BD StemFlow™ Human Pluripotent Stem Cell Sorting and Analysis Kit

Instruction Manual

Catalog No. 560461

BD

History

Revision	Date	Change Made
647928	08/09	Sodium azide warning update
646577	12/08	New document

BD flow cytometers are class I (1) laser products.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

Contents

1

About this kit

This section covers the following topics:

Purpose of the kit

About this topic

This topic explains the purpose of the BD StemFlow™ Human Pluripotent Stem Cell Sorting and Analysis Kit (Catalog No. 560461), and provides background for understanding the kit's components and how they work.

Uses of the kit

This kit provides the reagents necessary to perform multicolor flow cytometry on human pluripotent stem cells such as human embryonic stem cells (hESCs) and induced pluripotent stem (iPS) cells.

This kit can be used to analyze populations of live and fixed cells for expression of pluripotency markers and differentiation markers.

This kit can also be used to sort live populations of pluripotent stem cells and their derivatives.

Specific antibodies

Human pluripotent stem cells are characterized by the expression of specific cell surface markers.[1]

The BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit contains fluorescent antibodies to three markers. Two of these antibodies recognize markers specific to undifferentiated pluripotent stem cells: TRA-1-81 and SSEA-3 (stage-specific embryonic antigen-3). The third antibody recognizes a marker specific to differentiated cells: SSEA-1 (stage-specific embryonic antigen-1).

This combination of markers has been widely used to characterize and isolate hESCs and iPS cells.[2-4]

Isotype-control antibodies

This kit contains three isotype controls. Each isotype control is a non-specific antibody that is conjugated to the same fluorochrome as one of the specific antibodies.

The isotype controls are used to identify any non-specific (background) staining of the specific antibodies in the BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit.

This kit has been tested on multiple hESC lines (H9, H7, and HUES9), and no problematic background staining has been observed.

Control beads

This kit also contains BD™ CompBead Plus positive and negative beads to facilitate application setup for analysis or sorting of stem cells.

The positive beads are coated with antibodies that will bind to one of the specific antibodies in this kit. The negative beads have no binding capacity.

Once the beads have been stained with the specific antibodies, they provide distinct positive and negative populations that assist in optimizing photomultiplier tube (PMT) settings and calculating fluorescence compensation. Use of these beads ensures consistent application setup and conserves cells.

Use of other antibodies

The reagents in this kit and the methods described in this manual are compatible with the use of additional fluorochrome-conjugated antibodies specific to other surface antigens or intracellular proteins (eg, transcription factors and other proteins, cytokines, and cyclins) for studying developmental progression, activation, entry and progression through the cell cycle, and cell death.

Related topics

- Kit contents (page 4)

Kit contents

Reagent information

The BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit contains the following reagents.

Reagent	Details
BD Pharmingen™ FITC Mouse anti-SSEA-1	**Clone:** MC480 **Use:** Used to stain a terminal carbohydrate epitope (3-fucosyl-N-acetyllactosamine, or 3-FAL) on glycoproteins and lactose series glycolipids **Abbreviation:** FITC SSEA-1 **Quantity:** 1 vial (1.5 mL) **Amount for staining:** 20 µL/sample for analysis (for 1 x 10^6 cells) or 60 µL/sample for sorting (for 1 x 10^7 cells)

Reagent	Details
BD Pharmingen™ PE Rat anti-SSEA-3	**Clone:** MC631 **Use:** Used to stain a carbohydrate epitope on the major ganglioside, but not the neutral glycolipid, of mouse embryos and human teratocarcinoma cells **Abbreviation:** PE SSEA-3 **Quantity:** 1 vial (1.5 mL) **Amount for staining:** 20 µL/sample for analysis (for 1 x 10^6 cells) or 60 µL/sample for sorting (for 1 x 10^7 cells)
BD Pharmingen™ Alexa Fluor® 647 Mouse anti-Human TRA-1-81 Antigen	**Clone:** TRA-1-81 **Use:** Used to stain an epitope on a high-molecular-weight transmembrane glycoprotein that is specific to pluripotent stem cells **Abbreviation:** Alexa Fluor® 647 TRA-1-81 **Quantity:** 1 vial (1.5 mL) **Amount for staining:** 20 µL/sample for analysis (for 1 x 10^6 cells) or 60 µL/sample for sorting (for 1 x 10^7 cells)
BD Pharmingen™ FITC Mouse IgM, κ Isotype Control	**Clone:** G155-228 **Use:** Used as an isotype control for FITC SSEA-1 **Abbreviation:** FITC isotype control **Quantity:** 1 vial (0.5 mL) **Amount for staining:** 20 µL/sample (for 1 x 10^6 cells)
BD Pharmingen™ PE Rat IgM, κ Isotype Control	**Clone:** R4-22 **Use:** Used as an isotype control for PE SSEA-3 **Abbreviation:** PE isotype control **Quantity:** 1 vial (0.5 mL) **Amount for staining:** 20 µL/sample (for 1 x 10^6 cells)

Reagent	Details
BD Pharmingen™ Alexa Fluor® 647 Mouse IgM, κ Isotype Control	**Clone:** G155-228 **Use:** Used as an isotype control for Alexa Fluor® 647 TRA-1-81 **Abbreviation:** Alexa Fluor® 647 isotype control **Quantity:** 1 vial (0.5 mL) **Amount for staining:** 20 µL/sample (for 1 x 10^6 cells)
BD CompBead Plus Anti-Mouse Ig, κ	**Use:** Used to create control beads stained with either FITC SSEA-1 or Alexa Fluor® 647 TRA-1-81 (because beads bind any mouse kappa light-chain bearing immunoglobulin) **Abbreviation:** Anti-mouse beads **Quantity:** 1 vial (3.0 mL)
BD CompBead Plus Anti-Rat Ig, κ	**Use:** Used to create control beads stained with PE SSEA-3 (because beads bind any rat kappa light-chain bearing immunoglobulin) **Abbreviation:** Anti-rat beads **Quantity:** 1 vial (1.5 mL)
BD CompBead Plus Negative Control (PBS with 1% BSA)	**Use:** Used as negative control beads (because beads have no binding capacity) **Abbreviation:** Negative beads **Quantity:** 1 vial (6.0 mL)

Serum proteins

All components in this kit contain a small percentage of serum proteins. Source of all serum proteins is from USDA-inspected abattoirs located in the United States.

Related topics

- Purpose of the kit (page 2)
- Storage and safe handling (page 7)

Storage and safe handling

About this topic

This topic describes the requirements for kit storage and safe handling.

Storage

The entire BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit must be stored in the dark at 2° to 8°C. Do not freeze.

Warning

The reagents in this kit contain sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

Related topics

- Kit contents (page 4)

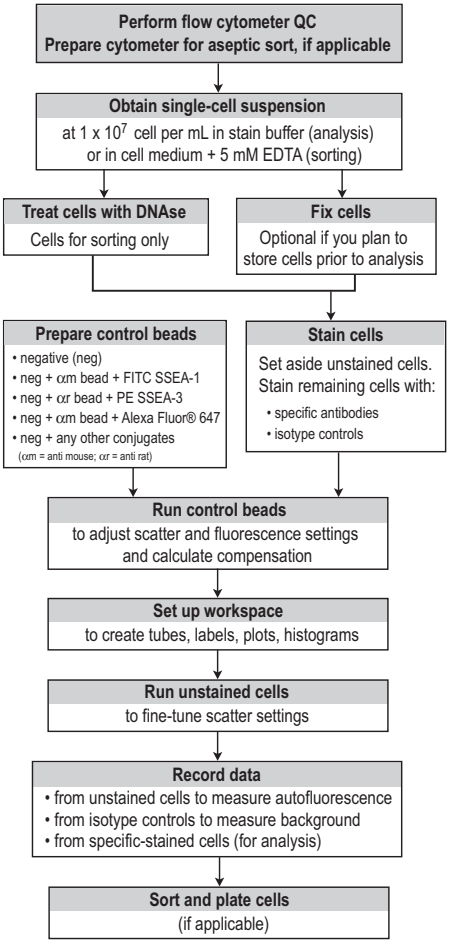
2

Before you begin

This section covers the following topics:

Workflow overview

About this topic This topic provides an overview of the steps involved in using the BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit.



Required materials

About this topic

This topic describes the reagents, consumables, and equipment that you will need to be able to use the kit for analyzing or sorting human pluripotent stem cells.

Materials for all applications

For analysis or sorting of cells, you will need:

- 1X PBS without Ca^{2+} or Mg^{2+} (sterile, if culturing live cells)
- Accutase™ Enzyme Cell Detachment Medium from Innovative Cell Technologies, or equivalent
- Microscope for confirming a single-cell suspension
- BD Falcon™ 70-µm cell strainer (Catalog No. 352350), or equivalent

  Note: The use of a cell strainer is optional for analysis and recommended for sorting.
- Hemocytometer or other cell counter
- BD Falcon round-bottom 12 x 75-mm polystyrene tubes with caps (Catalog No. 352058), or equivalent
- BD FACSAria™ II flow cytometer, or other flow cytometer equipped with a blue (488-nm) laser, a red (633-nm) laser, and detectors for FITC, PE, and APC/Alexa Fluor® 647

Materials for analysis

If you are analyzing cells, you will also need:

- BD Pharmingen™ stain buffer (FBS) (Catalog No. 554656), or equivalent (referred to throughout this manual as stain buffer)

If you are analyzing fixed cells, you will also need:

- BD Cytofix™ fixation buffer (4% PFA) (Catalog No. 554655), or equivalent

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Materials for sorting

If you are sorting then culturing live cells, you will also need:

- Biological safety cabinet, for aseptic sample preparation
- Ice
- 70% ethanol, if performing aseptic sort
- Sterile PBS, for sheath tank if performing aseptic sort
- Tissue-culture-grade buffered EDTA solution (available from Invitrogen)
- Sterile DNAse solution (available from Sigma-Aldrich)
- Antibiotics such as penicillin and streptomycin (available from Invitrogen)
- BD Falcon 6-well flat-bottom cell culture plates with lids (Catalog No. 353046), or equivalent
- BD Matrigel™ hESC-qualified matrix (Catalog No. 354277), or equivalent
- Complete mTESR™1 maintenance medium (basal + supplements) for human embryonic stem cells from StemCell Technologies, Inc., or equivalent (referred to throughout this manual as medium)

Related topics

- Kit contents (page 4)

Common cell-preparation techniques

About this topic

This topic describes how to perform common cell-preparation techniques that are part of the workflow for staining cells with the BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit.

Washing cells

Several of the procedures in this manual instruct you to wash the cell suspension.

To wash cells:

1. Add the specified volume of buffer or medium.

If staining cells for...	Add...
Analysis	2 mL of stain buffer
Sorting	2 mL of medium with 5 mM EDTA

2. Mix gently.
3. Centrifuge at 300*g* for 5 minutes.
4. Aspirate to remove the supernatant, being careful not to disturb the cells.
5. Resuspend as directed.

Adjusting cell concentration

After harvesting cells from culture, each of your samples will have a unique cell concentration. Several of the procedures in this manual require that you adjust your cell suspension to a specific cell concentration.

To adjust the cell concentration for each sample:

1. Determine the current cell concentration using the standard method for your hemocytometer or other cell counter.

2. Calculate the volume that would result in the required concentration (for example, 1 x 10^7 cells per mL). This is your target volume.

   For example, for 3 million cells, the target volume would be 300 µL to obtain a concentration of 1 x 10^7 cells per mL.

3. Adjust the concentration to achieve the target volume.

   If your cell suspension is too concentrated, add the appropriate buffer to bring the total volume up to the target volume.

   If your cell suspension is too dilute:

   a. Centrifuge the cells at 300*g* for 5 minutes.

   b. Aspirate to remove the supernatant, being careful not to disturb the cells.

   c. Resuspend in the target volume of the appropriate buffer or medium.

Related topics

- Preparation of cells and beads (page 15)

3

Preparation of cells and beads

This section covers the following topics:

Obtaining a single-cell suspension

About this topic

This topic explains how to obtain a single-cell suspension, and provides guidelines on the number of cells required for staining with the BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit.

Before you begin

Ensure that you have all of the necessary materials available. See Required materials (page 11) for details.

Ensure that your 1X PBS without Ca^{2+} or Mg^{2+} is at room temperature.

Aseptic technique

If you plan to use sorted cells in transplantation or tissue-culture experiments, we recommend that all sample-preparation steps be performed using an aseptic technique.

If you are performing an aseptic sort, ensure that your PBS is sterile.

Procedure

To obtain a single-cell suspension:

1. Wash the cells with room-temperature PBS.
2. Add the detachment enzyme (eg, Accutase) to the cells at the concentration recommended by the manufacturer.
3. Incubate at the recommended temperature and for the recommended duration, or until cells are detached.
4. Pipette the cells gently up and down to help disperse doublets.
5. Remove a small subset of the liquid and check it under a microscope to confirm the presence of single cells.

6. If you observe clumps of cells, collect the cell suspension and pass it through a 70-µm cell strainer.

   Note: Straining cells is optional for analysis and recommended for sorting.

7. Wash the cells in two to four volumes of PBS (if analyzing) or medium (if sorting), centrifuging at 300*g* for 5 minutes.

8. Resuspend the cells in a volume of PBS (if analyzing) or medium (if sorting) that is appropriate for cell counting.

9. Determine the cell concentration and total number of cells per sample using the standard method for your hemocytometer or other cell counter.

Guidelines for number of cells

Your research needs will determine how many cells you need for staining, depending upon the number of controls you decide to run.

For each cell type you will be analyzing, we recommend that you run a sample of unstained cells to measure autofluorescence, and an isotype control to measure non-specific staining. See Staining the cells (page 20) for more information about isotype controls.

Refer to the following guidelines.

Guideline	Value for analysis	Value for sorting
Required cell concentration for staining	1×10^7 cells/mL	1×10^7 cells/mL
Recommended cells per tube for staining	1×10^6 cells	1×10^7 cells

Guideline	Value for analysis	Value for sorting
Minimum cells per tube for staining	2.5 x 10^5 cells	5 x 10^6 cells
Recommended volume of cells per tube at the required concentration	100 µL	1,000 µL
Minimum volume of cells per tube at the required concentration	25 µL	500 µL

Next step

Proceed to Treating the cells with DNAse (page 18) if you are sorting, or Fixing the cells (page 19) if you are analyzing.

Treating the cells with DNAse

About this topic

This topic explains how to treat cells with DNAse before staining with the BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit.

These steps should be performed only if you are staining cells for sorting. If you are staining cells for analysis, skip this section and proceed to Fixing the cells (page 19).

Before you begin

Complete the steps described in Obtaining a single-cell suspension (page 16).

Prepare medium containing 100 units per mL of DNAse. Make enough to bring all of your samples to 1 x 10^7 cells per mL. See Required materials on page 11.

Procedure

To perform the DNAse treatment:

1. Centrifuge the cells at 300*g* for 5 minutes, and aspirate to remove the supernatant.

2. Resuspend the cells in medium containing 100 units per mL of DNAse to bring the concentration to 1 x 10^7 cells per mL.
3. Incubate the cells for 10 to 15 minutes at room temperature.

Next step

When the incubation is complete, proceed immediately to Staining the cells (page 20).

Related topics

- Required materials (page 11)

Fixing the cells

About this topic

This topic explains how to fix cells before staining with the BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit.

This procedure is optional, and is performed only if you are analyzing cells and need to store the cells prior to analysis.

Before you begin

Complete the steps described in Obtaining a single-cell suspension (page 16).

Procedure

To fix the cells:

1. Centrifuge at 300*g* for 5 minutes, and aspirate to remove the supernatant.
2. Add BD Cytofix fixation buffer (4% PFA) to bring the concentration to 1 x 10^7 cells per mL.
3. Incubate for 20 minutes at room temperature.

4. Wash cells twice.

   See Washing cells (page 13).

5. Resuspend in stain buffer to bring the concentration to 1 x 10^7 cells per mL.

Next step

Proceed to Staining the cells (page 20).

Related topics

- Workflow overview (page 10)
- Required materials (page 11)
- Common cell-preparation techniques (page 13)

Staining the cells

About this topic

This topic explains how to stain your prepared cells with the BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit.

This procedure includes specific staining, isotype-control staining, and creating a control tube of unstained cells.

Before you begin

First complete the steps described in Obtaining a single-cell suspension (page 16). Then, if you are sorting cells, complete the steps described in Treating the cells with DNAse (page 18). If you are analyzing fixed cells, complete the steps described in Fixing the cells (page 19).

To prepare unstained cells, add 100 µL of the single-cell suspension (1 x 10^6 cells) to a 12 x 75-mm polystyrene tube labeled “unstained” and place the tube in the dark at room temperature.

Isotype controls

We recommend setting up an isotype control to test for non-specific staining each time you test the kit on a new cell line. The use of isotype controls is optional after this initial testing.

This kit has been tested on multiple hESC lines (H9, H7, and HUES9), and no problematic background staining has been observed.

Workflow efficiency

To maximize your efficiency, you can stain the beads at the same time you stain your cells, or during the 30-minute incubation. See Preparing control beads on page 24.

Procedure

To stain the cells and set up unstained control cells:

1. For each of your samples, label one 12 x 75-mm polystyrene tube "specific stain," and one tube "isotype control" (if applicable).
2. Add the following to each tube. See the appropriate table for analysis or sorting.

(For Analysis) Component	Volume to add to tube for analysis	
	Specific stain	Isotype control
Cell suspension (at 1 x 10^7 cells per mL)	100 µL (1 x 10^6 cells)	100 µL (1 x 10^6 cells)
FITC SSEA-1	20 µL	—
PE SSEA-3	20 µL	—
Alexa Fluor® 647 TRA-1-81	20 µL	—
FITC isotype control	—	20 µL
PE isotype control	—	20 µL
Alexa Fluor® 647 isotype control	—	20 µL

(For Sorting) Component	Volume to add to tube for sorting	
	Specific stain	Isotype control
Cell suspension (at 1 x 10^7 cells per mL)	1 mL (1 x 10^7 cells)	100 µL (1 x 10^6 cells)
FITC SSEA-1	60 µL	—
PE SSEA-3	60 µL	—
Alexa Fluor® 647 TRA-1-81	60 µL	—
FITC isotype control	—	20 µL
PE isotype control	—	20 µL
Alexa Fluor® 647 isotype control	—	20 µL

3. Mix the tubes gently and incubate in the dark on ice for 30 minutes.

   Note: If you are working with fixed cells (or cells for analysis only), you can incubate on ice or at room temperature.

   Note: If you want to stain the control beads at this time, see Preparing control beads (page 24).

4. After the 30-minute incubation is complete, wash each tube twice as follows (centrifuging at 300*g* for 5 minutes).

If staining cells for...	Add...
Analysis	2 mL of stain buffer
Sorting	2 mL of medium with 5 mM EDTA

5. Resuspend the cells as follows.

Cells for...	Resuspend in...
Analysis	300 to 400 µL of stain buffer
Sorting	Medium with 5 mM EDTA • Unstained cells and isotype controls: 500 µL • Specific-stained cells: sufficient to obtain a concentration of 2 x 10^6 to 5 x 10^6 cells per mL (or the concentration recommended by your cell sorter manufacturer)

Storage

Storage of samples is not recommended. The stained cells should be analyzed or sorted immediately.

Next step

Proceed immediately to Running the control beads (page 28).

Related topics

- Kit contents (page 4)
- Required materials (page 11)
- Workflow overview (page 10)
- Common cell-preparation techniques (page 13)

Preparing control beads

About this topic

This topic explains how to prepare control beads for use with the BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit.

Aseptic technique

If you will be performing an aseptic sort, you may want to perform this procedure aseptically in a biological safety cabinet to minimize potential contamination of the flow cytometer prior to sorting.

Staining the beads

To stain beads for compensation controls:

1. Label four 12 x 75-mm polystyrene tubes as follows:
   - Negative
   - FITC
   - PE
   - Alexa Fluor® 647

   Note: If you plan to stain your cells with additional antibodies, prepare tubes for those antibodies as well so that you can calculate compensation for all relevant fluorochromes.

2. Add liquid to each tube as follows.

If staining beads for...	Add...
Analysis	100 µL of stain buffer
Sorting	100 µL of sterile medium or PBS

3. Add the following to each tube in the order shown. Vortex the beads *thoroughly* just before dispensing.

Component	Volume to add to tube			
	Negative	FITC	PE	Alexa Fluor® 647
Negative beads	1 drop	1 drop	1 drop	1 drop
Anti-mouse beads	—	1 drop	—	1 drop
Anti-rat beads	—	—	1 drop	—
FITC SSEA-1	—	20 µL	—	—
PE SSEA-3	—	—	20 µL	—
Alexa Fluor® 647 TRA-1-81	—	—	—	20 µL

4. Vortex the tubes.
5. Incubate at room temperature in the dark for 30 minutes.
6. After the incubation is complete, wash each tube twice as follows (centrifuge at 300*g* for 5 minutes).

If staining cells for...	Add...
Analysis	2 mL of stain buffer
Sorting	2 mL of sterile medium or PBS

7. Resuspend as follows:

If staining cells for...	Resuspend in...
Analysis	300 to 400 µL of stain buffer
Sorting	500 µL of medium with 5 mM EDTA

Next step

Proceed immediately to Running the control beads (page 28).

Storage of stained beads is not recommended. The beads should be run on the cytometer immediately after staining.

Related topics

- Kit contents (page 4)
- Required materials (page 11)
- Running the control beads (page 28)

4

Cytometer procedures

This section covers the following topics:

Running the control beads

About this topic

This topic explains how to use the prepared control beads to set up the application for analysis or sorting of stained human pluripotent stem cells.

Purpose of the procedure

The stained beads are used here for two purposes:

- To adjust the forward scatter (FSC), side scatter (SSC), and fluorescence settings so that hESCs or iPS cells will be on scale (this minimizes the adjustments you will have to make later, thereby preserving stained cells)
- To calculate compensation

If you are using this kit for the first time on a new cell type, running the beads establishes application settings that can be saved for future use. If you already have saved application settings, running the beads confirms these settings.

Before you begin

Ensure that your instrument configuration is appropriate for this assay. If necessary, add Alexa Fluor® 647 as a parameter. Alternatively, you can use the APC detector to detect Alexa Fluor® 647.

The guidelines and examples in this section use a BD FACSAria II flow cytometer and BD FACSDiva software. However, the fundamental approach to setup, acquisition, sorting, and analysis can be adapted to other flow cytometers. Note that the instrument adjustments in a given experiment might vary from one instrument to another and from one sample to another.

Ensure that you run the appropriate instrument setup and QC procedures for your flow cytometer. See your user's guide for more information. If you are performing an aseptic sort using the BD FACSAria II flow cytometer, follow the procedure outlined in the *BD FACSAria II User's Guide* to prepare for an aseptic sort.

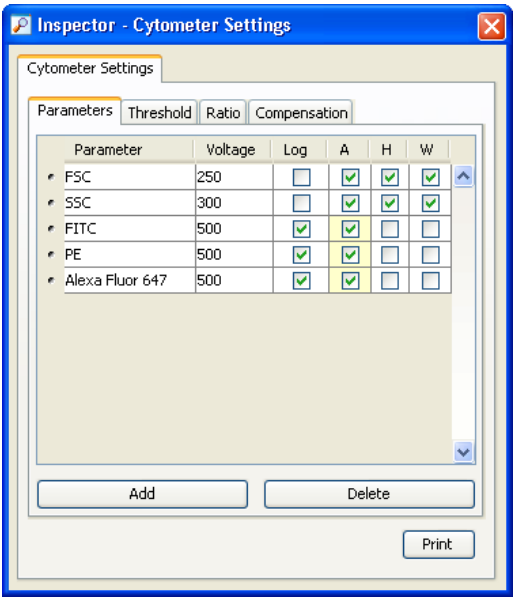
Complete the steps described in Preparing control beads (page 24).

Procedure

To run the prepared control beads:

1. Create a new experiment in BD FACSDiva software.
2. If you have saved application settings for use with this kit, apply the application settings, and proceed to step 4.

3. Delete all parameters except FSC, SSC, FITC, PE, and Alexa Fluor® 647 (or APC). Select the boxes for FSC-W, FSC-H, SSC-W, and SSC-H.



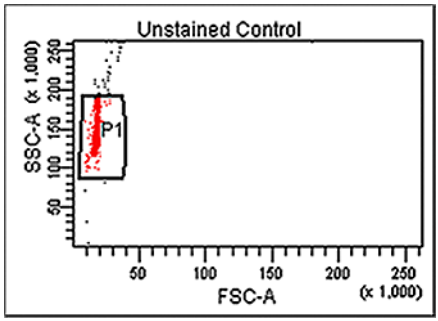
Note: The voltage settings that appear in this window will vary for each instrument.

4. Create compensation controls using the **Compensation Setup** feature in BD FACSDiva software.

5. Create a statistics view in the **Unstained Control** worksheet to display the FSC mean and SSC mean for the P1 population.

6. Place the tube of unstained (negative) beads on the cytometer and begin acquisition.

7. Set the P1 gate around the singlet bead population.



8. Adjust the FSC and SSC PMT voltages to obtain the following values.

Parameter	Mean of P1 bead population
FSC	10,000 to 20,000
SSC	85,000 to 105,000

Note: Adjusting the voltages to obtain these values should place your stem cells on scale.

9. Right-click **Cytometer Settings** and select **Application Settings > Create Worksheet.**

10. Check the settings for all parameters using the unstained cells.

11. Place each of the stained compensation control tubes on the cytometer in turn, and adjust the FITC, PE, and Alexa Fluor® 647 (or APC) PMT voltages so that the positive population is on scale. If any of the populations are off scale (too high), adjust the PMT voltages to bring the population to between 10^4 and 10^5. If a population is lower than 10^4, do not increase the PMT voltage.

12. If you have not already done so, save the application setting for future use.

    a. In the **Browser**, right-click **Cytometer Settings** and select **Application Settings > Save**.

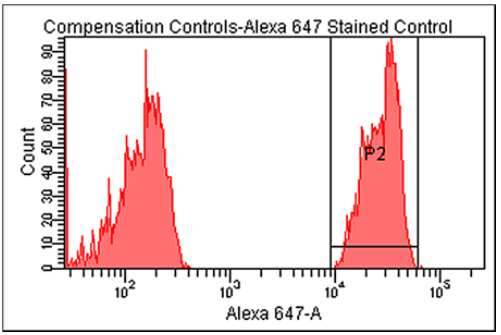
    b. Name the application, then click **OK**.

    Note: You can now use these application settings as a starting point for future experiments.

13. Return to the compensation worksheets.

14. Reinstall the tube of unstained beads and record data.

15. Record data for the remaining compensation control tubes. Make sure to adjust the P2 gates to fit the positive populations.



16. Calculate compensation.

    a. From the **Experiment** menu, select **Compensation Setup > Calculate Compensation**.

    b. Name the compensation setup, then click **Link and Save**.

Next step

Proceed to Setting up the workspace for running cells (page 34).

More information

See *Getting Started with BD FACSDiva Software* for information about creating and working with experiments.

See the *BD FACSDiva Software Reference Manual* for information about creating compensation controls, creating statistics views, acquiring data, and calculating compensation.

See the *BD Cytometer Setup and Tracking Application Guide* for information about applying application settings.

Related topics

- Troubleshooting (page 48)
- Example of bead and cell placement (page 50)

Setting up the workspace for running cells

About this topic

This topic explains how to set up a workspace in BD FACSDiva software for analyzing or sorting cells stained with the BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit.

Before you begin

Complete the steps in Running the control beads (page 28).

Check your cytometer configuration. If the configuration is not set up for Alexa Fluor® 647, use the APC parameter instead.

Your research needs will determine whether you run the isotype controls before or after you set up your gating strategy.

If you plan to run the isotype controls now, skip ahead to Running the cells (page 36) for instructions on how to run the controls, and then return to this procedure.

Procedure for analysis

To set up the workspace for running cells:

1. Create a new specimen in BD FACSDiva software.
2. Create tubes and label them appropriately for the unstained cells, isotype-control cells, and specific-stained cells.
3. If you have previously saved a template for use with this kit, import the template and proceed directly to Running the cells (page 36).
4. In the **Labels** tab of the **Experiment Layout** window, enter parameter labels for each marker in the experiment.

Plots for analysis

To set up a plots for analysis:

1. On a global worksheet, create the following contour plots:
   - FSC-A vs SSC-A
   - PE-A vs FITC-A
   - Alexa Fluor® 647-A (or APC-A) vs FITC-A
   - Alexa Fluor® 647-A (or APC-A) vs PE-A
2. Create the following histograms:
   - FITC-A
   - PE-A
   - Alexa Fluor® 647-A (or APC-A)
3. Select biexponential scaling for all fluorochrome axes.
4. Save this worksheet as a template for use in future experiments.

Plots for sorting

To set up a plots for sorting:

1. On a global worksheet, create the following plots:
   - FSC-A vs SSC-A
   - FSC-H vs FSC-W
   - SSC-H vs SSC-W
   - PE-A vs FITC-A
   - Alexa Fluor® 647-A (or APC-A) vs PE-A
2. Right-click the first plot and select **Show Population Hierarchy**.
3. Save this worksheet as a template for use in future experiments.

Next step

Proceed to Running the cells (page 36).

More information

See *Getting Started with BD FACSDiva Software* for information about working in the BD FACSDiva workspace.

See the *BD FACSDiva Software Reference Manual* for information about how to import analysis templates.

Related topics

- Workflow overview (page 10)
- Template examples (page 44)

Running the cells

About this topic

This topic explains how to fine-tune scatter settings on your flow cytometer using the unstained cells, and record data for unstained, isotype, and specific-stained cells.

See the instructions for your flow cytometer system for a detailed procedure.

Before you begin

Complete the steps described in Staining the cells (page 20) and Setting up the workspace for running cells (page 34).

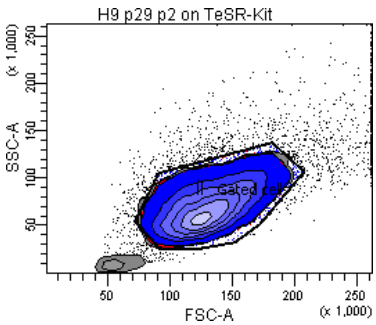
Retrieve the unstained cells that you set aside at the beginning of Staining the cells (page 20).

Procedure

To run the cells:

1. Place the tube of unstained cells on the cytometer and acquire a small subset of the unstained cells.
2. Adjust the FSC and SSC PMT voltages as needed to ensure that the cell population appears on scale in the scatter plot.

   Do not adjust the fluorescence settings at this stage. Adjusting the fluorescence settings now will invalidate your compensation calculations.
3. In the FSC-A vs SSC-A plot, gate on the cell population.



   Note: We recommend using a cluster-based approach for analyzing multicolor data, although single-parameter analysis can also be used.
4. Record data from the unstained cells.

   Record 10,000 cells (or more if you are interested in rare events).
5. Place the isotype control tubes on the cytometer and record the data.
6. Place the specific-stained cells on the cytometer and record the data.

Related topics

- Workflow overview (page 10)
- Template examples (page 44)
- Troubleshooting (page 48)
- Example of bead and cell placement (page 50)

Sorting cells

About this topic

This topic provides guidelines for setting up your flow cytometer for sorting, as well as an example procedure for how to sort stained pluripotent stem cells on a BD FACSAria II flow cytometer.

See the *BD FACSAria II User's Guide* for detailed instructions.

Before you begin

Complete the steps described in Setting up the workspace for running cells (page 34) and Running the cells (page 36).

If you are performing an aseptic sort, follow the procedure outlined in the *BD FACSAria II User's Guide* to prepare for an aseptic sort.

Cytometer setup guidelines

We recommend the following setup for sorting stem cells:

- A 100-µm nozzle
- A drop-drive frequency between 25 and 30 kHz
- Pressure of approximately 20 to 25 psi

Additional steps

Follow the instructions in you user's guide for optimizing the breakoff and drop delay.

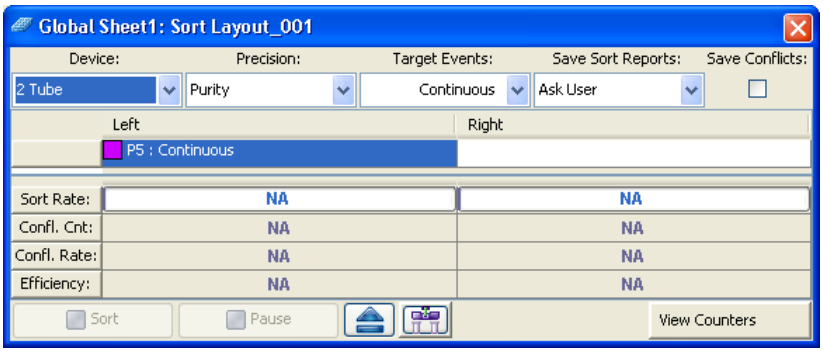
Warning

To avoid a potential aerosol hazard when using a BD FACSAria II cytometer and BD FACSDiva software versions 3.0 through 5.0, use the **Sort** button to stop the sort when replacing collection tubes. Do not use the **Sort Pause** button.

Procedure

To sort cells:

1. From the **Sort** menu, select **New Sort Layout**. The **Sort Layout** window opens.
2. Add gate *P5* to the appropriate sort layout selection (eg, left collection tube).

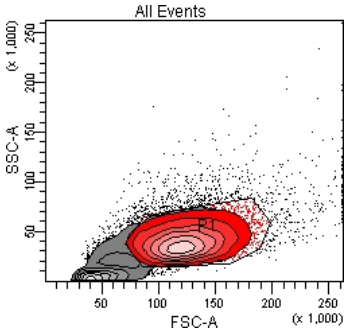


3. Fill the collection tubes with medium containing antibiotics.

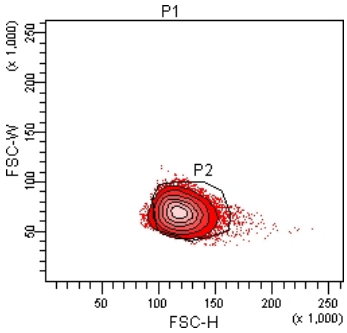
   Note: Consult the instructions for your cytometer to determine the appropriate volume of medium to use (typically 5 mL in a 15-mL conical tube).

4. Invert the tubes several times to coat the sides with medium, then install them on the cytometer.

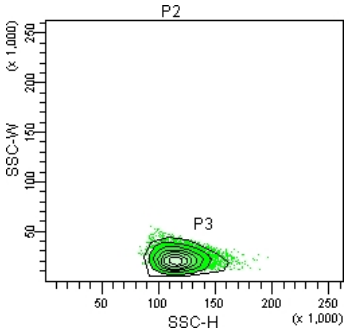
5. Place the tube of unstained cells on the cytometer and acquire a small subset of the unstained cells.
6. On the FSC-A vs SSC-A plot, gate on the cell population.



7. If necessary, increase the FSC threshold to exclude cell debris.
8. On the FSC-H vs FSC-W plot, gate the P2 population on P1-gated cells to discriminate doublets.

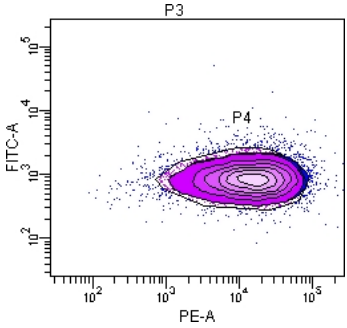


9. On the SSC-H vs SSC-W plot, gate the P3 population on P2-gated cells to discriminate doublets.

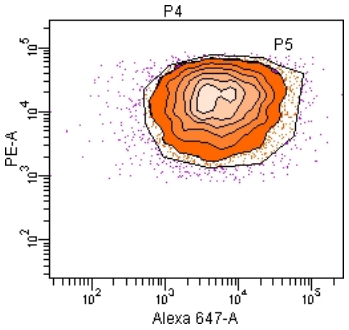


10. Place the tube of specific-stained cells on the cytometer and acquire a small subset of the specific-stained cells.

11. On the PE-A vs FITC-A plot, gate the P4 (SSEA-3^{+}SSEA-1^{-}) population on P3-gated cells.



12. On the Alexa Fluor® 647-A vs PE-A plot, gate the P5 (TRA-1-81^{+}SSEA-3^{+}) population on P4-gated cells.



13. Start the sort and monitor the stream during sorting.

Next step

Proceed to Plating cells (page 43).

Related topics

- Template examples (page 44)
- Troubleshooting (page 48)

Plating cells

About this topic

This topic describes how to plate cells that have been stained and sorted using the BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit.

Before you begin

Complete the steps described in Sorting cells (page 38).

Ensure that your cell culture plates are ready for plating.

Using antibiotics

You may want to culture sorted cells in medium containing antibiotics (penicillin/streptomycin) for at least one passage.

Procedure

To plate cells:

1. Centrifuge the sorted cells at 300*g* for 5 minutes.
2. Wash cells twice in medium containing antibiotics.
3. Plate cells onto the appropriate matrix medium or feeder layer.

   Note: For hESCs cultured in mTESR 1 medium on BD Matrigel hESC-qualified matrix or for hESCs cultured on mouse embryonic fibroblast feeder layers, we recommend plating about 2 x 10^5 cells per cm^2, or placing about 2 x 10^6 cells into one well of a 6-well dish.

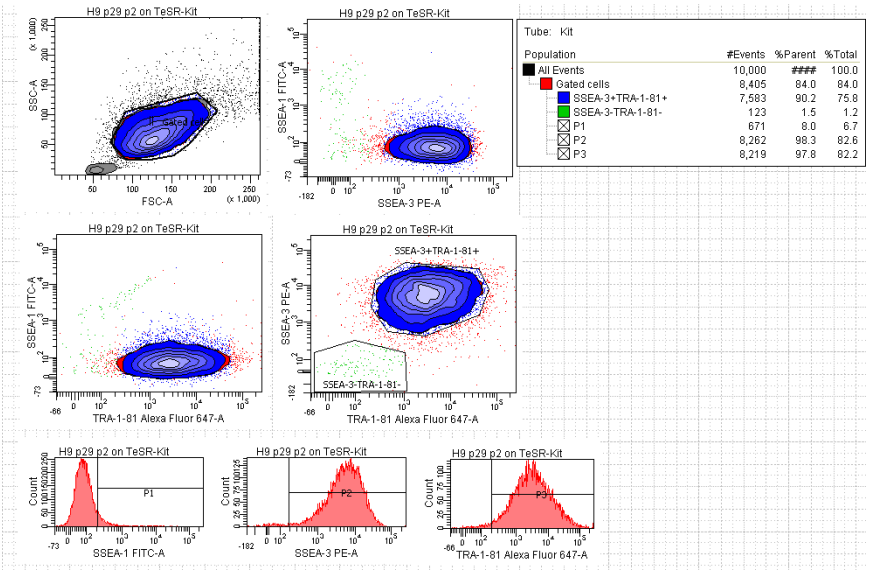
Template examples

About this topic

This topic contains examples of templates with defined gates, showing data from the H9 cell line stained with the BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit.

Example with undifferentiated cells

The following is an example of an analysis template, showing data from undifferentiated H9 cells.



Tube: Kit

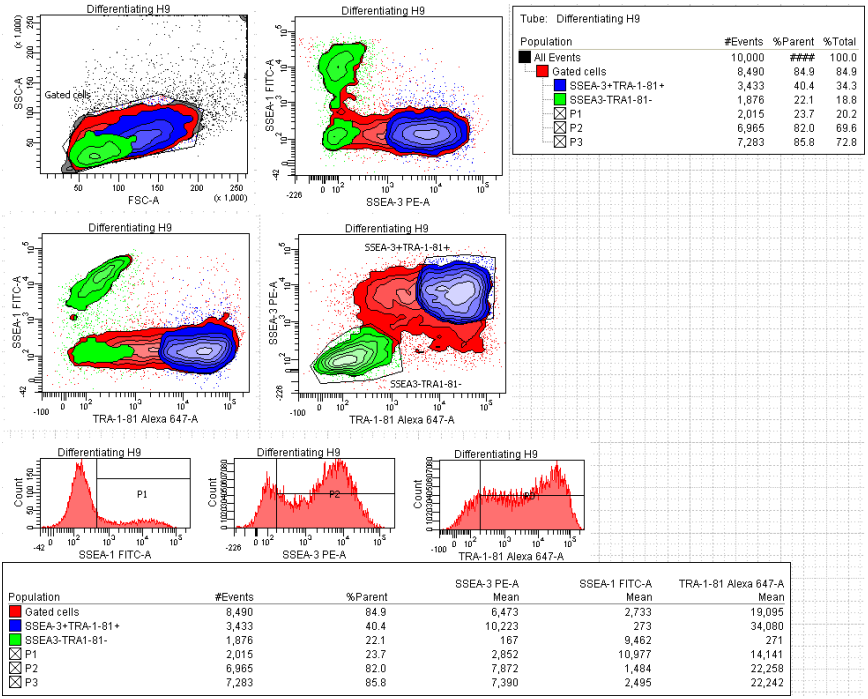
Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Gated cells	8,405	84.0	84.0
SSEA-3+TRA-1-81+	7,583	90.2	75.8
SSEA-3-TRA-1-81-	123	1.5	1.2
P1	671	8.0	6.7
P2	8,262	98.3	82.6
P3	8,219	97.8	82.2

Tube Name: Kit

Population	#Events	%Parent	SSEA-1 FITC-A Mean	SSEA-3 PE-A TRA-1-81 Alexa Fluor 647... Mean
Gated cells	8,405	84.0	148	7,759	7,933
SSEA-3+TRA-1-81+	7,583	90.2	100	7,662	6,299
SSEA-3-TRA-1-81-	123	1.5	2,133	63	230
P1	671	8.0	961	7,081	9,906
P2	8,262	98.3	104	7,893	8,013
P3	8,219	97.8	144	7,863	8,110

Example with differentiating cells

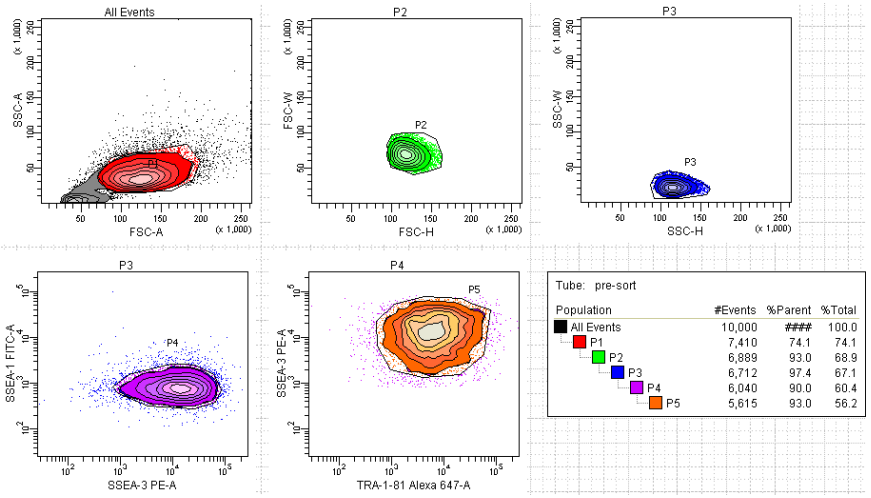
The following is an example of an analysis template, showing data from differentiating H9 cells.



Population	#Events	%Parent	SSEA-3 PE-A Mean	SSEA-1 FITC-A Mean	TRA-1-81 Alexa 647-A Mean
Gated cells	8,490	84.9	6,473	2,733	19,095
SSEA-3+TRA-1-81+	3,433	40.4	10,223	273	34,080
SSEA3-TRA1-81-	1,876	22.1	167	9,462	271
P1	2,015	23.7	2,852	10,977	14,141
P2	6,965	82.0	7,872	1,484	22,258
P3	7,283	85.8	7,390	2,495	22,242

Example for sorting

The following is an example of a sorting template, showing data from undifferentiated H9 cells.



Related topics

- Running the cells (page 36)

5

Reference

This section covers the following topics:

Troubleshooting

About this topic This topic provides assistance for specific problems that you might encounter while using the BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit.

Recommended actions These are the actions we recommend you take if you encounter the following specific problems.

Problem	Recommended actions
Sorting pauses automatically on a BD FACSAria II flow cytometer	See *BD FACSAria II User's Guide* for troubleshooting information.
Sorting stops automatically on a BD FACSAria II flow cytometer	See *BD FACSAria II User's Guide* for troubleshooting information.
Cells clump during acquisition	Ensure that you stain a single-cell suspension. Add EDTA to a concentration of 5mM to your staining buffer. Pass the cells through a 70-µm cell strainer. Treat the cells with DNAse before staining if considerable cell death is observed.
High background fluorescence during sorting	Use medium without phenol red. Serum components are known to sometimes bind non-specifically to the surface of some cells and result in high background. Use BD Pharmingen stain buffer (FBS) or a pre-screened serum to supplement your stain buffer.
Low rates of cell survival and attachment after plating sorted cells	Incubate your sorted cells in Y27632 (Rho-associated kinase inhibitor) at a concentration of 10 µM for 24 hours.[5,6] Plate your sorted cells at a higher density.

Problem	Recommended actions
Difficulty sorting collagenase IV passaged cells	• Adapt the cells to single-cell dissociation (eg, Accutase).[7] • Plate the sorted cells at a higher density. • Continue to feed cells for two weeks after sorting (collagenase IV–passaged cells may take longer to attach after sorting). • Use cell lines that can be routinely passaged to single cells (eg, HUES lines).

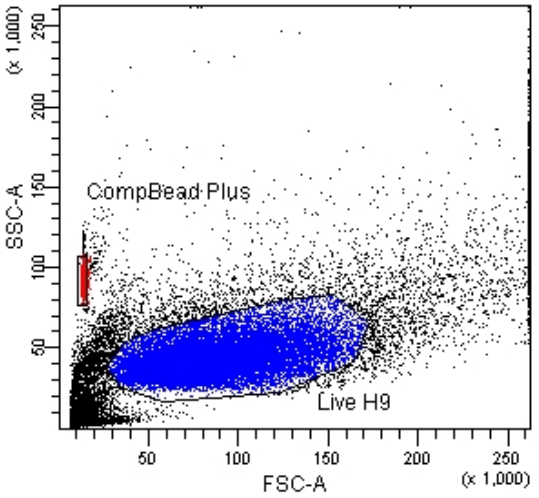
Example of bead and cell placement

About this topic

This topic gives an example of an FSC-A vs SSC-A dot plot, showing where BD CompBead Plus beads appear relative to a single-cell suspension of undifferentiated stem cells.

Example of bead and cell placement

The following plot shows BD CompBead Plus beads run together with a single-cell suspension of live cells from the H9 hESC line.



Note: If you are running differentiated cells and/or fixed cells, you might need to adjust both FSC and SSC to accommodate your cell populations.

Related topics

- Running the control beads (page 28)

References

About this topic This topic contains a list of the publications cited in this manual.

References

1. Draper JS, Pigott C, Thomson JA, Andrews PW. Surface antigens of human embryonic stem cells: changes upon differentiation in culture. *J Anat*. 2002;200:249-258.
2. Xu C. Characterization and evaluation of human embryonic stem cells. *Methods Enzymol*. 2006;420:18-37.
3. Adewumi O, Aflatoonian B, Ahrlund-Richter L, et al. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol*. 2007;25:803-816.
4. Lowry WE, Richter L, Yachechko R, et al. Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc Natl Acad Sci U S A*. 2008;105:2883-2888.
5. Watanabe K, Ueno M, Kamiya D, et al. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat Biotechnol*. 2007;25:681-686.
6. Park IH, Zhao R, West JA, et al. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature*. 2008;451:141-146.
7. Bajpai R, Lesperance J, Kim M, Terskikh AV. Efficient propagation of single cells Accutase-dissociated human embryonic stem cells. *Mol Reprod Dev*. 2008;75:818-827.

Further information

Additional information about the software and cytometers recommended for this application can be found in the Training section of the BD Biosciences website:

bdbiosciences.com/immunocytometry_systems/support/training

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0120.8555.90

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Latin America/Caribbean
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