BD Human Pluripotent Stem Cell Sorting and Analysis Kit

Instruction Manual

History

Revision	Date	Change Made
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™ 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 researchers with the reagents necessary to perform multicolor flow cytometry on human pluripotent stem cells such as human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSs).

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 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 used and reported widely in the literature 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 fluorophore as one of the specific antibodies.

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

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

Control beads

This kit also contains BD CompBead Plus positive and negative beads to facilitate cytometer 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 used to optimize cytometer settings, including fluorescence compensation. Use of these beads ensures consistent 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 fluorescent antibodies specific to other cellular molecules.

The kit is therefore compatible with the use of antibodies to cell 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, or cell death.

Kit contents

About this topic This topic describes the contents of the BD Human Pluripotent Stem Cell Sorting and Analysis Kit.

Kit contents The kit contains the following:

Vial	Reagent information
1	**Reagent.** 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)
2	**Reagent.** 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)
3	**Reagent.** 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)

Vial	Reagent information
4	**Reagent.** 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)
5	**Reagent.** 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)
6	**Reagent.** 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)

Vial	Reagent information
7	**Reagent.** 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 κ light-chain bearing immunoglobulin) **Abbreviation.** anti-mouse beads **Quantity.** 1 vial (3.0 mL)
8	**Reagent.** BD CompBead Plus Anti-Rat Ig, κ **Use.** Used to create control beads stained with PE SSEA-3 (because beads bind any rat κ light-chain bearing immunoglobulin) **Abbreviation.** anti-rat beads **Quantity.** 1 vial (1.5 mL)
9	**Reagent.** 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)

Related topics

- Storage and handling (page 12)

Storage and handling

About this topic

This topic describes the requirements for kit storage and handling.

Storage

The entire BD Human Pluripotent Stem Cell Sorting and Analysis Kit must be stored in the dark at 4°C. Do not freeze.

Warning

The reagents in this kit contain sodium azide. Sodium azide is harmful if swallowed (R22). Keep away from foodstuffs, beverages, and feed. Keep container tightly closed (S7). Do not breathe gas, fumes, vapor, or spray (S23). Wear suitable protective clothing, gloves, and eye/face protection (S36/37/39). In case of contact with eyes, rinse immediately with plenty of water and seek medical advice (S26). Take off immediately all contaminated clothing (R27). Contact with acids liberates very toxic gas (R32). This material and its container must be disposed of as hazardous waste (S60). Contact with acidic solutions and metal compounds over time may form potentially explosive metal azides. Should any of this material be introduced into a sanitary sewer system, flush with copious amounts of water.

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.

2

Before you begin

This section covers the following topics:

Planning your work

About this topic

This topic provides some guidelines for planning the work involved in using the BD Human Pluripotent Stem Cell Sorting and Analysis Kit to analyze or sort cells.

Before you begin

Ensure that you run the appropriate setup and QC procedures for your cytometer before you begin the cytometer methods described in this manual.

Analysis vs sorting

To plan your work, you must first decide whether you will be analyzing live cells, analyzing fixed cells (if you need to store the cells prior to analysis), or sorting live cells.

The required materials, incubation times, and specific procedures differ for each of these applications.

Required conditions

If you plan to sort cells, all sample-preparation steps must be performed under aseptic conditions.

Workflow stages

Whether you are preparing cells for analysis or for sorting, the overall workflow consists of three stages, each of which is described in its own chapter:

1. Preparing the cells (page 21)
2. Setting up the cytometer (page 35)
3. Processing samples (page 59)

Overview of preparing cells

Preparing the cells includes the following steps:

Step	Description
1	Obtaining a single-cell suspension (page 22)
2	Determining the target volume (page 24)
3	Treating the cells with DNAse (page 26) **Note:** Perform only if sorting cells.
4	Adjusting the cell concentration (page 27)
5	Fixing the cells (page 28) **Note:** Perform only if analyzing fixed cells.
6	Staining the cells (page 29)
7	Washing the cells (page 32)

Overview of setting up the cytometer

Setting up the cytometer includes the following steps:

Step	Description
1	Preparing control beads (page 36)
2	Creating a new experiment (page 40)
3	Running beads for cytometer setup (page 42)
4	Setting up for sorting (page 47) **Note:** Perform only if sorting cells.
5	Creating a new specimen (page 48)
6	Optimizing the settings (page 49)
7	Setting up a gating strategy (page 50)

Overview of processing samples

Processing samples includes the following steps:

Step	Description
1	Acquiring data (page 60) **Note:** If analyzing samples, this is the end of your workflow.
2	Sorting cells (page 61)
3	Plating cells (page 63)

Incubation times

To help you plan your work, the incubation times are listed in the following table:

Procedure	Incubation time
Obtaining a single-cell suspension	As recommended by the enzyme manufacturer
Treating the cells with DNAse **Note:** Only if sorting cells	10 to 15 minutes
Fixing the cells **Note:** Only if analyzing fixed cells	10 minutes
Staining the cells	30 minutes
Preparing control beads	30 minutes

Workflow efficiency

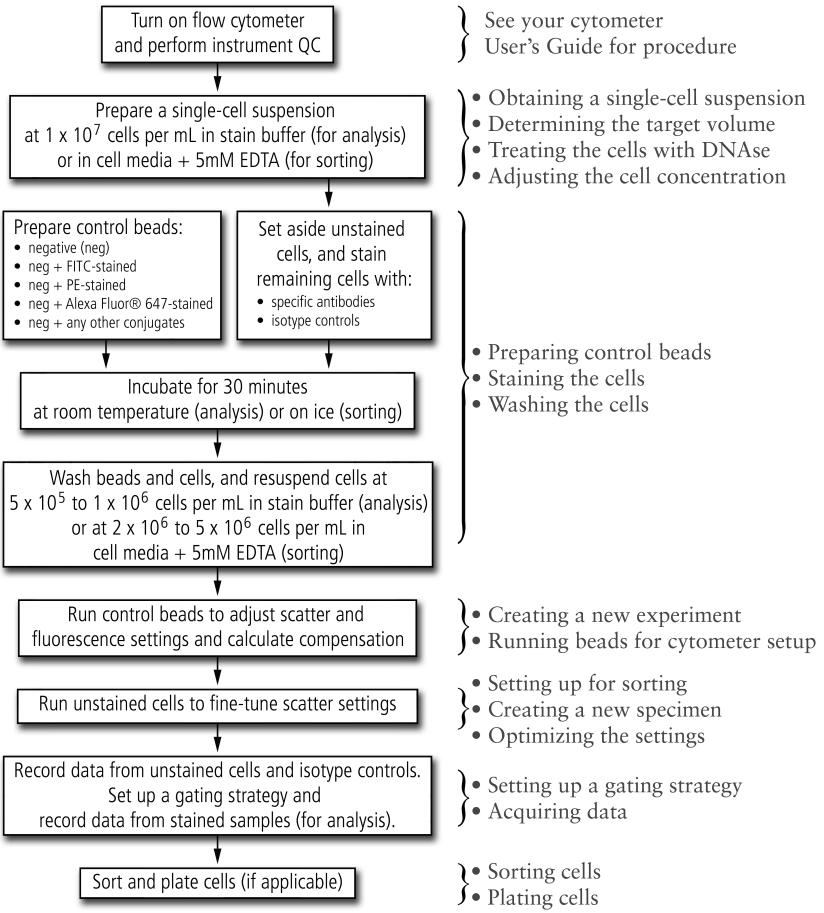
Some procedures must be performed sequentially, and this is noted in the text. For example, you must stain the beads before you can run them on the flow cytometer.

For efficiency, you can perform other procedures in parallel. For example, you might decide to stain the beads at the same time as you stain your cells.

Example workflow

The following example shows a typical workflow where steps are performed in parallel for efficiency.

The procedure objectives are shown in boxes, while the specific procedures required to obtain those objectives are listed on the right.



Required materials and conditions

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 for sorting)
- 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™ 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)

Materials for analyzing fixed cells

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

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

Materials for sorting

If you are sorting cells, you will also need:

- Tissue culture hood (for aseptic sample preparation)
- Ice
- Tissue-culture-grade buffered EDTA solution (available from Gibco)
- Sterile DNAse solution (available from Sigma)
- Antibiotics such as penicillin and streptomycin (available from Gibco)
- 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
- mTESR™1 maintenance medium for human embryonic stem cells from Stem Cell Technologies, Inc., or equivalent (referred to throughout this manual as media)

Related topics

- Kit contents (page 9)

3

Preparing the cells

This section covers the following topics:

Obtaining a single-cell suspension

About this topic

This topic explains how to obtain a single-cell suspension before staining with the BD Human Pluripotent Stem Cell Sorting and Analysis Kit.

There are two steps involved in obtaining a single-cell suspension: detaching the cells from their growth matrix, and then removing any cell clumps.

Before you begin

Ensure that you have all of the necessary materials available. See Required materials and conditions (page 18) for details.

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

Aseptic conditions

If you are preparing cells for sorting, all sample-preparation steps must be done aseptically in a tissue culture hood.

Aseptic conditions are not required if you are preparing cells for analysis only.

Detaching cells

To detach the cells from their growth matrix:

1. Wash the cells with room-temperature PBS.
2. Add 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.

Removing cell clumps

To remove cell clumps by gently breaking them apart or filtering them out:

1. Pipette the cells gently up and down with a pipette.
2. Remove a small subset of the liquid and check it under a microscope to confirm the presence of single cells.
3. If you observed 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.
4. Add two to four volumes of PBS (if analyzing) or media (if sorting) to wash the cells.
5. Centrifuge at 300*g* for 5 minutes.
6. Aspirate to remove the supernatant, being careful not to disturb the cells.
7. Resuspend the cells in a volume of PBS (if analyzing) or media (if sorting) that is appropriate for cell counting.

Next step

Proceed to Determining the target volume (page 24).

Determining the target volume

About this topic

This topic explains how to determine the target volume for each individual sample that you will stain. It also provides guidelines about target volumes to yield enough cells for analysis or sorting.

Purpose of this procedure

After completing the steps described in Obtaining a single-cell suspension (page 22), each of your samples will have a unique cell concentration.

For the next several procedures, your cell samples all need to be at a concentration of 1×10^7 cells per mL.

To obtain this uniform concentration, you must add a different target volume of liquid to each sample.

Procedure

To determine the target volume:

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 a concentration of 1×10^7 cells per mL. This is your target volume. Write this number down so that you can refer to it later.
3. Repeat steps 1 and 2 for each sample.

Target volumes for cell analysis

If you plan to stain cells for analysis, we recommend that you use 1×10^6 cells per sample, but you might be successful with as few as 2.5×10^5.

For each cell line you will be analyzing, we also recommend that you run a tube of unstained cells along with your stained cells. In addition, you might decide to run an isotype control for each cell line.

This means that if you plan to run an isotype control (ie, three tubes per sample), it is best to have a target volume of at least 300 µL (3 x 10^6 cells), and no less than 75 µL (7.5 x 10^5 cells). If you do not plan to run an isotype control (ie, two tubes per sample), it is best to have a target volume of at least 200 µL (2 x 10^6 cells), and no less than 50 µL (5 x 10^5 cells).

Target volumes for sorting

If you plan to stain cells for sorting, we recommend that you use at least 5 x 10^6 cells per sample.

For each cell line you will be sorting, we recommend that you also run a tube of roughly 1 x 10^6 unstained cells. In addition, you might decide to run an isotype control for each cell line.

This means that if you plan to run an isotype control (ie, three tubes per sample), it is best to have a target volume of at least 700 µL (5 x 10^6 cells for sorting plus 2 x 10^6 cells for controls). If you do not plan to run an isotype control, it is best to have a target volume of at least 600 µL (5 x 10^6 cells for sorting plus 1 x 10^6 cells for controls).

Next step

Proceed to Treating the cells with DNAse (page 26) if you are preparing to sort cells. If you are preparing to analyze cells, proceed directly to Adjusting the cell concentration (page 27).

Further information

See Staining the cells (page 29) for more information about isotype controls.

Treating the cells with DNAse

About this topic

This topic explains how to treat cells with DNAse before staining with the BD 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 Adjusting the cell concentration (page 27).

Before you begin

Complete the steps described in Determining the target volume (page 24).

Make media containing 100 units per mL of DNAse. Make enough to supply all target volumes calculated in the previous step. See Required materials and conditions on page 18.

Procedure

To perform the DNAse treatment:

1. Centrifuge the cells at 300*g* for 5 minutes.
2. Aspirate to remove the supernatant, being careful not to disturb the cells.
3. Resuspend the cells in media containing 100 units per mL of DNAse to bring up to the volume calculated in Determining the target volume (page 24).
4. Incubate the cells for 10-15 minutes at room temperature.

Next step

When the incubation is complete, proceed immediately to Adjusting the cell concentration (page 27).

Related topics

- Required materials and conditions (page 18)

Adjusting the cell concentration

About this topic

This topic explains how to adjust the cell concentration to 1 x 10^7 cells per mL before staining with the BD Human Pluripotent Stem Cell Sorting and Analysis Kit.

Before you begin

Complete the steps described in Determining the target volume (page 24). If you are preparing cells for sorting, complete the steps described in Treating the cells with DNAse (page 26).

Procedure for analysis

To adjust the cell concentration if you will be staining for analysis:

1. Centrifuge the cells at 300*g* for 5 minutes.
2. Aspirate to remove the supernatant, being careful not to disturb the cells.
3. Resuspend in stain buffer to bring to the volume calculated in Determining the target volume (page 24).

Procedure for sorting

To adjust the cell concentration if you will be staining for sorting:

1. Centrifuge the cells at 300*g* for 5 minutes.
2. Aspirate to remove the supernatant, being careful not to disturb the cells.
3. Resuspend in media with 5mM EDTA to bring to the volume calculated in Determining the target volume (page 24).

Next step

If you plan to analyze fixed cells, proceed to Fixing the cells (page 28). If you plan to analyze live cells or if you plan to sort cells, proceed directly to Staining the cells (page 29).

Related topics

- Required materials and conditions (page 18)

Fixing the cells

About this topic

This topic explains how to fix cells before staining with the BD 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 Adjusting the cell concentration (page 27).

Procedure

To fix the cells:

1. Centrifuge at 300*g* for 5 minutes.
2. Aspirate to remove the supernatant, being careful not to disturb the cells.
3. Add BD Cytofix fixation buffer (4% PFA) to bring to the volume calculated in Determining the target volume (page 24).
4. Incubate for 10 minutes.
5. Add two to four volumes of PBS to wash the cells.
6. Centrifuge at 300*g* for 5 minutes.

7. Aspirate to remove the supernatant, being careful not to disturb the cells.
8. Repeat steps 5 to 7.
9. Resuspend in stain buffer to bring to the volume calculated in Determining the target volume (page 24).

Next step Proceed to Staining the cells (page 29).

Related topics
- Required materials and conditions (page 18)

Staining the cells

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

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

Use of 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 significant background staining has been observed.

Before you begin Complete the steps described in Adjusting the cell concentration (page 27). If you are preparing to analyze fixed cells, complete the steps described in Fixing the cells (page 28).

Workflow efficiency

To maximize your efficiency, you might decide to stain the beads at the same time as you stain your cells. See Preparing control beads on page 36.

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 "unstained cells" and one "isotype control" (if applicable).
2. Add 100 µL (1 x 10^6 cells) of your prepared cell suspension to each labeled tube.
3. For the tube labeled "unstained cells":

If staining cells for...	Place the tube...
Analysis	In the dark at room temperature or on ice
Sorting	In the dark on ice

4. If you are using isotype controls, add each of the following reagents to the "isotype control" tube:
   - 20 µL of FITC isotype control
   - 20 µL of PE isotype control
   - 20 µL of Alexa Fluor® 647 isotype control
5. Mix gently and set aside in the dark.
6. For each of your samples, label one 12 x 75-mm polystyrene tube for specific staining.

7. Add cells from your prepared cell suspension to each specific-staining tube as follows:

If staining cells for...	Add...
Analysis	100 µL (1×10^6 cells)
Sorting	500 µL to 1 mL (5×10^6 to 1×10^7 cells)

8. Add reagents to each specific-staining tube as follows:

If staining cells for...	Add...
Analysis	• 20 µL of FITC SSEA-1 • 20 µL of PE SSEA-3 • 20 µL of Alexa Fluor® 647 TRA-1-81
Sorting	• 60 µL of FITC SSEA-1 • 60 µL of PE SSEA-3 • 60 µL of Alexa Fluor® 647 TRA-1-81

9. Mix gently.

10. Incubate your isotype control tubes and specific-staining tubes as follows:

If staining cells for...	Incubate...
Analysis	In the dark at room temperature or on ice for 30 minutes
Sorting	In the dark on ice for 30 minutes

Next step Proceed immediately to Washing the cells (page 32).

Related topics

- Kit contents (page 9)
- Required materials and conditions (page 18)

Washing the cells

About this topic

This topic explains how to wash the cells obtained in Staining the cells (page 29).

Timing of this procedure

Perform this procedure immediately after the completion of the staining incubation.

Procedure

To wash the cells:

1. Add the following to each tube:

If staining cells for...	Add...
Analysis	2 mL of stain buffer
Sorting	2 mL of media with 5mM 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. Repeat steps 1 through 4.

6. Resuspend as follows:

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

Storage

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

Related topics

- Required materials and conditions (page 18)

4

Setting up the cytometer

This section covers the following topics:

Preparing control beads

About this topic

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

This procedure has two steps: staining and then washing the BD CompBead Plus beads.

Required conditions

If you will be sorting cells, perform this procedure aseptically in a tissue culture hood 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
   - 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 fluorophores.

2. Add liquid to each tube as follows:

If staining cells for...	Add...
Analysis	100 µL of stain buffer
Sorting	100 µL of sterile PBS

3. Vortex the beads *thoroughly* just before adding the following to each tube:

Into the tube labeled...	Add...
Negative	• 1 drop of negative beads
FITC	• 1 drop of negative beads • 1 drop of anti-mouse beads • 20 µL of FITC SSEA-1
PE	• 1 drop of negative beads • 1 drop of anti-rat beads • 20 µL of PE SSEA-3
647	• 1 drop of negative beads • 1 drop of anti-mouse beads • 20 µL of Alexa Fluor® 647 TRA-1-81

4. Vortex the tubes.
5. Incubate at room temperature in the dark for 30 minutes.

Washing the beads

To wash the beads:

1. Add the following to each tube:

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

2. Mix gently.
3. Centrifuge at 300*g* for 5 minutes.

4. Aspirate to remove the supernatant, being careful not to disturb the beads.
5. Repeat steps 1 through 4.
6. Resuspend as follows:

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

Storage

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

Next step

Proceed immediately to Creating a new experiment (page 40).

Related topics

- Kit contents (page 9)
- Required materials and conditions (page 18)
- Running beads for cytometer setup (page 42)
- About spectral overlap and compensation (page 68)

Example cytometer methods

About this topic

This topic explains that the cytometer methods in this manual are examples that use one specific cytometer system, and it provides information about using other systems.

Example system

This manual provides example methods for using a BD FACSAria flow cytometer and BD FACSDiva software with the BD Human Pluripotent Stem Cell Sorting and Analysis Kit.

Other cytometer systems

While this manual does not provide instructions for using other systems, the fundamental methods can be adapted for research labs with other flow cytometers.

Note that the required instrument adjustments might vary between instruments and between individual samples in a given experiment.

Related topics

- Creating a new experiment (page 40)
- Running beads for cytometer setup (page 42)
- Setting up for sorting (page 47)
- Creating a new specimen (page 48)
- Optimizing the settings (page 49)
- Setting up a gating strategy (page 50)
- Processing samples (page 59)

Creating a new experiment

About this topic

This topic explains how to create a new BD FACSDiva experiment for setting up a BD FACSAria flow cytometer for use with the BD Human Pluripotent Stem Cell Sorting and Analysis Kit.

Before you begin

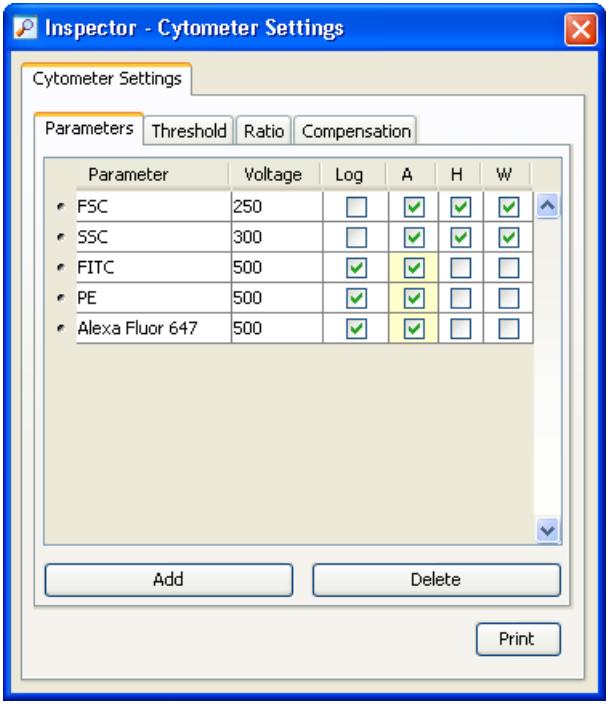
Ensure that you have run the appropriate setup and QC procedures for your cytometer.

Procedure

To create a new experiment for cytometer setup:

1. Start BD FACSDiva software.
2. From the **Experiment** menu, select **New Experiment**. A new experiment appears in the **Browser**.
3. From the **View** menu, select **Inspector**, then **Worksheet**, then **Acquisition Dashboard**.
4. Open the new experiment in the **Browser**, then click **Cytometer Settings**. The **Inspector - Cytometer Settings** window appears.

5. 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.



Next step

Proceed to Running beads for cytometer setup (page 42).

Related topics

- Example cytometer methods (page 39)

Running beads for cytometer setup

About this topic

This topic explains how to use the control beads prepared in Preparing control beads (page 36) to set up the cytometer for analysis or sorting of stained human pluripotent stem cells.

There are three steps in this procedure: creating the controls, acquiring the beads, then saving the settings.

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 iPSs will be on scale (this minimizes the adjustments you will have to make later, thereby preserving stained cells)
- To calculate compensation

Before you begin

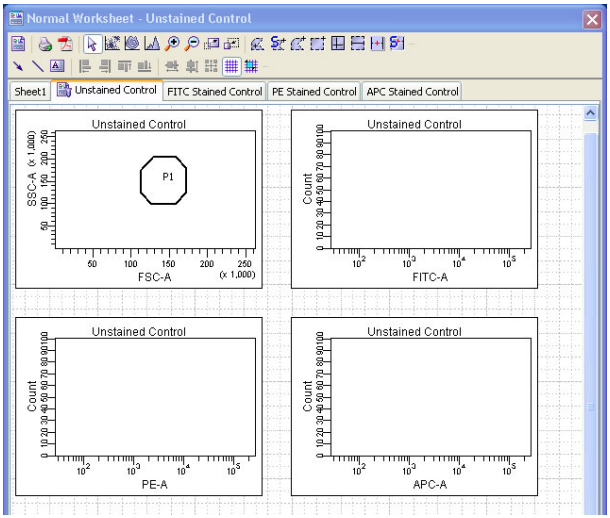
Complete the steps described in Preparing control beads (page 36) and Creating a new experiment (page 40).

Creating the controls

To create the controls in BD FACSDiva software:

1. From the **Experiment** menu, select **Compensation Setup > Create Compensation Controls**.
2. In the **Create Compensation Control** window, click **OK**.
3. In the **Browser**, click **Compensation Controls** to expand.

4. Double-click **Unstained Control**. The worksheet for the unstained control appears.



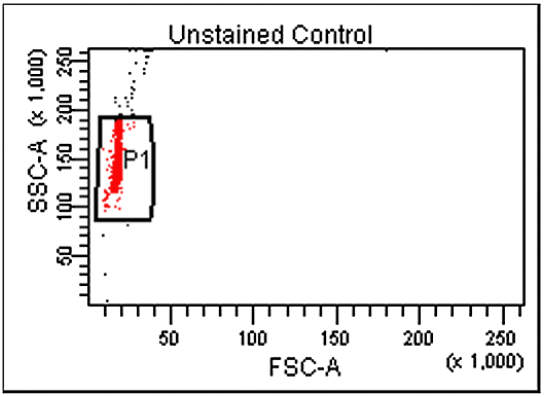
Acquiring the beads

1. Place the tube of unstained (negative) beads on the cytometer.
2. On the **Application Dashboard**, click **Acquire Data**. Events appear in the dot plot.
3. Adjust the FSC and SSC photomultiplier tube (PMT) voltages to obtain the following values:

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

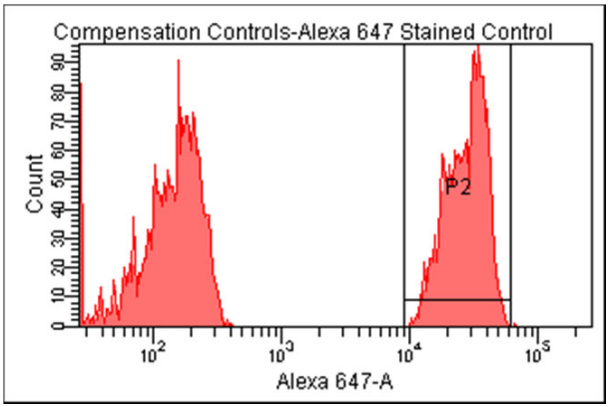
Note: Adjusting the voltages to obtain these values should place your stem cells on scale. See Example of bead and cell placement on page 45.

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



5. Place the FITC-stained compensation control tube on the cytometer.
6. Adjust the PMT voltage for FITC so that the positive population is between 10^4 and 10^5.
7. Adjust the PMT voltage for PE so that the positive population is between 10^4 and 10^5.
8. Adjust the PMT voltage for Alexa Fluor® 647 or APC so that the positive population is between 10^4 and 10^5.
9. Reinstall the tube of unstained beads and record data. Record data for the remaining compensation

control tubes. Make sure to adjust the P2 gates to fit the positive populations.



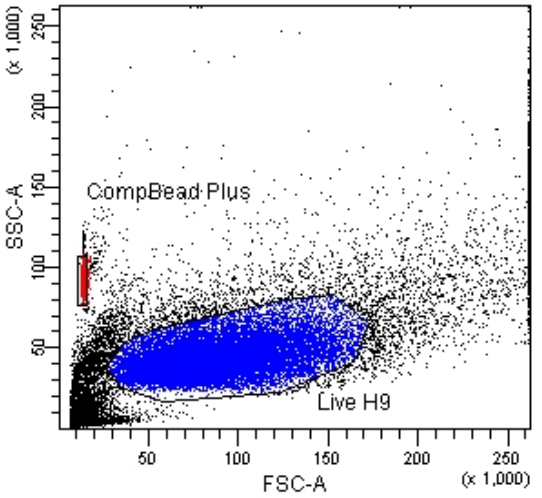
Saving settings

1. In the **Browser**, right-click on **Cytometer Settings** and select **Application Settings > Save**.
2. Name the application, then click **OK**.

   Note: You can now use these application settings as a baseline for future experiments.
3. From the **Experiment** menu, select **Compensation Setup > Calculate Compensation**. The **Single Stained Setup** dialog appears.
4. Name the compensation setup, then click **Link and Save**.

Example of bead and cell placement

The following plot is an example that demonstrates where the BD CompBead Plus beads appear relative to 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 the FSC and SSC to accommodate your cell populations. See Optimizing the settings (page 49).

Next step

Proceed to Setting up for sorting (page 47) if you will be sorting cells. If you will be analyzing cells, proceed directly to Creating a new specimen (page 48).

Related topics

- Example cytometer methods (page 39)
- Troubleshooting (page 66)
- About spectral overlap and compensation (page 68)

Setting up for sorting

About this topic

This topic gives general guidelines for setting up your flow cytometer for sorting. For specific procedures, see the User's Guide for your cytometer.

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 your User's Guide for optimizing the breakoff and drop delay.

Next step

Proceed to Creating a new specimen (page 48).

Related topics

- Sorting cells (page 61)

Creating a new specimen

About this topic

This topic explains how to create a new specimen for analyzing or sorting stained pluripotent stem cells using BD FACSDiva software.

Procedure

To create a new specimen:

1. From the **Experiment** menu, select **New Specimen**. A new specimen appears in the **Browser**.
2. Click the specimen to expand it.
3. Select the first tube and name it appropriately for your sample.
4. Add and name any additional tubes that you will need.

   Note: You can edit the properties of each tube using either the **Experiment Layout** or the **Inspector**.

Next step

Proceed to Optimizing the settings (page 49).

Related topics

- Example cytometer methods (page 39)

Optimizing the settings

About this topic

This topic explains how to optimize your flow cytometer settings for analyzing or sorting stained pluripotent stem cells using BD FACSDiva software.

Before you begin

Complete the steps described in Creating a new specimen (page 48).

Retrieve the unstained cells that you set aside at the beginning of Staining the cells (page 29).

Caution Do not adjust the fluorescence settings at this stage. Adjusting the fluorescence settings now will invalidate your compensation calculations.

Procedure

To optimize the settings:

1. Place the tube of unstained cells on the cytometer.
2. On the **Application Dashboard**, click **Acquire Data**. Events appear in the dot plot.
3. Adjust the FSC and SSC PMT voltages as needed to ensure that your cell population appears on scale in the scatter plot.
4. Stop acquiring.

Next step

Proceed to Setting up a gating strategy (page 50).

Related topics

- Example cytometer methods (page 39)

Setting up a gating strategy

About this topic

This topic provides examples of how to set up a gating strategy for either analysis or sorting of cells stained with the BD Human Pluripotent Stem Cell Sorting and Analysis Kit.

Before you begin

Complete the steps described in Optimizing the settings (page 49).

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

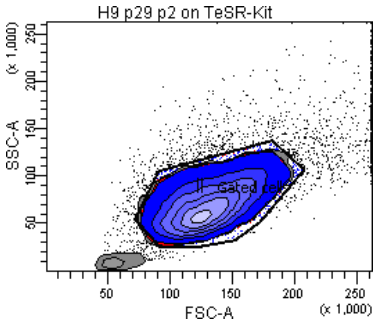
If you plan to run your isotype controls now, skip ahead to Acquiring data (page 60) for instructions on how to run the controls, and then return to this procedure.

Procedure for analysis

To set up a gating strategy for analysis:

1. On a global worksheet, create the following 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. Generate the following histograms:
   - FITC-A
   - PE-A
   - Alexa Fluor® 647-A (or APC-A)
3. Acquire a small subset of your unstained cells.

4. In the FSC-A vs SSC-A plot, gate on your cell population.



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

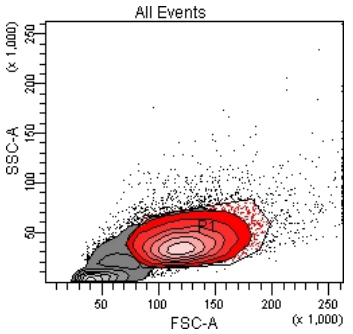
5. See the *BD FACSDiva Software Reference Manual* for instructions on how to save this worksheet as a template for use in future experiments.

Procedure for sorting

To set up a gating strategy for sorting:

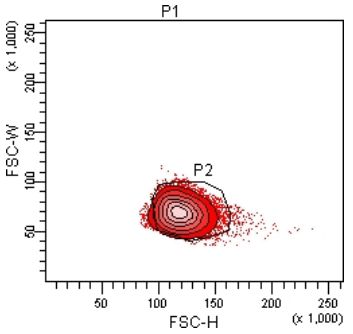
1. On a global worksheet, create the following plots:
   - FSC-A vs SSC-A
   - FSC-H vs FSC-W
   - SSC-H vs SSC-W
   - PE-A vs FITC-A
   - Alexa Fluor® 647-A (or APC-A) vs PE-A
2. Right-click on the first plot and select **Show Population Hierarchy.**
3. Acquire a small subset of your specific-stained cells.

4. On the FSC-A vs SSC-A plot, gate on your cell population.

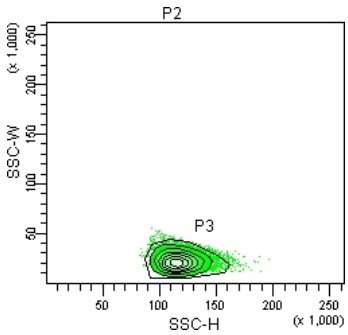


5. If necessary, increase the FSC threshold to exclude cell debris.

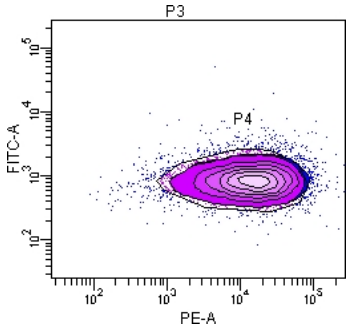
6. On the FSC-H vs FSC-W plot, gate the P2 population on P1-gated cells to discriminate doublets.



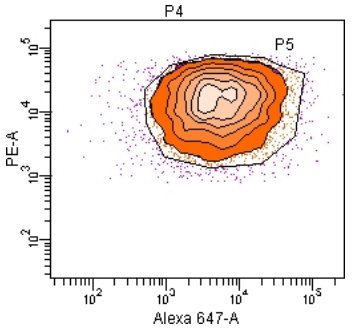
7. On the SSC-H vs SSC-W plot, gate the P3 population on P2-gated cells to discriminate doublets.



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



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



10. See the *BD FACSDiva Software Reference Manual* for instructions on how to save this worksheet as a template for use in future experiments.

Next step

Proceed to Processing samples (page 59).

Related topics

- Example cytometer methods (page 39)
- Template examples (page 55)

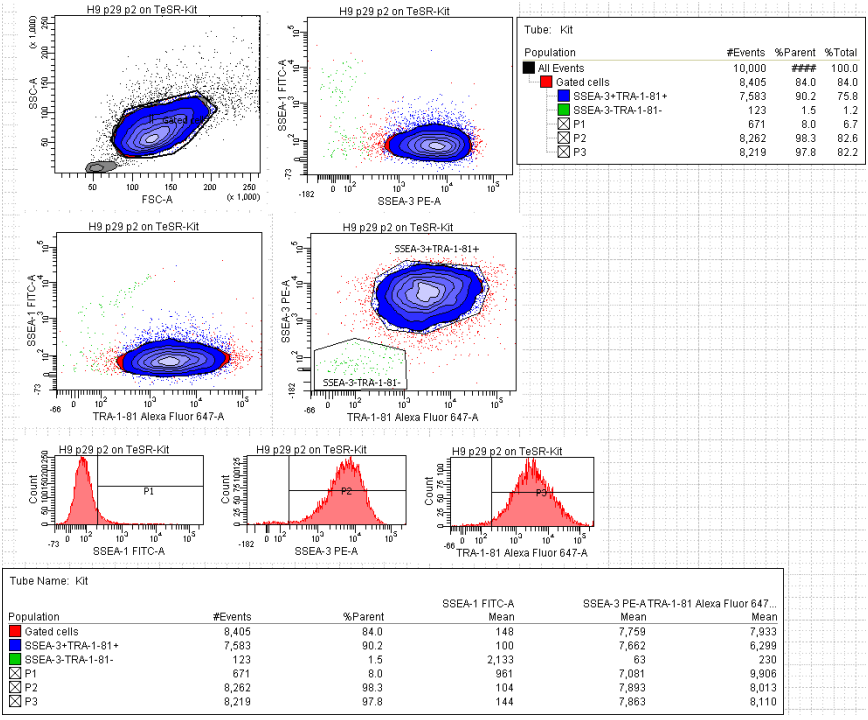
Template examples

About this topic

This topic contains examples of templates with defined gates, showing data from the H9 cell line stained with the BD 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:

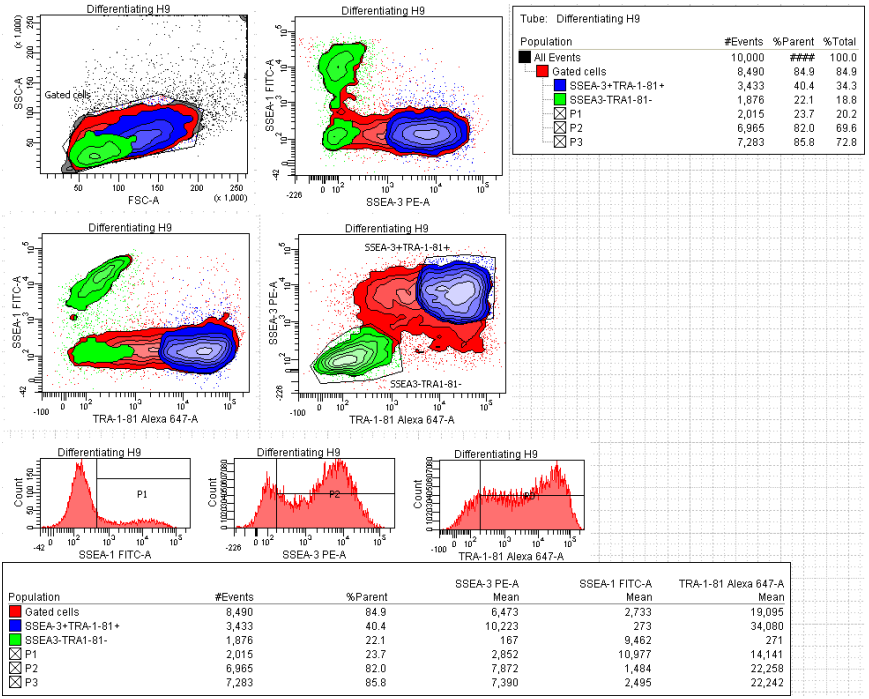


Tube Name: Kit

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

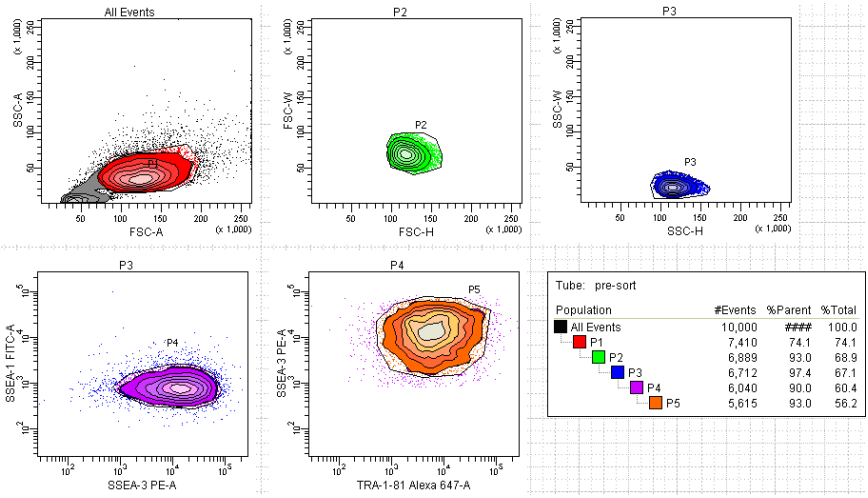
The following is an example of an analysis template showing data from differentiating H9 cells:



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 template for sorting, showing data from undifferentiated H9 cells:



Related topics

- Setting up a gating strategy (page 50)

5

Processing samples

This section covers the following topics:

Acquiring data

About this topic

This topic provides an example of how to acquire data using BD FACSDiva software.

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

Before you begin

Complete the steps described in Preparing the cells (page 21) and Setting up the cytometer (page 35).

Procedure

To acquire data:

1. Place the tube of unstained cells on the cytometer.
2. On the **Application Dashboard**, click **Acquire Data**. Events appear in the dot plot.
3. Click **Record Data** to begin recording data to the database.
4. Stop recording at 10,000 cells (or more if interested in rare events).
5. Repeat steps 1 to 4 for the isotype control tubes.
6. If you stained samples for analysis, repeat steps 1 to 3 for your sample tubes.

Related topics

- Example cytometer methods (page 39)
- Troubleshooting (page 66)

Sorting cells

About this topic

This topic provides an example procedure for how to sort stained pluripotent stem cells on a BD FACSAria flow cytometer.

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

Before you begin

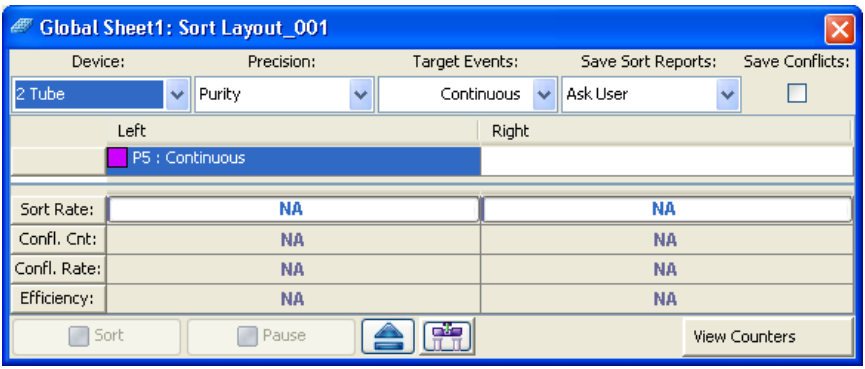
Complete the steps described in Setting up the cytometer (page 35).

Complete the steps described in Acquiring data (page 60).

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 your collection tubes with media containing antibiotics.

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

4. Invert the tubes several times to coat the sides with media, and install them on the cytometer.
5. Start the sort and monitor the stream during sorting.

Warning

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

Next step

Proceed to Plating cells (page 63).

Related topics

- Example cytometer methods (page 39)
- Troubleshooting (page 66)

Plating cells

About this topic

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

Before you begin

Complete the steps described in Sorting cells (page 61).

Ensure that your cell culture plates are ready for plating.

Using antibiotics

We recommend that you grow sorted cells in media containing antibiotics (penicillin/streptomycin) for at least one passage, to safeguard against contamination from sorting.

Procedure

To plate cells:

1. Pool your sorted cell samples.
2. Centrifuge at 300*g* for 5 minutes.
3. Wash cells twice in growth media containing antibiotics.
4. Plate cells onto the appropriate matrices and media.

   Note: For hESCs grown in mTESR™1 medium on BD Matrigel hESC-qualified matrix or for hESCs grown 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 six-well dish.

6

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 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 flow cytometer	Wait for sorting to continue. If this happens often, stop the sort, turn off the Sweet Spot, and see the *BD FACSAria User's Guide* for instructions on troubleshooting an unstable breakoff.
Sorting stops automatically on a BD FACSAria flow cytometer	Turn on the stream and see if Drop 1 returns to the original value. • If Drop 1 returns to the original value, then set the Sweet Spot and continue sorting. • If Drop 1 returns to a different value but the breakoff still looks good, set the Sweet Spot, optimize the drop delay, and continue sorting. • If the stream is unstable or leaking, follow the directions in the *BD FACSAria User's Guide* to clean a clogged nozzle. When finished, set the Sweet Spot, optimize the drop delay, and continue sorting.
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.

Problem	Recommended actions
High background fluorescence during sorting	Use media without phenol red.
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.
Difficulty sorting collagenase IV passaged cells	Adapt your cells to single-cell dissociation (eg, Accutase).[7] Plate your sorted cells at a higher density. Continue to feed cells for two weeks after sorting (collagenase IV passaged cells may take longer to appear after sorting). Use cell lines that can be routinely passaged to single cells (eg, HUES lines).

About spectral overlap and compensation

About this topic

This topic provides background on why you must calculate compensation before running samples stained with the BD Human Pluripotent Stem Cell Sorting and Analysis Kit.

Spectral overlap

The spectral overlap values for a given fluorophore are the fluorescence values above background in all detectors relative to the primary detector for that fluorophore.

For example, the fluorescence of a PE-stained sample is defined as 100% in the PE detector, and its spectral overlap values could be up to 1% in the FITC detector, and up to 20% in the APC/Alexa Fluor® 647 detector.

Compensation

Compensation is the process by which spectral overlap is removed so that the fluorescence value for a parameter reflects only the fluorescence in the primary detector.

To calculate compensation, the spectral overlap values are measured for each of the fluorophores to be used in an experiment.

Related topics

- Preparing control beads (page 36)
- Running beads for cytometer setup (page 42)

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/

United States
877.232.8995

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888.259.0187

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32.2.400.98.95

Japan
0120.8555.90

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65.6861.0633

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