BD Mouse Pluripotent Stem Cell Transcription Factor Analysis Kit

Instruction Manual

Catalog No. 560585

BD

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History

Revision	Date	Change Made
647623 Rev. A	5/2009	New document
23-12788-00 Rev. 01	10/2010	BD Perm/Wash™ Buffer packaging update
Rev. 02	04/2015	Warnings section update

Contents

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About this kit

This section covers the following topics:

Purpose of the kit

Uses of the kit

The BD™ Mouse Pluripotent Stem Cell Transcription Factor Analysis Kit (Catalog No. 560585) provides the reagents necessary to perform multicolor flow cytometry on mouse pluripotent stem cells such as mouse embryonic stem cells (mESCs) 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

Mouse pluripotent stem cells are characterized by the expression of specific intracellular transcription factors.[1] The BD Mouse Pluripotent Stem Cell Transcription Factor Analysis Kit contains three fluorochrome-conjugated antibodies that distinguish and identify mouse pluripotent cells. The three conjugated antibodies included in this kit (Nanog, Oct3/4 [or POU5F1], and Sox2) recognize transcription factors that are expressed in mouse pluripotent stem cells and have been termed the "core" pluripotency factors in mESCs.[2–7] This combination of markers has been widely used to characterize mESCs and iPS cells.[1,8]

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 Mouse Pluripotent Stem Cell Transcription Factor Analysis Kit.

This kit has been tested on the mouse ATCC CRL-1821™ ES-E14TG2a embryonic stem cell line, 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 39).

Kit contents

Reagent information

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

Reagent	Details
BD Pharmingen™ PE Mouse anti-mouse Nanog	**Clone:** M55-312 **Use:** The M55-312 monoclonal antibody reacts with mouse Nanog, which is a homeobox transcription factor required for the maintenance of the undifferentiated state of pluripotent stem cells. **Abbreviation:** PE mNanog **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™ 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)

Reagent	Details
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 mNanog **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)
BD Pharmingen™ 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)

Reagent	Details
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 mNanog, 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 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:** 1 bottle (60 mL)

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.

Storage and safe handling

Storage

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

Warning

Danger

BD Cytofix™ Fixation Buffer (component 51-9006276) contains 4.2% formaldehyde (w/w).

Hazard statements

Harmful if inhaled.

Causes skin irritation.

Causes serious eye damage.

May cause an allergic skin reaction.

Suspected of causing genetic defects.

May cause cancer. Route of exposure: Inhalative.

May cause respiratory irritation.

Precautionary statements

Wear protective clothing / eye protection.

Wear protective gloves.

Do not breathe mist/vapours/spray.

IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do.

Continue rinsing.

If skin irritation or rash occurs: Get medical advice/ attention.

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.

2

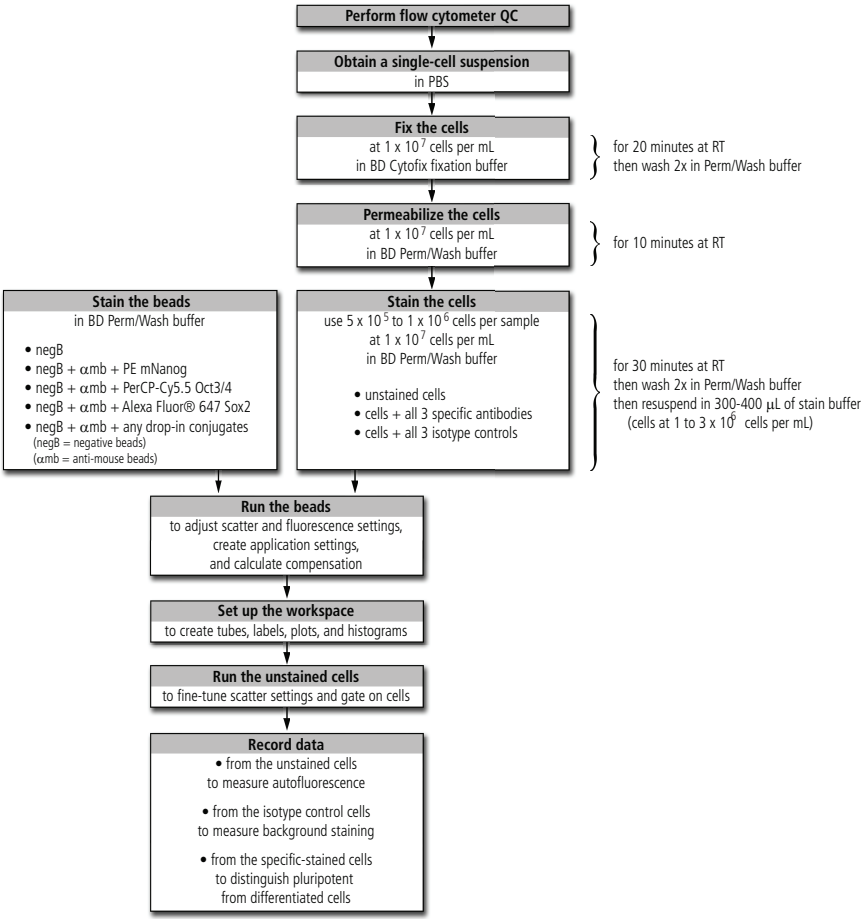
Before you begin

This section covers the following topics:

Workflow overview

Workflow

Following is an overview of the steps involved in using the BD Mouse Pluripotent Stem Cell Transcription Factor Analysis Kit to analyze cells.



Required materials

Materials list

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

- 1X PBS without Ca^{2+} or Mg^{2+}
- TrypLE™ Express stable trypsin replacement enzyme from Invitrogen, or equivalent (if analyzing mESCs)
- Microscope for confirming a single-cell suspension
- 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
- 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

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.

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Preparing cells and beads

This section covers the following topics:

Obtaining a single-cell suspension

Before you begin

Ensure that you have all of the necessary materials available. See Required materials (page 17) for details.

As a detachment enzyme, we recommend using TrypLE Express stable trypsin replacement enzyme if analyzing mESCs.

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 26) for more information about isotype controls.

Refer to the following guidelines.

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 24) unless you are adding a drop-in conjugate before fixing. See Adding drop-in conjugates (page 40) for more information about this option.

Fixing the cells

Before you begin

Complete the steps in Obtaining a single-cell suspension (page 22).

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 to bring to 1 x 10^7 cells per mL.
3. Incubate for 20 minutes at room temperature.
4. Proceed as follows.

If you will...	Then...
Stain cells the same day	Proceed immediately to Permeabilizing the cells (page 25)
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 25).

Permeabilizing the cells

Before you begin

Complete the steps in Fixing the cells (page 24).

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 26). Permeabilized cells cannot be stored and must be stained immediately.

Staining

Before you begin

Complete the steps in Permeabilizing the cells (page 25).

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 the ES-E14TG2a mESC line, 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.

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 mNanog	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 40).

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).

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 mNanog	—	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 1×10^6 cells per mL and 3×10^6 cells per mL.

Next step

Proceed to Running the beads (page 30).

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

4

Cytometer procedures

This section provides guidelines for application setup and data acquisition for analysis of cells stained with the BD Mouse 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

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 mESCs 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.

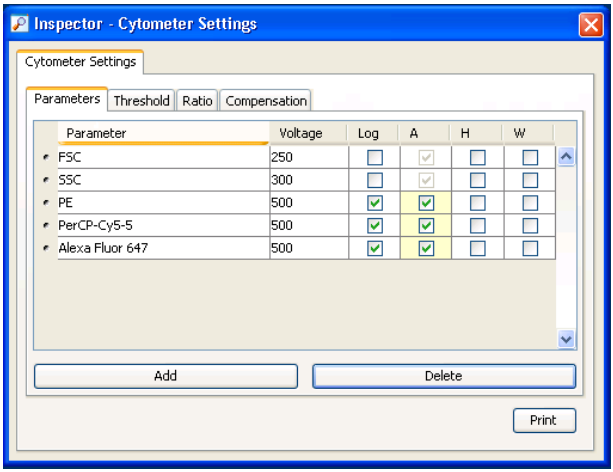
Complete the steps in Preparing cells and beads (page 21).

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

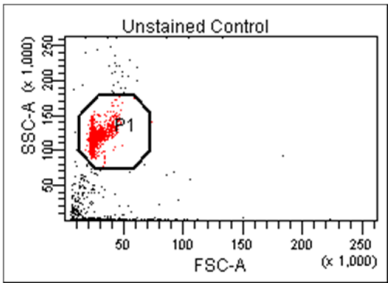
the cells with additional conjugates, include all relevant fluorescence parameters.



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.



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

Cell type	Parameter	Mean of bead population
mESCs	FSC-A	20,000 to 30,000
mESCs	SSC-A	130,000 to 155,000

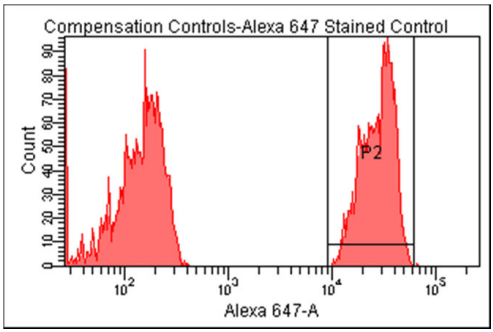
Note: Adjusting the voltages to obtain these values should place the stem cells on scale.

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

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.



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 34).

Related documents

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.

Setting up the workspace for running cells

Before you begin

Complete the steps in Running the beads (page 30).

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 it and proceed directly to Running the cells (page 36).
4. In the **Labels** tab of the **Experiment Layout** window, enter parameter labels for each marker in the experiment, 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 36).

Related documents

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.

Running the cells

Before you begin Complete the steps in Setting up the workspace for running cells (page 34).

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.

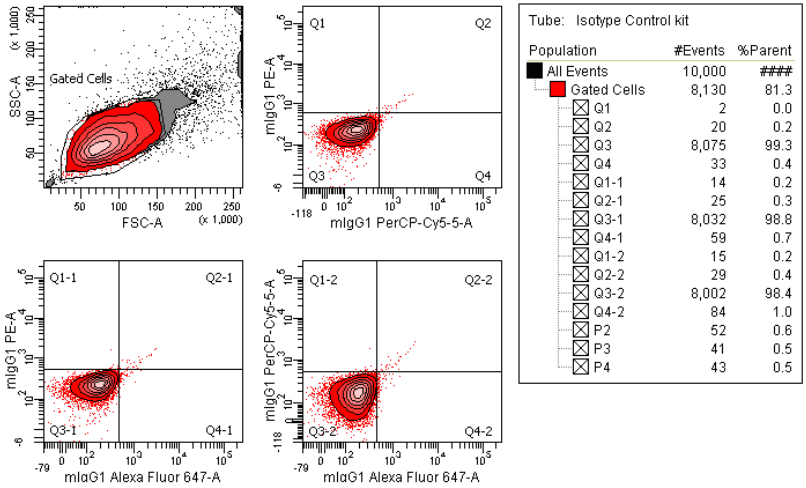
   Note: Do not adjust the fluorescence settings at this stage. Adjusting the fluorescence settings now will invalidate the compensation calculations.
3. Create a P1 gate on the population in the FSC-A vs SSC-A plot.

   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.

Template examples

Example with isotype-control mESCs

The following is an example of an analysis template showing isotype-control data from undifferentiated mESCs from the ES-E14TG2a cell line.



Tube: Isotype Control kit

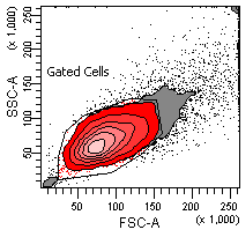
Population	#Events	%Parent
All Events	10,000	####
Gated Cells	8,130	81.3
Q1	2	0.0
Q2	20	0.2
Q3	8,075	99.3
Q4	33	0.4
Q1-1	14	0.2
Q2-1	25	0.3
Q3-1	8,032	98.8
Q4-1	59	0.7
Q1-2	15	0.2
Q2-2	29	0.4
Q3-2	8,002	98.4
Q4-2	84	1.0
P2	52	0.6
P3	41	0.5
P4	43	0.5

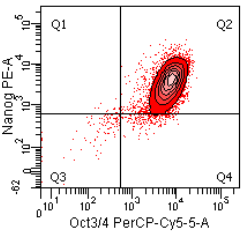
Tube Name: Isotype Control kit

Population	%Parent	mIgG1 PE-A Mean	mIgG1 PerCP-Cy5-5-A Mean	mIgG1 Alexa Fluor 647-A Mean
Gated Cells	####	232	182	192
P2	0.6	740	751	844
P3	0.5	731	939	940
P4	0.5	682	807	1,087

Example with specific-stained mESCs

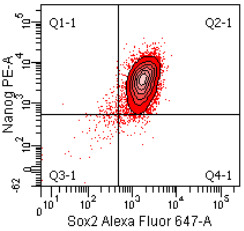
The following is an example of an analysis template showing data from specific-stained undifferentiated mESCs from the ES-E14TG2a cell line.

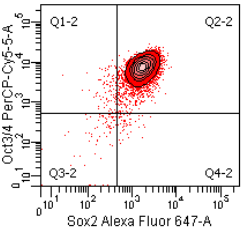


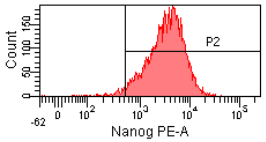


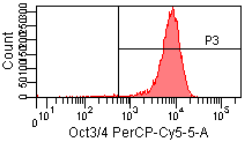
Tube: Kit

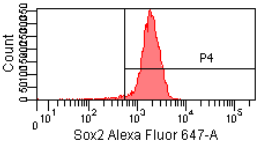
Population	#Events	%Parent
All Events	10,000	####
Gated Cells	8,165	81.6
Q1	18	0.2
Q2	7,822	95.8
Q3	101	1.2
Q4	224	2.7
Q1-1	98	1.2
Q2-1	7,829	95.9
Q3-1	98	1.2
Q4-1	140	1.7
Q1-2	107	1.3
Q2-2	7,933	97.2
Q3-2	74	0.9
Q4-2	51	0.6
P2	7,947	97.3
P3	8,037	98.4
P4	7,905	96.8











Tube Name: Kit

Population	%Parent	Nanog PE-A Mean	Oct3/4 PerCP-Cy5-5-A Mean	Sox2 Alexa Fluor 647-A Mean
Gated Cells	####	4,153	7,838	1,932
P2	97.3	4,258	8,006	1,965
P3	98.4	4,213	7,958	1,955
P4	96.8	4,253	8,024	1,984

5

Drop-in conjugates

This section covers the following topics:

Adding drop-in conjugates

Purpose of adding drop-ins

This topic provides information about adding "drop-in" conjugates: staining cells with fluorochrome-conjugated antibodies in addition to those provided in the kit. To obtain more information about the cells, you may decide to add antibody conjugates to surface or intra-cellular markers not already recognized by the antibodies in the BD Mouse 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 26).

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 21).

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 mNanog, PerCP-Cy5.5 Oct3/4, and Alexa Fluor® 647 Sox2) plus the matched isotype of the drop-in.

Compensation

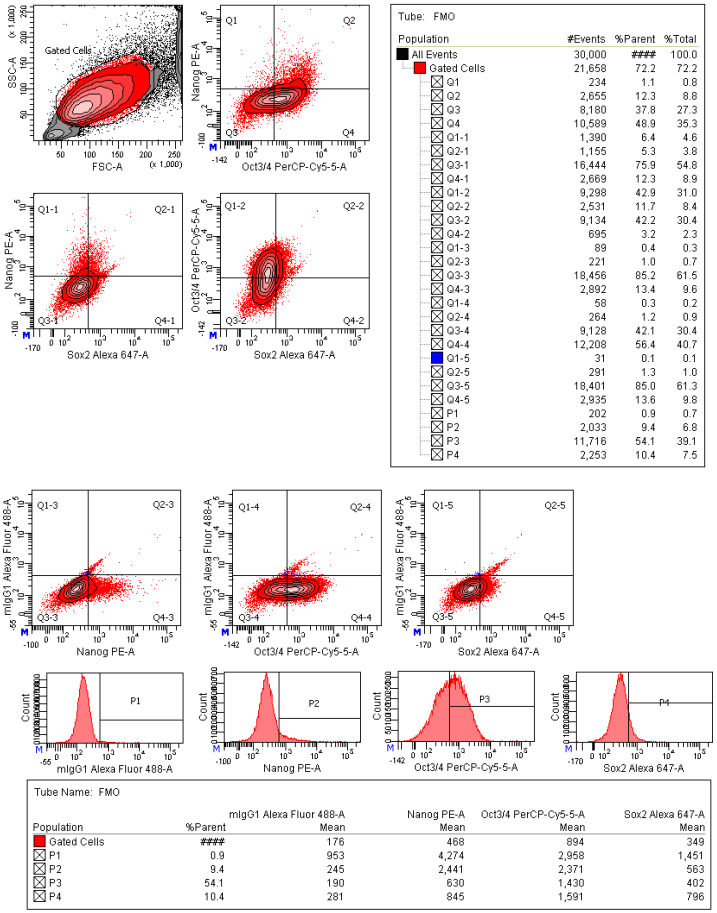
When staining beads, ensure that you prepare an additional tube for calculating compensation for the fluorochrome of each drop-in conjugate.

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

Examples of data with drop-in conjugates

Example of FMO control for GATA4 drop-in

The following is an example of an analysis template showing data from differentiating mouse ES-E14TG2a stem cells that were treated with retinoic acid (10 µM) for 2 days and then stained with the kit antibodies plus mIgG$_1$ Alexa Fluor® 488 (the matching isotype for Alexa Fluor® 488 Mouse anti-GATA4).



Tube: FMO

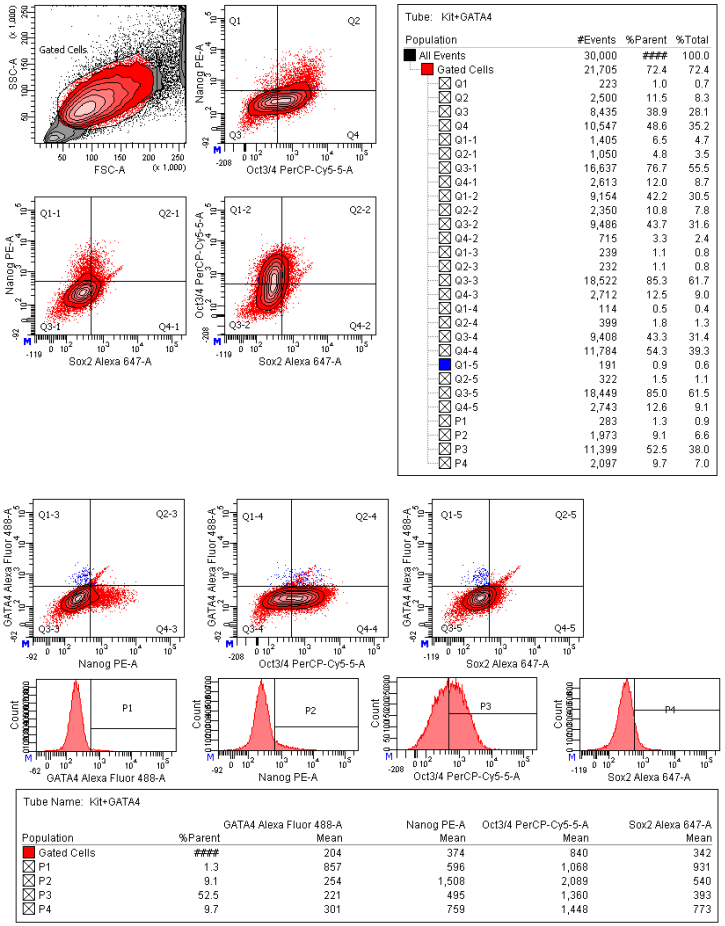
Population	#Events	%Parent	%Total
All Events	30,000	####	100.0
Gated Cells	21,658	72.2	72.2
Q1	234	1.1	0.8
Q2	2,655	12.3	8.8
Q3	8,180	37.8	27.3
Q4	10,589	48.9	35.3
Q1-1	1,390	6.4	4.6
Q2-1	1,155	5.3	3.8
Q3-1	16,444	75.9	54.8
Q4-1	2,669	12.3	8.9
Q1-2	9,298	42.9	31.0
Q2-2	2,531	11.7	8.4
Q3-2	9,134	42.2	30.4
Q4-2	695	3.2	2.3
Q1-3	89	0.4	0.3
Q2-3	221	1.0	0.7
Q3-3	18,456	85.2	61.5
Q4-3	2,892	13.4	9.6
Q1-4	58	0.3	0.2
Q2-4	264	1.2	0.9
Q3-4	9,128	42.1	30.4
Q4-4	12,208	56.4	40.7
Q1-5	31	0.1	0.1
Q2-5	291	1.3	1.0
Q3-5	18,401	85.0	61.3
Q4-5	2,935	13.6	9.8
P1	202	0.9	0.7
P2	2,033	9.4	6.8
P3	11,716	54.1	39.1
P4	2,253	10.4	7.5

Tube Name: FMO

Population	%Parent	mIgG1 Alexa Fluor 488-A Mean	Nanog PE-A Mean	Oct3/4 PerCP-Cy5-5-A Mean	Sox2 Alexa 647-A Mean
Gated Cells	####	176	468	894	349
P1	0.9	953	4,274	2,958	1,451
P2	9.4	245	2,441	2,371	563
P3	54.1	190	630	1,430	402
P4	10.4	281	845	1,591	796

Example of GATA4 drop-in

The following is an example of an analysis template showing data from differentiating mouse ES-E14TG2a stem cells that were treated with retinoic acid (10 µM) for 2 days and then stained with the kit antibodies plus Alexa Fluor® 488 Mouse anti-GATA4, a marker for mesoderm and definitive endoderm (Catalog No. 560330).



Tube: Kit+GATA4

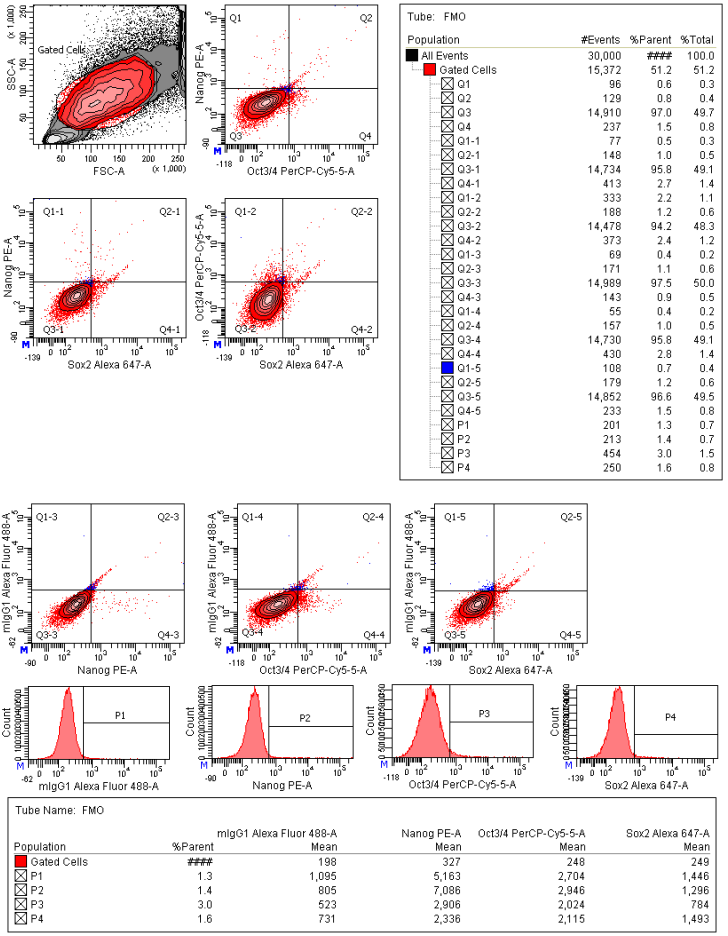
Population	#Events	%Parent	%Total
All Events	30,000	###	100.0
Gated Cells	21,705	72.4	72.4
Q1	223	1.0	0.7
Q2	2,500	11.5	8.3
Q3	8,435	38.9	28.1
Q4	10,547	48.6	35.2
Q1-1	1,405	6.5	4.7
Q2-1	1,050	4.8	3.5
Q3-1	16,637	76.7	55.5
Q4-1	2,613	12.0	8.7
Q1-2	9,154	42.2	30.5
Q2-2	2,350	10.8	7.8
Q3-2	9,486	43.7	31.6
Q4-2	715	3.3	2.4
Q1-3	239	1.1	0.8
Q2-3	232	1.1	0.8
Q3-3	18,522	85.3	61.7
Q4-3	2,712	12.5	9.0
Q1-4	114	0.5	0.4
Q2-4	399	1.8	1.3
Q3-4	9,408	43.3	31.4
Q4-4	11,784	54.3	39.3
Q1-5	191	0.9	0.6
Q2-5	322	1.5	1.1
Q3-5	18,449	85.0	61.5
Q4-5	2,743	12.6	9.1
P1	283	1.3	0.9
P2	1,973	9.1	6.6
P3	11,399	52.5	38.0
P4	2,097	9.7	7.0

Tube Name: Kit+GATA4

Population	%Parent	GATA4 Alexa Fluor 488-A Mean	Nanog PE-A Mean	Oct3/4 PerCP-Cy5-5-A Mean	Sox2 Alexa 647-A Mean
Gated Cells	###	204	374	840	342
P1	1.3	857	596	1,068	931
P2	9.1	254	1,508	2,089	540
P3	52.5	221	495	1,360	393
P4	9.7	301	759	1,448	773

Example of FMO control for GATA4 drop-in

The following is an example of an analysis template showing data from differentiating mouse ES-E14TG2a stem cells that were treated with retinoic acid (10 µM) for 5 days and then stained with the kit antibodies plus mIgG$_1$ Alexa Fluor® 488 (the matching isotype for Alexa Fluor® 488 Mouse anti-GATA4).



Tube: FMO

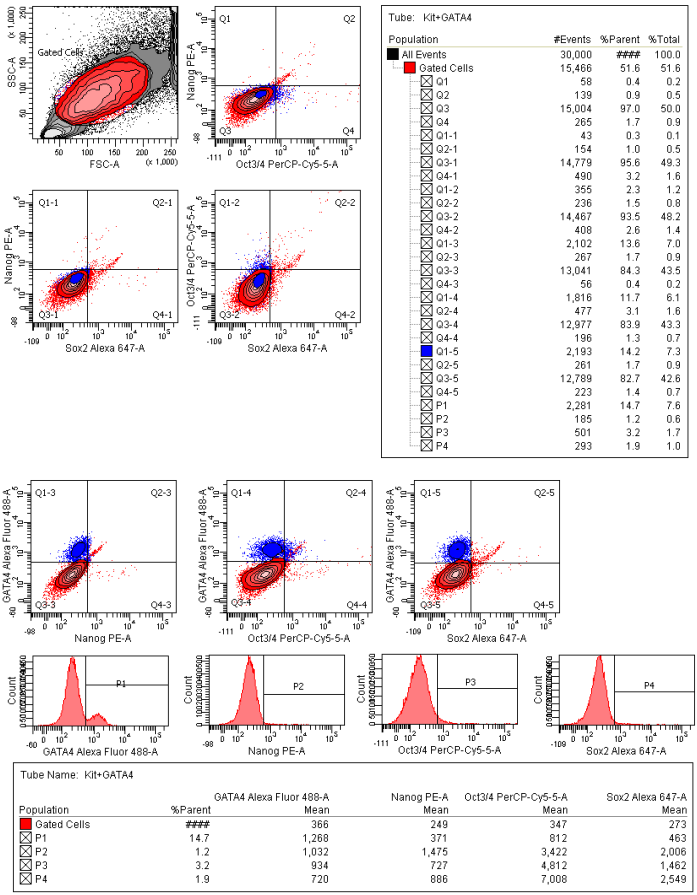
Population	#Events	%Parent	%Total
All Events	30,000	####	100.0
Gated Cells	15,372	51.2	51.2
Q1	96	0.6	0.3
Q2	129	0.8	0.4
Q3	14,910	97.0	49.7
Q4	237	1.5	0.8
Q1-1	77	0.5	0.3
Q2-1	148	1.0	0.5
Q3-1	14,734	95.8	49.1
Q4-1	413	2.7	1.4
Q1-2	333	2.2	1.1
Q2-2	188	1.2	0.6
Q3-2	14,478	94.2	48.3
Q4-2	373	2.4	1.2
Q1-3	69	0.4	0.2
Q2-3	171	1.1	0.6
Q3-3	14,989	97.5	50.0
Q4-3	143	0.9	0.5
Q1-4	55	0.4	0.2
Q2-4	157	1.0	0.5
Q3-4	14,730	95.8	49.1
Q4-4	430	2.8	1.4
Q1-5	108	0.7	0.4
Q2-5	179	1.2	0.6
Q3-5	14,852	96.6	49.5
Q4-5	233	1.5	0.8
P1	201	1.3	0.7
P2	213	1.4	0.7
P3	454	3.0	1.5
P4	250	1.6	0.8

Tube Name: FMO

Population	%Parent	mIgG1 Alexa Fluor 488-A Mean	Nanog PE-A Mean	Oct3/4 PerCP-Cy5-5-A Mean	Sox2 Alexa 647-A Mean
Gated Cells	####	198	327	248	249
P1	1.3	1,095	5,163	2,704	1,446
P2	1.4	805	7,086	2,946	1,296
P3	3.0	523	2,906	2,024	784
P4	1.6	731	2,336	2,115	1,493

Example of GATA4 drop-in

The following is an example of an analysis template showing data from differentiating mouse ES-E14TG2a stem cells that were treated with retinoic acid (10 µM) for 5 days and then stained with the kit antibodies plus Alexa Fluor® 488 Mouse anti-GATA4, a marker for mesoderm and definitive endoderm (Catalog No. 560330).



Tube: Kit+GATA4

Population	#Events	%Parent	%Total
All Events	30,000	###	100.0
Gated Cells	15,466	51.6	51.6
Q1	58	0.4	0.2
Q2	139	0.9	0.5
Q3	15,004	97.0	50.0
Q4	265	1.7	0.9
Q1-1	43	0.3	0.1
Q2-1	154	1.0	0.5
Q3-1	14,779	95.6	49.3
Q4-1	490	3.2	1.6
Q1-2	355	2.3	1.2
Q2-2	236	1.5	0.8
Q3-2	14,467	93.5	48.2
Q4-2	408	2.6	1.4
Q1-3	2,102	13.6	7.0
Q2-3	267	1.7	0.9
Q3-3	13,041	84.3	43.5
Q4-3	56	0.4	0.2
Q1-4	1,816	11.7	6.1
Q2-4	477	3.1	1.6
Q3-4	12,977	83.9	43.3
Q4-4	196	1.3	0.7
Q1-5	2,193	14.2	7.3
Q2-5	261	1.7	0.9
Q3-5	12,789	82.7	42.6
Q4-5	223	1.4	0.7
P1	2,281	14.7	7.6
P2	185	1.2	0.6
P3	501	3.2	1.7
P4	293	1.9	1.0

Tube Name: Kit+GATA4

Population	%Parent	GATA4 Alexa Fluor 488-A Mean	Nanog PE-A Mean	Oct3/4 PerCP-Cy5-5-A Mean	Sox2 Alexa 647-A Mean
Gated Cells	###	366	249	347	273
P1	14.7	1,268	371	812	463
P2	1.2	1,032	1,475	3,422	2,006
P3	3.2	934	727	4,812	1,462
P4	1.9	720	886	7,008	2,549

6

Reference

This section covers the following topics:

Troubleshooting

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

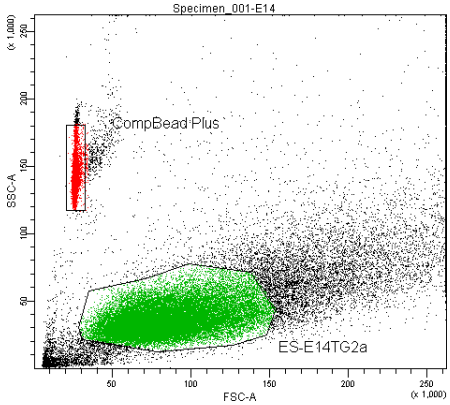
Problem	Recommended actions
Too few events during acquisition	Try one or more of the following: • Start with 1 x 10^6 cells per tube (some cell loss is expected during washes). • Centrifuge at a higher speed. • Centrifuge for a longer period of time. • Aspirate gently after centrifugation to avoid disturbing 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.

Problem	Recommended actions
High background staining	Decrease the amount of antibody used. **Note:** This kit has been tested on mouse (ATCC CRL-1821, ES-E14TG2a) 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.

Examples of bead and cell placement

Example with mESCs

The following plot shows BD CompBead Plus beads run together with a single-cell suspension of fixed cells from the ES-E14TG2a mESC line.



About spectral overlap and compensation

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.

References

Cited publications

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3. Okamoto K, Okazawa H, Okuda A, Sakai M, Muramatsu M, Hamada H. A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. *Cell*. 1990;60:461–472.

4. Loh Y, Wu Q, Chew J, et al. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nature Genetics*. 2006;38:431–440.

5. Masui S, Nakatake Y, Toyooka,Y, et al. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nature Cell Biology*. 2007;9:625–635.

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7. Kim J, Chu J, Shen X, Wang J, Orkin SH. An extended transcriptional network for pluripotency of embryonic stem cells. *Cell*. 2008;312:1049–1061.

8. Yamanaka S. Pluripotency and nuclear reprogramming. *Philos Trans R Soc Lond B Biol Sci*. 2008;363:2079–2087.

Notes

Notes

Notes

United States
877.232.8995

Canada
800.268.5430

Europe
32.2.400.98.95

Japan
0120.8555.90

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65.6861.0633

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