BD Human Pluripotent Stem Cell Transcription Factor Analysis Kit

Instruction Manual

Catalog No. 560589

BD

History

Revision	Date	Change Made
647624 Rev A	5/09	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 Transcription Factor Analysis Kit (Catalog No. 560589), 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 embryonic stem cells (hESCs) and induced pluripotent stem (iPS) cells.

This kit can be used to analyze cells for expression of intracellular pluripotency markers. We also designed this kit to give you the option to add additional antibodies (for some surface or intracellular markers) that fluoresce in any open channel (for example, the FITC channel), or to analyze cells expressing green fluorescent protein (GFP).

Specific antibodies

Human pluripotent stem cells are characterized by the expression of specific intracellular transcription factors.[1,2] The BD Human Pluripotent Stem Cell Transcription Factor Analysis Kit contains three fluorochrome-conjugated antibodies that distinguish and identify human pluripotent cells. The three conjugated antibodies included in this kit (Nanog, Oct3/4 [or POU5F1], and Sox2) recognize transcription factors that are expressed in human pluripotent stem cells and have been termed the "core" pluripotency factors in hESCs.[3-5] This combination of markers has been widely used to characterize hESCs and iPS cells.[2,6,7]

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, and is bottled at the same concentration as the specific antibodies.

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

This kit has been tested on human H9 and H7 (WiCell, Madison, WI) embryonic stem cell lines, 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 of stained 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 cellular molecules (for example, surface antigens, transcription factors, cytokines, etc).

For more information about this option, see Drop-in conjugates (page 37).

Related topics

- Kit contents (page 4)

Kit contents

Reagent information

The BD Human Pluripotent Stem Cell Transcription Factor Analysis Kit contains the following reagents.

{at-anchortable}

Reagent	Details
BD Pharmingen™ PE Mouse anti-human Nanog	**Clone:** N31-355 **Use:** The N31-355 monoclonal antibody reacts with human Nanog, which is a homeobox transcription factor required for the maintenance of the undifferentiated state of pluripotent stem cells. **Abbreviation:** PE hNanog **Quantity:** 1 vial (1.5 mL) **Amount for staining:** 20 µL per sample (for 5 x 10^5 to 1 x 10^6 cells)

Reagent	Details
BD Pharmingen™ PerCP-Cy™5.5 Mouse anti-Oct3/4	**Clone:** 40/Oct-3 **Use:** The 40/Oct-3 monoclonal antibody reacts with Oct3/4, a transcription factor that plays an important role in determining early steps of embryogenesis and differentiation. **Abbreviation:** PerCP-Cy5.5 Oct3/4 **Quantity:** 1 vial (1.5 mL) **Amount for staining:** 20 µL per sample (for 5 x 10^5 to 1 x 10^6 cells)
BD Pharmingen™ Alexa Fluor® 647 Mouse anti-Sox2	**Clone:** 245610 **Use:** The monoclonal antibody 245610 recognizes the Sox2 transcription factor. Complexes of Sox2 with the homeobox transcription factors Oct3/4 and/or Nanog bind to the promoters of a network of genes that are involved in the maintenance of pluripotency and self renewal in stem cells. **Abbreviation:** Alexa Fluor® 647 Sox2 **Quantity:** 1 vial (1.5 mL) **Amount for staining:** 20 µL per sample (for 5 x 10^5 to 1 x 10^6 cells)
BD Pharmingen™ PE Mouse IgG_1, κ Isotype Control	**Clone:** MOPC-21 **Use:** Used as an isotype control for PE hNanog **Abbreviation:** PE isotype control **Quantity:** 1 vial (1.0 mL) **Amount for staining:** 20 µL per sample (for 5 x 10^5 to 1 x 10^6 cells)

Reagent	Details
BD™ PerCP-Cy5.5 Mouse IgG_1, κ Isotype Control	**Clone:** X40 **Use:** Used as an isotype control for PerCP-Cy5.5 Oct3/4 **Abbreviation:** PerCP-Cy5.5 isotype control **Quantity:** 1 vial (1.0 mL) **Amount for staining:** 20 µL per sample (for 5 x 10^5 to 1 x 10^6 cells)
BD Pharmingen™ Alexa Fluor® 647 Mouse IgG_{2a}, κ Isotype Control	**Clone:** MOPC-173 **Use:** Used as an isotype control for Alexa Fluor® 647 Sox2 **Abbreviation:** Alexa Fluor® 647 isotype control **Quantity:** 1 vial (1.0 mL) **Amount for staining:** 20 µL per sample (for 5 x 10^5 to 1 x 10^6 cells)
BD™ CompBead Plus Anti-Mouse Ig, κ	**Use:** Used to create control beads stained with PE hNanog, PerCP-Cy5.5 Oct3/4, or Alexa Fluor® 647 Sox2 (because beads bind any mouse kappa light-chain-bearing immunoglobulin) **Abbreviation:** Anti-mouse beads **Quantity:** 1 vial (6.0 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)
BD Cytofix™ fixation buffer	**Use:** To fix cells **Quantity:** 1 bottle (50 mL)
BD Perm/Wash™ buffer (10X)	**Use:** To permeabilize and wash cells **Quantity:** 2 bottles (30 mL each)

Serum proteins

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 Human Pluripotent Stem Cell Transcription Factor Analysis Kit must be stored in the dark at 2° to 8°C. Do not freeze.

Warning

The BD Cytofix fixation buffer in this kit contains 4% paraformaldehyde and is classified as harmful and a suspected carcinogen. The following risk and safety precautions should be followed when handling this product:

> Limited evidence of a carcinogenic effect (R40). May cause sensitization by skin contact (R43). Keep out of the reach of children (S2). Keep away from food, drink, and animal feedingstuffs (S13). Wear suitable protective clothing (S36). Wear suitable gloves (S37). If swallowed, seek medical attention immediately and show this container or label (S46). Not recommended for interior use on large surface areas (S52).

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

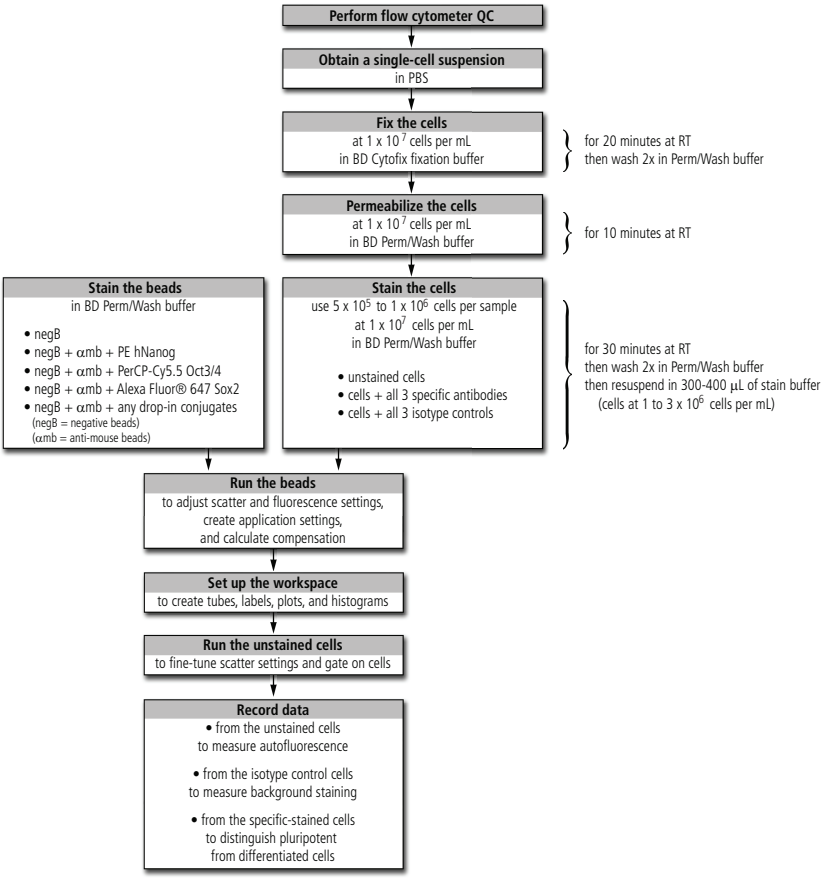
Before you begin

This section covers the following topics:

Workflow overview

About this topic This topic provides an overview of the steps involved in using the BD Human Pluripotent Stem Cell Transcription Factor Analysis Kit to analyze cells.

{aw-anchorwide: anchored frame width=4.5"}



Required materials

Materials list

The following reagents, consumables, and equipment are required for use with the BD Human Pluripotent Stem Cell Transcription Factor Analysis Kit:

- 1X PBS without Ca^{2+} or Mg^{2+}
- Accutase™ Enzyme Cell Detachment Medium from Innovative Cell Technologies, or equivalent (if analyzing hESCs)
- Microscope for confirming a single-cell suspension
- BD Falcon™ 70-µm cell strainer (Catalog No. 352350), or equivalent (optional, but recommended)
- Hemocytometer or other cell counter
- Deionized water, or equivalent
- BD Pharmingen™ stain buffer (FBS) (Catalog No. 554656), or equivalent
- BD Falcon™ round-bottom 12 x 75-mm polystyrene tubes with caps (Catalog No. 352058), or equivalent
- BD™ LSR II flow cytometer, BD FACSCanto™ II flow cytometer, BD FACSCalibur™ flow cytometer, or other flow cytometer equipped with a blue (488-nm) laser, a red (633-nm) laser, and detectors for PE, PerCP-Cy5.5, and Alexa Fluor® 647

Common cell-preparation techniques

About this topic

This topic describes how to perform two common cell-preparation techniques that are part of the workflow for staining cells with the BD Human Pluripotent Stem Cell Transcription Factor 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.
2. Centrifuge for 5 minutes at the specified speed.

   Note: Centrifuge cells at 300*g* before they are fixed, and at 500*g* after they are fixed.
3. Aspirate the supernatant, being careful not to disturb the cells.
4. 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, 1×10^7 cells per mL).

   This is your target volume.

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 for 5 minutes at 300*g* (for unfixed cells) or 500*g* (for fixed cells).

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

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

      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.

Related topics

- Preparing cells and beads (page 15)

3

Preparing 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 Human Pluripotent Stem Cell Transcription Factor Analysis Kit.

Before you begin

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

As a detachment enzyme, we recommend using Accutase for analyzing hESCs.

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

Procedure

To obtain a single-cell suspension:

1. Wash the cells with room-temperature PBS.
2. Add the detachment enzyme to the cells at the concentration recommended by the manufacturer.
3. Incubate at the recommended temperature and for the recommended duration.
4. If required, neutralize the enzyme.
5. Pipette the cells gently up and down.
6. Remove a small subset of the liquid and check it under a microscope to confirm the presence of single cells.
7. If you observe clumps of cells, collect the cell suspension and pass it through a 70-µm cell strainer.
8. Wash the cells in two to four volumes of PBS (centrifuging at 300*g* for 5 minutes).

9. Resuspend the cells in a volume of PBS that is appropriate for cell counting (for example, 5 mL of PBS for one confluent 6-well culture dish).
10. 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 (page 7) for more information about isotype controls.

Refer to the following guidelines.

{at-anchortable}

Guideline	Value
Required cell concentration for staining	1 x 10^7 cells/mL
Recommended cells per tube for staining	1 x 10^6 cells
Minimum cells per tube for staining	5 x 10^5 cells
Recommended volume of cells per tube at the required concentration	100 µL
Minimum volume of cells per tube at the required concentration	50 µL

Next step

Proceed immediately to Fixing the cells (page 4) unless you are adding a drop-in conjugate before fixing. See Adding drop-in conjugates (page 38) for more information about this option.

Related topics

- Workflow overview (page 10)
- Required materials (page 11)
- Common cell-preparation techniques (page 12)

Fixing the cells

About this topic

This topic explains how to fix cells before staining with the BD Human Pluripotent Stem Cell Transcription Factor Analysis Kit.

Before you begin

Complete the steps in Obtaining a single-cell suspension (page 2).

Decide whether you will need to store the fixed cells. For best results, we recommend that you plan to stain and acquire samples within 24 to 48 hours of fixing.

Procedure

To fix the cells:

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

4. Proceed as follows.

{at-anchortable}

If you will...	Then...
Stain cells the same day	Proceed immediately to Permeabilizing the cells (page 5)
Store the fixed cells	1. Wash the cells twice in two to four volumes of PBS (centrifuging at 500*g* for 5 minutes). 2. Resuspend in PBS at 1 x 10^7 cells per mL. 3. Store at 4°C for 24 to 48 hours. 4. Proceed to Permeabilizing the cells (page 5).

Related topics

- Kit contents (page 4)
- Workflow overview (page 10)
- Required materials (page 11)
- Common cell-preparation techniques (page 12)

Permeabilizing the cells

About this topic

This topic explains how to permeabilize cells before staining with the BD Human Pluripotent Stem Cell Transcription Factor Analysis Kit.

Before you begin

Complete the steps in Fixing the cells (page 4).

Prepare 1X BD Perm/Wash buffer by diluting the 10X BD Perm/Wash buffer in deionized water. You will need approximately 4.5 to 5.0 mL of 1X Perm/Wash buffer per one million cells, plus 2.1 mL for each bead tube

(each experiment has at least four bead tubes, plus one for each drop-in conjugate).

Note: If you are co-staining with surface markers, we do not recommend the use of methanol-based perm buffers.

Procedure

To permeabilize the cells:

1. Wash cells twice in approximately 1 mL of 1X BD Perm/Wash buffer for each 1 x 10^7 cells (centrifuging at 500*g* for 5 minutes).
2. Resuspend the cells in 1X BD Perm/Wash buffer at 1 x 10^7 cells per mL.
3. Incubate for 10 minutes at room temperature.

Next step

Proceed immediately to Staining (page 7). Permeabilized cells cannot be stored and must be stained immediately.

Related topics

- Kit contents (page 4)
- Workflow overview (page 10)
- Required materials (page 11)
- Common cell-preparation techniques (page 12)
- Troubleshooting (page 46)

Staining

About this topic

This topic explains how to stain both the prepared cells and the BD CompBead Plus beads with the antibodies provided in the BD Human Pluripotent Stem Cell Transcription Factor Analysis Kit.

Before you begin

Complete the steps in Permeabilizing the cells (page 5).

We recommend setting aside a sample of unstained cells to measure autofluorescence for each cell type.

To prepare unstained cells, add 100 µL of permeabilized cells (1 x 10^6 cells) to a labeled 12 x 75-mm polystyrene tube 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. Once you have determined the background for a particular cell type, the use of isotype controls is optional.

This kit has been tested on H9 and H7 hESC lines and no problematic background staining has been observed.

Procedure

To stain cells and beads:

1. For each of your cell types, label one 12 x 75-mm polystyrene tube “specific stain” and one tube “isotype control” (if applicable).

2. Add the following to each tube.

{at-anchortable}

Component	Volume to add to tube labeled	
	Specific stain	Isotype control
Permeabilized cells (at 1 x 10^7 cells per mL)	100 µL (1 x 10^6 cells)	100 µL (1 x 10^6 cells)
PE hNanog	20 µL	—
PerCP-Cy5.5 Oct3/4	20 µL	—
Alexa Fluor® 647 Sox2	20 µL	—
PE isotype control	—	20 µL
PerCP-Cy5.5 isotype control	—	20 µL
Alexa Fluor® 647 isotype control	—	20 µL

If you are adding additional antibodies at this stage, see Adding drop-in conjugates (page 38).

3. Mix the tubes gently and incubate at room temperature in the dark for 30 minutes.

4. Immediately after starting the cell-stain incubation, label four 12 x 75-mm polystyrene tubes for the beads as follows:

   - Negative
   - PE
   - PerCP-Cy5.5
   - Alexa 647

   Note: If you stained the cells with additional fluorochrome-conjugated antibodies, prepare stained beads for those antibodies as well so that you can calculate compensation for all relevant fluorochromes.

5. Add the following to each tube in the order shown (vortex the beads thoroughly immediately before dispensing drops).

{at-anchortable}

Component	Volume to add to the tube labeled			
	Negative	PE	PerCP-Cy5.5	Alexa 647
Perm/Wash buffer (1X)	100 µL	100 µL	100 µL	100 µL
Negative beads	1 drop	1 drop	1 drop	1 drop
Anti-mouse beads	—	1 drop	1 drop	1 drop
PE hNanog	—	20 µL	—	—
PerCP-Cy5.5 Oct3/4	—	—	20 µL	—
Alexa Fluor® 647 Sox2	—	—	—	20 µL

6. Vortex the tubes and incubate at room temperature in the dark for 30 minutes.
7. After the 30-minute incubation is complete for both the cells and the beads, wash each tube twice in 1 mL of 1X Perm/Wash buffer (centrifuging at 500*g* for 5 minutes).
8. Resuspend the cells and beads in 300 to 400 µL of BD Pharmingen stain buffer (FBS).

   Resuspend the cells at a concentration between 1 x 10^6 cells per mL and 3 x 10^6 cells per mL.

Next step

Proceed to Running the beads (page 26).

Storage is not recommended. Run stained beads and cells within 1 to 2 hours of staining.

Related topics

- Kit contents (page 4)
- Workflow overview (page 10)
- Required materials (page 11)
- Common cell-preparation techniques (page 12)
- Adding drop-in conjugates (page 38)

4

Cytometer procedures

This section provides guidelines for application setup and data acquisition for analysis of cells stained with the BD Human Pluripotent Stem Cell Transcription Factor Analysis Kit.

The guidelines and examples in this section use BD FACSDiva™ software and BD FACS™ flow cytometers. However, the fundamental approach to setup and acquisition can be adapted for research labs with other flow cytometers.

This section covers the following topics:

Running the beads

About this topic

This topic describes how to use the prepared control beads to set up the application for analyzing cells stained with the BD Human Pluripotent Stem Cell Transcription Factor Analysis Kit.

Purpose of the procedure

The stained beads are run 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.

Ensure that you run the appropriate instrument setup and QC procedures for your flow cytometer. See your user's guide for more information.

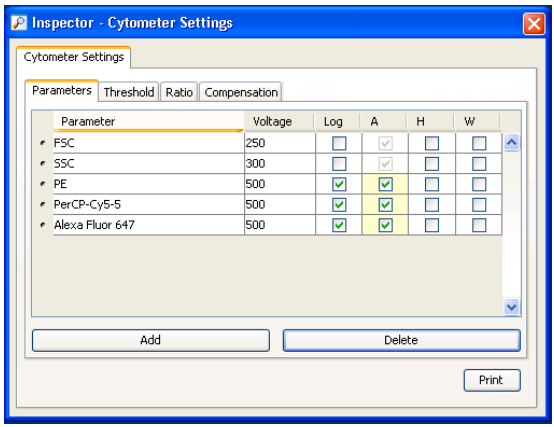
Complete the steps in Preparing cells and beads (page 15).

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.
3. Delete all parameters except FSC, SSC, PE, PerCP-Cy5.5, and Alexa Fluor® 647. If you stained the cells with additional conjugates, include all relevant fluorescence parameters.

{a1-anchor1: anchored frame width=3.15"}

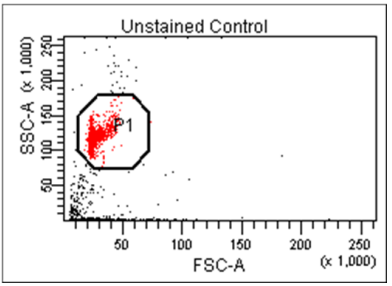


Note: The voltage settings that appear in this window will vary with each instrument.

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.

{a1-anchor1: anchored frame width=3.15"}



8. Adjust the FSC and SSC photomultiplier tube (PMT) voltages to obtain the following values.

{at-anchortable}

Cell type	Parameter	Mean of bead population
hESCs	FSC-A	10,000 to 20,000
hESCs	SSC-A	90,000 to 115,000

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

9. Place each of the stained compensation control tubes on the cytometer in turn, and adjust the PMT voltages as follows.

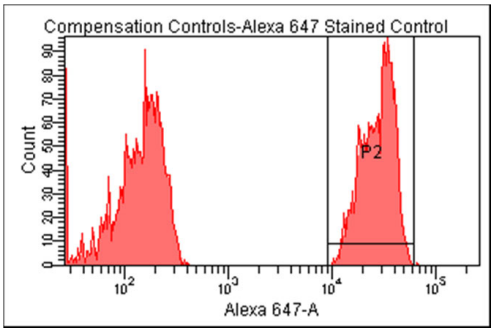
{at-anchortable}

Adjust the PMT voltage for...	Until the mean of the positive population is...
PE	Between 10^4 and 10^5
PerCP-Cy5.5	Between 10^4 and 10^5
Alexa Fluor® 647	Between 10^4 and 10^5

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

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

{a1-anchor1: anchored frame width=3.15"}



12. If you have not already done so, save the application settings for future use.
    a. In the **Browser**, right-click **Cytometer Settings** and select **Application Settings > Save**.
    b. Name the application, then click **OK**.
13. 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 6).

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

- Workflow overview (page 10)
- Examples of bead and cell placement (page 47)
- About spectral overlap and compensation (page 49)

Setting up the workspace for running cells

About this topic

This topic explains how to set up the BD FACSDiva workspace in preparation for running control cells and specific-stained cells.

Before you begin

Complete the steps in Running the beads (page 2).

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

Procedure

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 8).
4. In the **Labels** tab of the **Experiment Layout** window, enter parameter labels for each marker in the experiment, including any drop-in conjugates.
5. On a global worksheet, create the following plots (we recommend acquiring data with dot plots and analyzing data with contour plots):
   - FSC-A vs SSC-A
   - PE-A vs PerCP-Cy5.5-A
   - Alexa Fluor® 647-A vs PerCP-Cy5.5-A
   - PE-A vs Alexa Fluor® 647-A
6. Create the following histograms:
   - PE-A
   - PerCP-Cy5.5-A
   - Alexa Fluor® 647-A
7. Select biexponential scaling for all fluorochrome axes.
8. Save this worksheet as a template for use in future experiments.

Next step

Proceed to Running the cells (page 8).

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 9)

Running the cells

About this topic

This topic explains how to:

- Fine-tune scatter settings on your flow cytometer by running the unstained cells
- Detect autofluorescence by recording data from the unstained cells
- Identify background staining by recording data from the isotype control cells
- Record data from the specific-stained cells

Before you begin

Complete the steps in Setting up the workspace for running cells (page 6).

Procedure

To run the cells:

1. Place the tube of unstained cells on the cytometer and begin acquiring.

2. Adjust the FSC and SSC PMT voltages as needed to ensure that the cell population appears on scale in the scatter plot.

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

3. Create a P1 gate on the population in the FSC-A vs SSC-A plot.

   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.
5. Place the isotype control tube on the flow cytometer and record data.
6. Place the specific-stained cells on the cytometer and record data.

Related topics

- Workflow overview (page 10)
- Template examples (page 9)
- Troubleshooting (page 46)
- Examples of bead and cell placement (page 47)

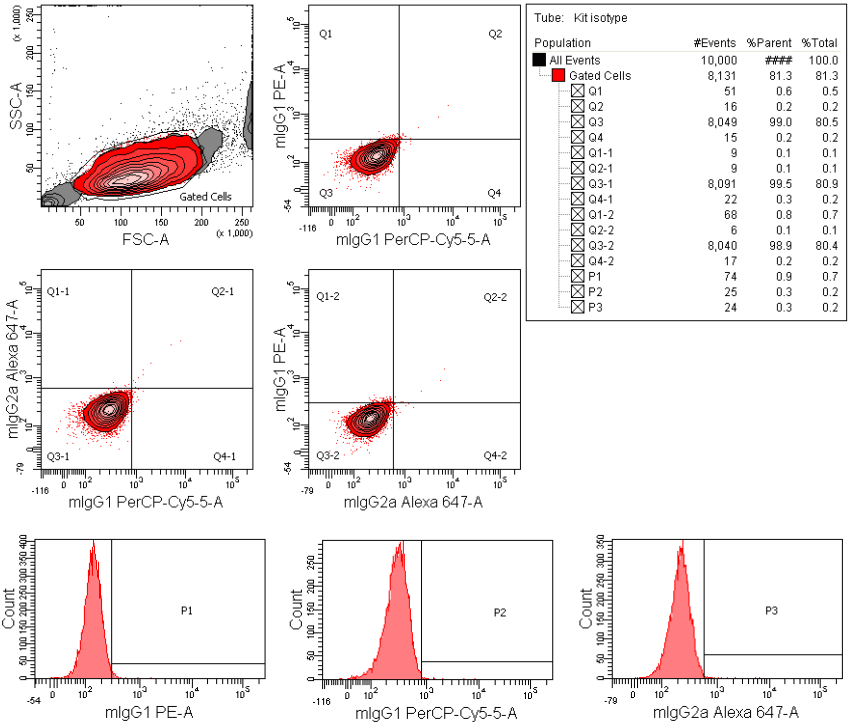
Template examples

About this topic This topic contains examples of templates with defined gates, showing data from cell lines stained with the BD Human Pluripotent Stem Cell Transcription Factor Analysis Kit.

Example with isotype-control hESCs

The following is an example of an analysis template showing isotype-control data from undifferentiated hESCs from the H9 cell line.

{aw-anchorwide: anchored frame width=4.5"}



Tube: Kit isotype

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Gated Cells	8,131	81.3	81.3
Q1	51	0.6	0.5
Q2	16	0.2	0.2
Q3	8,049	99.0	80.5
Q4	15	0.2	0.2
Q1-1	9	0.1	0.1
Q2-1	9	0.1	0.1
Q3-1	8,091	99.5	80.9
Q4-1	22	0.3	0.2
Q1-2	68	0.8	0.7
Q2-2	6	0.1	0.1
Q3-2	8,040	98.9	80.4
Q4-2	17	0.2	0.2
P1	74	0.9	0.7
P2	25	0.3	0.2
P3	24	0.3	0.2

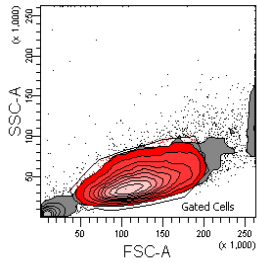
Tube Name: Kit isotype

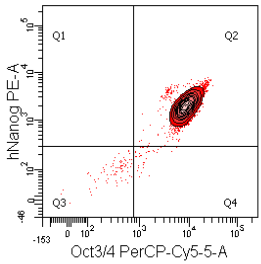
Population	#Events	%Parent	mIgG1 PE-A Mean	mIgG1 PerCP-Cy5-5-A Mean	mIgG2a Alexa 647-A Mean
Gated Cells	8,131	81.3	144	315	230
P1	74	0.9	354	831	588
P2	25	0.3	382	1,434	1,069
P3	24	0.3	332	1,188	1,234

Example with specific-stained hESCs

The following is an example of an analysis template showing data from specific-stained undifferentiated hESCs from the H9 cell line.

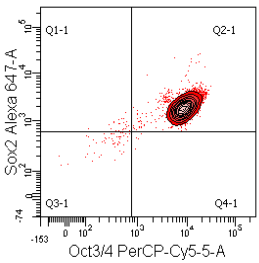
{aw-anchorwide: anchored frame width=4.5"}

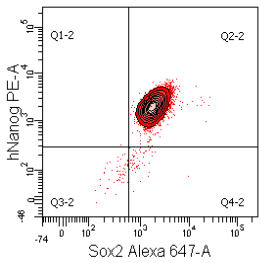


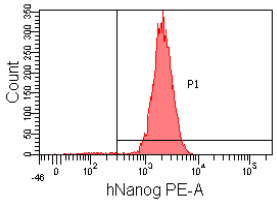


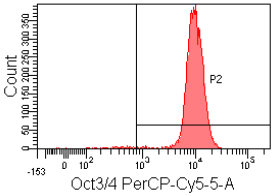
Tube: Kit

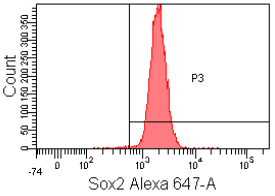
Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Gated Cells	8,037	80.4	80.4
Q1	1	0.0	0.0
Q2	7,921	98.6	79.2
Q3	80	1.0	0.8
Q4	35	0.4	0.4
Q1-1	25	0.3	0.2
Q2-1	7,951	98.9	79.5
Q3-1	56	0.7	0.6
Q4-1	5	0.1	0.0
Q1-2	1	0.0	0.0
Q2-2	7,921	98.6	79.2
Q3-2	58	0.7	0.6
Q4-2	57	0.7	0.6
P1	7,922	98.6	79.2
P2	7,956	99.0	79.6
P3	7,978	99.3	79.8











Tube Name: Kit

Population	#Events	%Parent	hNanog PE-A Mean	Oct3/4 PerCP-Cy5-5-A Mean	Sox2 Alexa 647-A Mean
Gated Cells	8,037	80.4	2,175	9,764	2,054
P1	7,922	98.6	2,204	9,895	2,073
P2	7,956	99.0	2,196	9,859	2,069
P3	7,978	99.3	2,190	9,833	2,066

Related topics

- Setting up the workspace for running cells (page 6)

5

Drop-in conjugates

This section covers the following topics:

Adding drop-in conjugates

About this topic

This topic provides information about adding "drop-in" conjugates: staining cells with fluorochrome-conjugated antibodies in addition to those provided in the kit.

Purpose of adding drop-ins

To obtain more information about the cells, you may decide to add antibody conjugates to surface or intracellular markers not already recognized by the antibodies in the BD Human Pluripotent Stem Cell Transcription Factor Analysis Kit.

Criteria for conjugate choice

Ensure that:

- The drop-in conjugate will fluoresce in an open channel (for example, the FITC channel), and your flow cytometer is equipped with the appropriate detector
- The drop-in conjugate has a Mouse Ig, κ isotype so that the anti-mouse beads provided with this kit can be used for compensation
- You know the optimal concentration for the drop-in conjugate and have calculated the correct amount of antibody to add to the sample tubes

When to add drop-ins

If you can confirm that the drop-in will appropriately stain cells that have been fixed and permeabilized, simply add the correct amount of antibody along with the kit antibodies as described in Staining (page 21).

If the drop-in conjugate recognizes a surface marker and will not work with fixed and permeabilized cells, try the following steps:

1. Stain live cells with the antibody for 20 to 30 minutes.

2. Wash the cells twice in PBS.
3. Fix, permeabilize, and stain the cells with the rest of the antibodies in the kit as described in Preparing cells and beads (page 15).

Isotype controls

When using drop-in conjugates, we recommend using fluorescence minus one (FMO) controls to reveal any non-specific binding that either the additional antibody or the fluorochrome on this antibody might have with the kit antibodies.

To create an FMO control, include a tube of cells in which you add the three kit antibodies (PE hNanog, PerCP-Cy5.5 Oct3/4, and Alexa Fluor® 647 Sox2) plus the matched isotype of the drop-in.

Compensation

When staining beads, ensure that you prepare an additional tube for calculating compensation for the fluorochrome of each drop-in conjugate.

When creating a new experiment, ensure that you include all relevant fluorescence parameters, and calculate compensation for all tubes.

Related topics

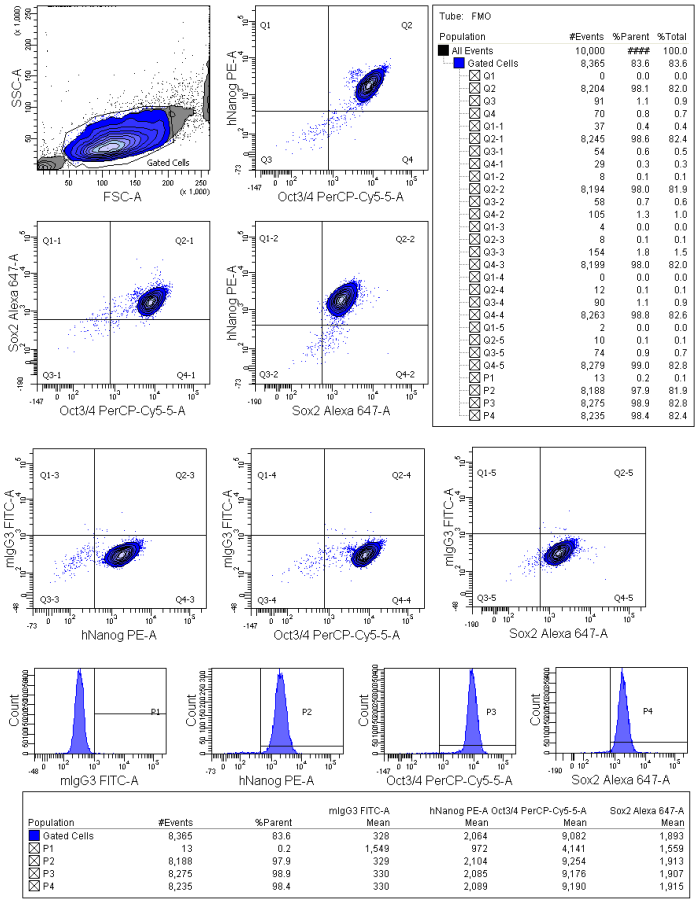
- Purpose of the kit (page 2)
- Staining (page 21)
- Examples of data with drop-in conjugates (page 4)
- Troubleshooting (page 46)

Examples of data with drop-in conjugates

Example of FMO control for SSEA-4 drop-in on undifferentiated H9s

The following is an example of an analysis template showing data from human H9 ES stained with the kit antibodies plus mIgG3 FITC (the matching isotype for FITC anti-SSEA-4).

{aw-anchorwide: anchored frame width=4.5"}

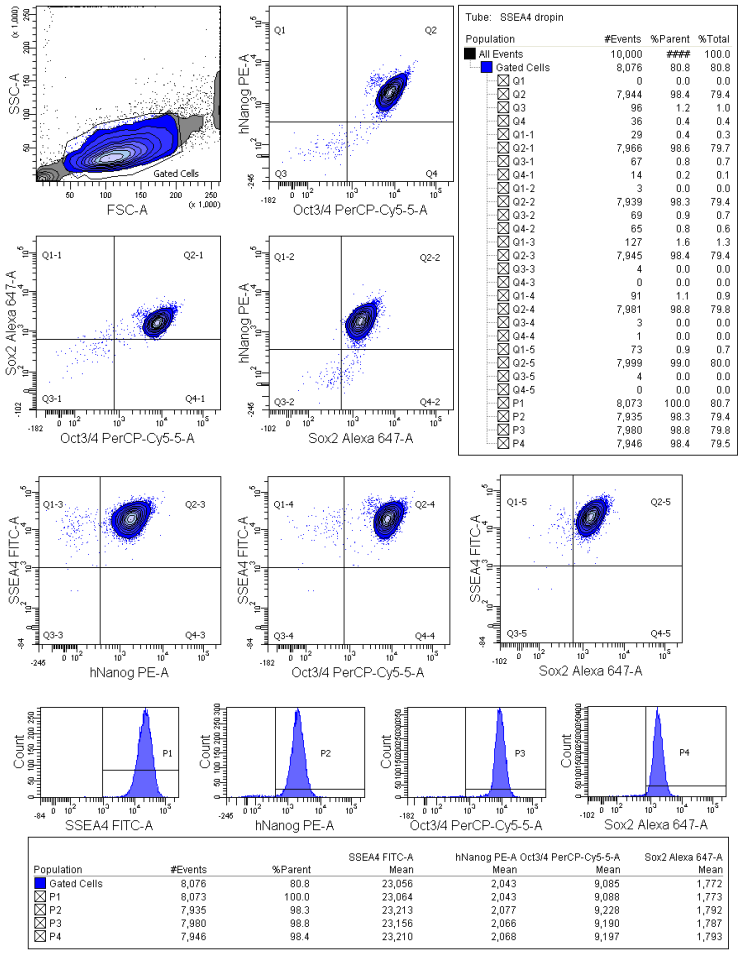


Population	#Events	%Parent	mIgG3 FITC-A Mean	hNanog PE-A Mean	Oct3/4 PerCP-Cy5-5-A Mean	Sox2 Alexa 647-A Mean
Gated Cells	8,365	83.6	328	2,064	9,082	1,893
P1	13	0.2	1,549	972	4,141	1,559
P2	8,188	97.9	329	2,104	9,254	1,913
P3	8,275	98.9	330	2,085	9,176	1,907
P4	8,235	98.4	330	2,089	9,190	1,915

Example of SSEA-4 drop-in on undifferentiated H9s

The following is an example of an analysis template showing data from undifferentiated human H9 ES cells that were stained with the kit antibodies plus FITC anti-SSEA-4, a pluripotency surface marker for hESCs (Catalog No. 560126).

{aw-anchorwide: anchored frame width=4.5"}

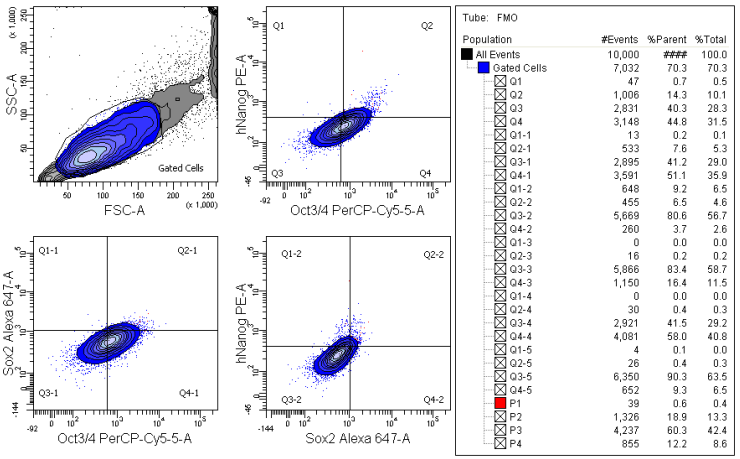


Population	#Events	%Parent	SSEA4 FITC-A Mean	hNanog PE-A Mean	Oct3/4 PerCP-Cy5-5-A Mean	Sox2 Alexa 647-A Mean
Gated Cells	8,076	80.8	23,056	2,043	9,085	1,772
P1	8,073	100.0	23,064	2,043	9,088	1,773
P2	7,935	98.3	23,213	2,077	9,228	1,792
P3	7,980	98.8	23,156	2,066	9,190	1,787
P4	7,946	98.4	23,210	2,068	9,197	1,793

Example of FMO control for SSEA-1 drop-in on differentiating H9s

The following is an example of an analysis template showing data from Day 3, differentiating human H9 ES cells that were treated with retinoic acid (10 µM) for 3 days and then stained with the kit antibodies plus mIgM FITC (the matching isotype for FITC anti-SSEA-1).

{aw-anchorwide: anchored frame width=4.5"}



Tube: FMO

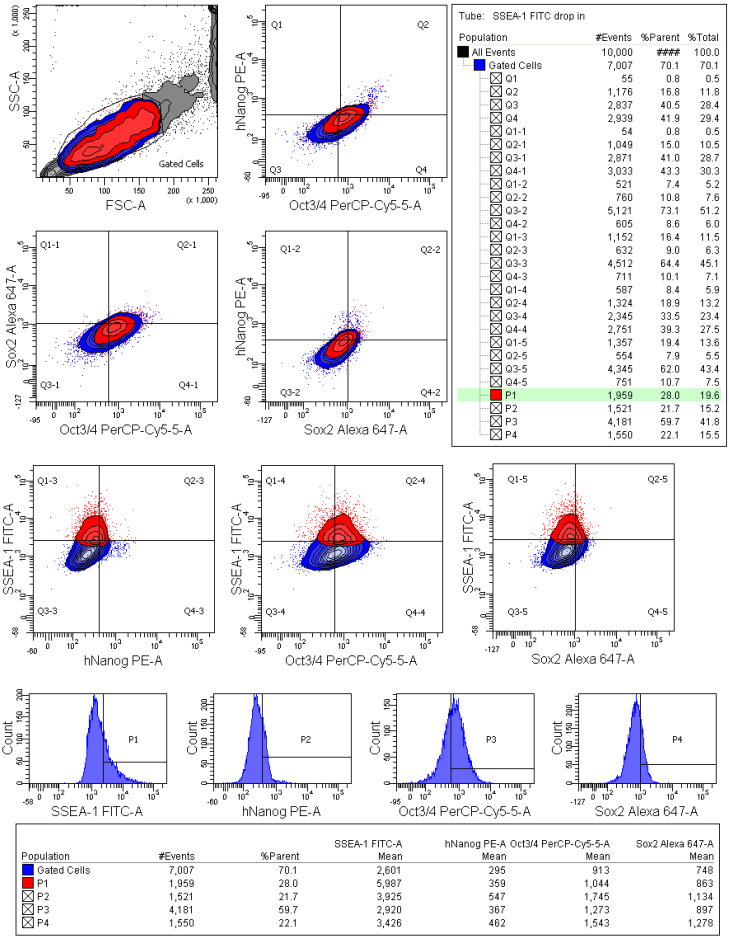
Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Gated Cells	7,032	70.3	70.3
Q1	47	0.7	0.5
Q2	1,006	14.3	10.1
Q3	2,831	40.3	28.3
Q4	3,148	44.8	31.5
Q1-1	13	0.2	0.1
Q2-1	533	7.6	5.3
Q3-1	2,895	41.2	29.0
Q4-1	3,591	51.1	35.9
Q1-2	648	9.2	6.5
Q2-2	455	6.5	4.6
Q3-2	5,669	80.6	56.7
Q4-2	260	3.7	2.6
Q1-3	0	0.0	0.0
Q2-3	16	0.2	0.2
Q3-3	5,866	83.4	58.7
Q4-3	1,150	16.4	11.5
Q1-4	0	0.0	0.0
Q2-4	30	0.4	0.3
Q3-4	2,921	41.5	29.2
Q4-4	4,081	58.0	40.8
Q1-5	4	0.1	0.0
Q2-5	26	0.4	0.3
Q3-5	6,350	90.3	63.5
Q4-5	652	9.3	6.5
P1	39	0.6	0.4
P2	1,326	18.9	13.3
P3	4,237	60.3	42.4
P4	855	12.2	8.6

Population	#Events	%Parent	mIgM FITC-A Mean	hNanog PE-A Mean	Oct3/4 PerCP-Cy5-5-A Mean	Sox2 Alexa 647-A Mean
Gated Cells	7,032	70.3	1,026	286	920	626
P1	39	0.6	2,672	1,129	2,104	1,309
P2	1,326	18.9	1,567	560	1,816	963
P3	4,237	60.3	1,189	352	1,280	741
P4	855	12.2	1,575	522	1,743	1,199

Example of SSEA-1 drop-in on differentiating H9s

The following is an example of an analysis template showing data from Day 3, differentiating human H9 ES cells that were treated with retinoic acid (10 µM) for 3 days and then stained with the kit antibodies plus FITC anti-SSEA-1, a marker for differentiation of hESCs (Catalog No. 560127).

{aw-anchorwide: anchored frame width=4.5"}



Related topics

- Adding drop-in conjugates (page 2)
- Troubleshooting (page 46)

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 Transcription Factor Analysis Kit.

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

{at-anchortable}

Problem	Recommended actions
Too few events during acquisition	Try one or more of the following: • Start with 1 x 10^6 cells per tube (some cell loss is expected during washes). • Centrifuge at a higher speed. • Centrifuge for a longer period of time. • Aspirate gently after centrifugation to avoid disturbing the cell pellet. • See the user's guide for your flow cytometer.
Dim staining of drop-in conjugates	Try one or more of the following: • Stain and record data the same day you fix and permeabilize the cells. • Stain fresh cells with your drop-in conjugate and compare staining of fresh cells with staining of fixed and permeabilized cells (to determine whether fixing and permeabilizing has a deleterious effect on staining). • Increase the staining time to 1 hour at room temperature. • Increase the amount of fluorescent antibody. • For surface-marker drop-ins, ensure that you use an appropriate detachment reagent to harvest cells so that epitopes on the cell surface are not destroyed. • Make sure to mix well after adding antibody conjugate to the tube.

{at-anchortable}

Problem	Recommended actions
High background staining	Decrease the amount of antibody used. **Note:** This kit has been tested on human (H9, H7) embryonic stem cell lines, and no problematic background staining has been observed.
Insoluble precipitate observed in 10X Perm/Wash buffer	A small amount of precipitate is common and does not affect product performance. You can filter the solution with a 0.45-micron filter before using it.

Related topics

- Preparing cells and beads (page 15)
- Running the cells (page 32)
- Adding drop-in conjugates (page 38)

Examples of bead and cell placement

About this topic

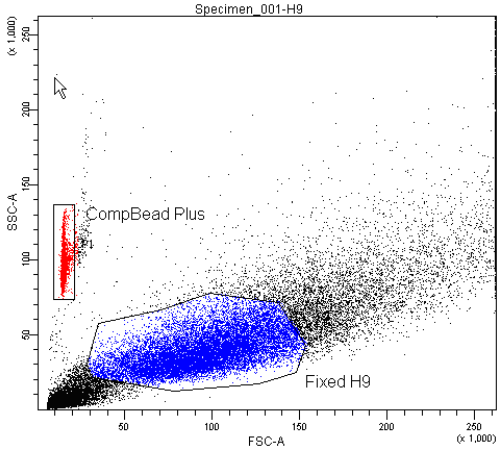
This topic gives two examples of FSC-A vs SSC-A dot plots, showing where the BD CompBead Plus beads appear relative to a single-cell suspension of undifferentiated stem cells.

These plots were obtained by running beads and cells together in the same tube.

Example with hESCs

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

{a0-anchor:anchored frame width=3.4"}



Related topics

- Running the beads (page 26)

About spectral overlap and compensation

About this topic

This topic explains why you must calculate compensation before running samples stained with the BD Human Pluripotent Stem Cell Transcription Factor Analysis Kit.

Spectral overlap

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

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 or 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 fluorochromes to be used in an experiment.

Related topics

- Staining (page 21)
- Running the beads (page 26)

References

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

References

1. Xu C. Characterization and evaluation of human embryonic stem cells. *Methods Enzymol.* 2006;420:18–37.
2. International Stem Cell Initiative. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnology.* 2007;25:803–816.
3. Boyer LA, Lee TI, Cole MF, et al. Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell.* 2005;122:1–10.
4. Pan G, Thomson JA. Nanog and transcriptional networks in embryonic stem cell pluripotency. *Cell Res.* 2007;17:42–49.
5. Boiani M, Schöler HR. Regulatory networks in embryo-derived pluripotent stem cells. *Nat Rev Mol Cell Biol.* 2005;6:872–884.
6. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science.* 2007;318(5858):1917–1920.
7. Yamanaka S. Pluripotency and nuclear reprogramming. *Philos Trans R Soc Lond B Biol Sci.* 2008;363:2079–2087.

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

Canada
888.259.0187

Europe
32.2.400.98.95

Japan
0120.8555.90

Asia/Pacific
65.6861.0633

Latin America/Caribbean
55.11.5185.9995

BD Biosciences
2350 Qume Drive
San Jose, CA 95131-1807
Toll free: 877.232.8995 (US)
Tel: 408.432.9475
Fax: 408.954.2347
bdbiosciences.com

647624 Rev A