analysis.

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**ISCI/SOP/005 – ES cells for Indirect Immunofluorescence (refer to additional advice given below - to be read in conjunction with this protocol)**

**Purpose:**
To provide hES cells for analysis using indirect immunofluorescence.

**Items Required:**
- Gelatin solution – 0.1% gelatin dissolved in PBS containing Ca\(^{2+}\) and Mg\(^{2+}\).
- 70% ethanol
- Methanol
- Sterile distilled water
- Sterilizing oven
- QuadriPerm dishes, supplied by Sartorius, cat no. IV76077308
- Ethanol resistant multitest slide with 12 wells, supplied by ICN, cat no. 604120E

**Procedure:**
1. Prepare slides by washing with 70% ethanol, followed by two rinses in sterile distilled water, and finally sterilize using dry heat at 160°C for 1 hour.
2. Pre-treat slides by immersing in gelatin solution for 1 hour at 4°C.
3. Add feeder cells, at the appropriate density, 1-5 days prior to addition of hES cells. Each well has a diameter of 7mm.
4. Transfer hES cells as intact clumps or clusters of cells (using the standard methodology in your lab) to the multitest slides, which are placed into the QuadriPerm dish. The dish holds 4 slides. (See diagram below.) Please prepare four slides per cell line.
5. Cultivate hES cells for 3 days to allow intermediate sized colonies to form.
6. Rinse slides in PBS, followed by a brief rinse in methanol, to minimize salt precipitation.
7. Fix slides in methanol at -20°C for 5 minutes, and then air dry slides.
8. Slides can be stored for at least 6 months at -20°C, prior to shipment (on dry ice) for analysis.

*QuadriPerm dish with 4 multitest slides*
### Additional advice for ISCI/SOP/005

There are 2 ways of plating cells in Quadriperm dishes.

1. This method requires a larger number of MEF and hES cells. The surface area of 1 chamber of a Quadriperm is 24.6 cm\(^2\). Put slides in Quadriperm chambers and add enough gelatine to cover the surface (3 - 5ml). Aspirate gelatine and plate MEFs in 5-7ml medium. For collagenase-treated cultures we plate 2 \(\times\) 10\(^5\) cm\(^2\) i.e. 4.9 \(\times\) 10\(^5\) chamber. Plate hES at the same density as in 25 cm\(^2\) flask.

2. This is our method of choice. It requires less MEFs and medium and can be used for collagenase-treated and for cut hES cells. The surface area of 1 well of a 12-well slide 0.5 cm\(^2\). It holds a 50 µl volume. Put slides in Quadriperm chambers and add 50 µl of gelatine/well (with a 3 ml plastic transfer pipette). Make sure you don’t wet the blue area of the slide. Aspirate gelatine and plate MEFs at required density in 50 µl drops. For collagenase-treated cultures we plate 2 \(\times\) 10\(^5\) cm\(^2\), i.e. 1.2 \(\times\) 10\(^5\) 12 wells. For cut pieces we plate 6 \(\times\) 10\(^5\) cm\(^2\), i.e. 3.6 \(\times\) 10\(^5\) 12 wells. Leave the MEFs to attach overnight and plate the hES cells next day.

To plate cut pieces: replace MEFs medium with 50 µl hES medium. Plate 1 – 2 pieces per well. Incubate overnight. Next day flood the chamber with 5 – 7 ml medium.

To plate collagenase-treated cells: the surface area of 12 wells is 6 cm\(^2\). Prepare hES cell suspension at appropriate density in 800 µl of hES medium/slide. Aspirate MEF medium, wash each well with 50 µl PBS and plate hES in 50 µl droplets. Next day flood the the chamber with 5 – 7 ml medium.