BD Stemflow™ Human and Mouse Pluripotent Stem Cell Analysis Kit

Instruction Manual

Catalog No. 560477

Trademarks

Cy is a trademark of Amersham Biosciences Corp. This product is subject to proprietary rights of Amersham Biosciences Corp. and Carnegie Mellon University and made and sold under license from Amersham Biosciences Corp. This product is licensed for sale only for research. It is not licensed for any other use. If you require a commercial license to use this product and do not have one return this material, unopened to BD Biosciences, 2350 Qume Drive, San Jose, CA, 95131-1807 and any money paid for the material will be refunded.

Accutase is a trademark of Innovative Cell Technologies.

Alexa Fluor® is a registered trademark of Molecular Probes, Inc. The Alexa Fluor®, Pacific Blue™, and Cascade Blue® dye antibody conjugates in this product are sold under license from Molecular Probes, Inc., for research use only, excluding use in combination with microarrays, or as analyte specific reagents. The Alexa Fluor® dyes (except for Alexa Fluor® 430), Pacific Blue™ dye, and Cascade Blue® dye are covered by pending and issued patents.

ATCC CRL-1821 is a trademark of American Type Culture Collection.

Falcon® is a registered trademark of Corning Incorporated.

TrypLE is a trademark of Invitrogen Corp.

BD flow cytometers are Class 1 Laser Products.
For Research Use Only. Not for use in diagnostic or therapeutic procedures.

History

Revision	Date	Change made
647095	03/09	New document
23-10865-00	08/09	Warnings section update
23-10865-01 Rev. 01	11/2010	BD Perm/Wash™ packaging update
Rev. 02	04/2015	Warnings section update

Contents

1

About this kit

This section covers the following topics:

Purpose of the kit

Uses of the kit

The BD Stemflow™ Human and Mouse Pluripotent Stem Cell Analysis Kit (Catalog No. 560477) provides the reagents necessary to perform multicolor flow cytometry on human embryonic stem cells (hESCs), mouse embryonic stem cells (mESCs), and induced pluripotent stem (iPS) cells.

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

Specific antibodies

Human and mouse pluripotent stem cells are characterized by the expression of specific cell surface markers and intracellular transcription factors.[1,2]

The BD Stemflow Human and Mouse Pluripotent Stem Cell Analysis Kit contains three fluorochrome-conjugated antibodies that distinguish pluripotent cells from differentiated cells. One of the antibodies recognizes Oct3/4 (POU5F1), a transcription factor expressed in pluripotent stem cells in both humans and mice.[3,4] The second antibody recognizes SSEA-1 (stage-specific embryonic antigen-1), a differentiation marker on human cells and a pluripotency marker on mouse cells.[1,5] The third antibody recognizes SSEA-4 (stage-specific embryonic antigen-4), which is a pluripotency marker on human cells and can be a differentiation marker on mouse cells.[6,7] This combination of markers has been widely used to characterize mESCs, hESCs, and iPS cells.[1,8-10]

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 Stemflow Human and Mouse Pluripotent Stem Cell Analysis Kit.

This kit has been tested on human H9 and H7 (WiCell, Madison, WI) and mouse ATCC CRL-1821™ ES-E14TG2a 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 29).

Kit contents

Reagent information

The BD Stemflow Human and Mouse Pluripotent Stem Cell Analysis Kit contains the following reagents.

Reagent	Details
BD Pharmingen™ PerCP-Cy™5.5 Mouse anti-Oct3/4	**Clone:** 40/Oct-3 **Use:** Used to stain the intracellular transcription factor Oct3/4 (POU5F1) **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™ PE 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:** PE SSEA-1 **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-SSEA-4	**Clone:** MC813 **Use:** Used to stain a carbohydrate epitope on the major ganglioside, but not the neutral glycolipid of human teratocarcinoma cells and undifferentiated human pluripotent stem cells **Abbreviation:** Alexa Fluor® 647 SSEA-4 **Quantity:** 1 vial (1.5 mL) **Amount for staining:** 20 µL per sample (for 5×10^5 to 1×10^6 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 5×10^5 to 1×10^6 cells)
BD Pharmingen™ PE Mouse IgM, κ Isotype Control	**Clone:** G155-228 **Use:** Used as an isotype control for PE SSEA-1 **Abbreviation:** PE isotype control **Quantity:** 1 vial (1.0 mL) **Amount for staining:** 20 µL per sample (for 5×10^5 to 1×10^6 cells)
BD Pharmingen™ Alexa Fluor® 647 Mouse IgG_3, κ Isotype Control	**Clone:** J606 **Use:** Used as an isotype control for Alexa Fluor® 647 SSEA-4 **Abbreviation:** Alexa Fluor® 647 isotype control **Quantity:** 1 vial (1.0 mL) **Amount for staining:** 20 µL per sample (for 5×10^5 to 1×10^6 cells)

Reagent	Details
BD™ CompBead Plus Anti-Mouse Ig, κ	**Use:** Used to create control beads stained with PerCP-Cy5.5 Oct3/4, PE SSEA-1, or Alexa Fluor® 647 SSEA-4 (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 Stemflow Human and Mouse Pluripotent Stem Cell 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

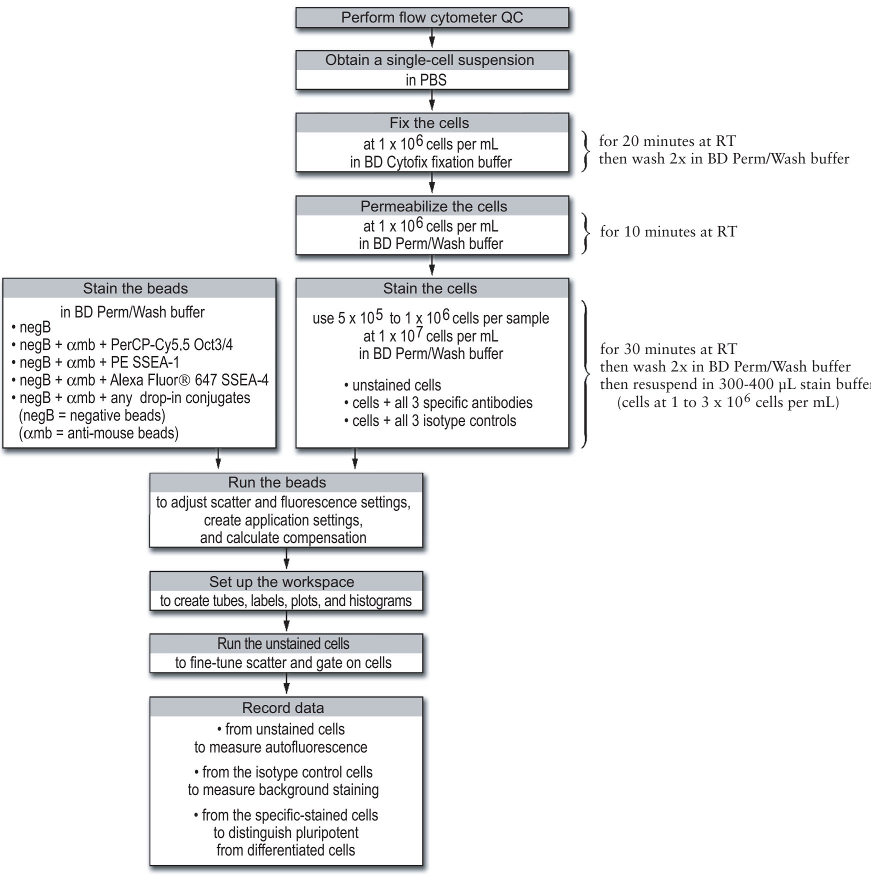
Before you begin

This section covers the following topics:

Workflow overview

Workflow

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



Required materials

Materials list

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

- 1X PBS without Ca^{2+} or Mg^{2+}
- Accutase™ Enzyme Cell Detachment Medium from Innovative Cell Technologies, or equivalent (if analyzing hESCs)
- 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 PerCP-Cy5.5, PE, 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 the 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.

3

Preparation of 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 Accutase enzyme if analyzing hESCs, or TrypLE Express stable trypsin replacement enzyme if analyzing mESCs.

Ensure that your 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.

See the following guidelines.

Guideline	Value
Required cell concentration for staining	1×10^7 cells/mL
Recommended cells per tube for staining	1×10^6 cells
Minimum cells per tube for staining	5×10^5 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 30) 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 your fixed cells. For best results we recommend that you plan to stain samples within 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/mL. 3. Store at 4°C for up 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 4.5–5.0 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: We do not recommend the use of methanol-based perm buffers with this kit.

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 H9 and H7 hESC lines and 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	
	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)
PerCP-Cy5.5 Oct3/4	20 µL	—
PE SSEA-1	20 µL	—
Alexa Fluor® 647 SSEA-4	20 µL	—
PerCP-Cy5.5 isotype control	—	20 µL
PE isotype control	—	20 µL
Alexa Fluor® 647 isotype control	—	20 µL

If your are adding additional antibodies at this stage, see Adding drop-in conjugates (page 30).

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
   - PerCP
   - PE
   - Alexa 647

   Note: If you stained your 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 tube			
	Negative	PerCP	PE	Alexa 647
BD 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
PerCP-Cy5.5 Oct3/4	—	20 µL	—	—
PE SSEA-1	—		20 µL	—
Alexa Fluor® 647 SSEA-4	—	—	—	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 BD 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 40).

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

4

Drop-in conjugates

This section covers the following topics:

Adding drop-in conjugates

Purpose of adding drop-ins

To obtain more information about your cells, you may decide to add fluorochrome-conjugated antibodies to surface or intra-cellular markers not already recognized by the antibodies in the BD Stemflow Human and Mouse Pluripotent Stem Cell 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 your 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 Preparation of 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 SSEA-1, PerCP-Cy5.5 Oct3/4, and Alexa Fluor® 647 SSEA-4) plus the matched isotype of the drop-in.

Compensation

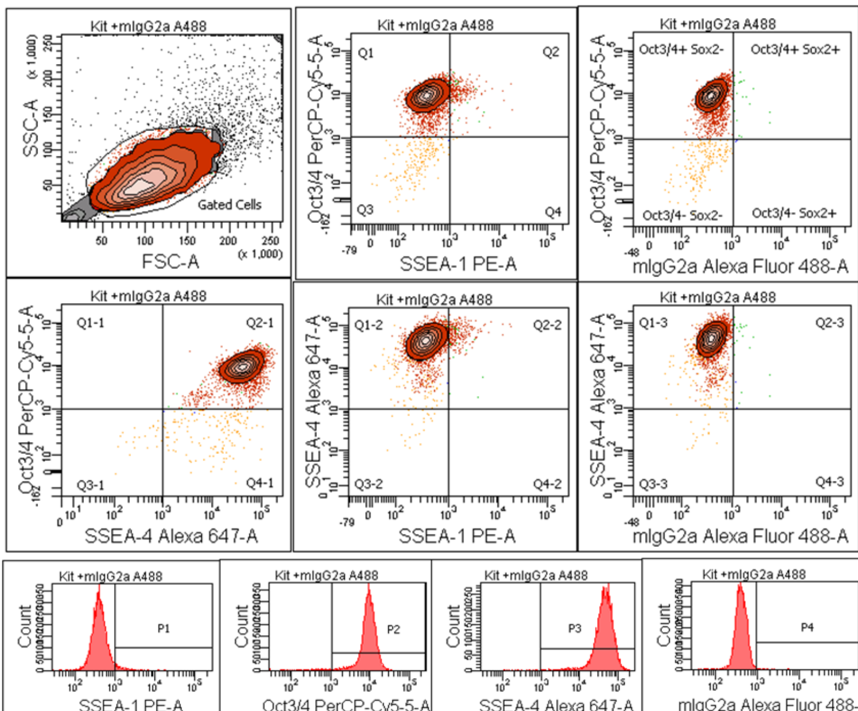
When staining beads, ensure that you prepare an additional tube for calculating compensation for the fluorochrome of each drop-in conjugate.

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 with FMO control for Sox2 drop-in

The following is an example of an analysis template showing data from undifferentiated H9 hESCs stained with the kit antibodies plus mIgG$_{2a}$ Alexa Fluor® 488 (the matching isotype for Alexa Fluor® 488 Mouse anti-Sox2).



Tube Name: Kit +mIgG2a A488

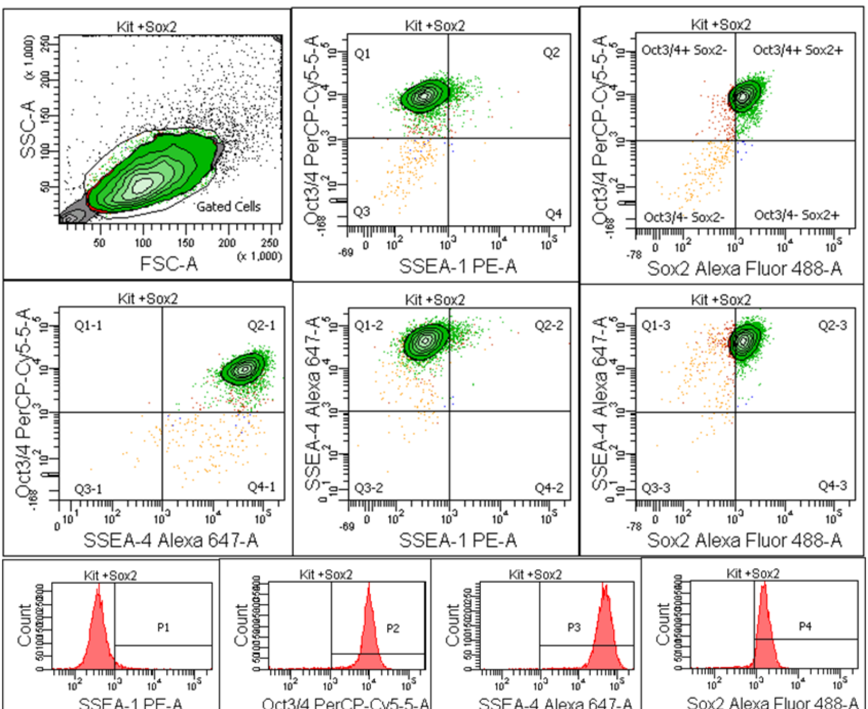
Population	Oct3/4 PerCP-Cy5-5-A Mean	SSEA-1 PE-A Mean	SSEA-4 Alexa 647-A Mean
Gated Cells	9,689	467	49,053
Oct3/4+ Sox2-	9,884	468	49,728
Oct3/4+ Sox2+	12,321	1,554	56,798
Oct3/4- Sox2-	404	286	17,196
Oct3/4- Sox2+	882	947	2,616
P1	12,386	1,785	71,680
P2	9,897	471	49,770
P3	9,715	467	49,188
P4	12,216	1,296	53,389

Tube: Kit +mIgG2a A488

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Gated Cells	8,254	82.5	82.5
Oct3/4+ Sox2-	8,058	97.6	80.6
Oct3/4+ Sox2+	21	0.3	0.2
Oct3/4- Sox2-	173	2.1	1.7
Oct3/4- Sox2+	2	0.0	0.0
P1	304	3.7	3.0
P2	8,073	97.8	80.7
P3	8,231	99.7	82.3
P4	37	0.4	0.4

Example with Sox2 drop-in

The following is an example of an analysis template showing data from undifferentiated H9 hESCs stained with the kit antibodies plus Alexa Fluor® 488 Mouse anti-Sox2, a marker for pluripotent hESCs and neural stem cells (Catalog No. 560301).



Tube Name: Kit +Sox2

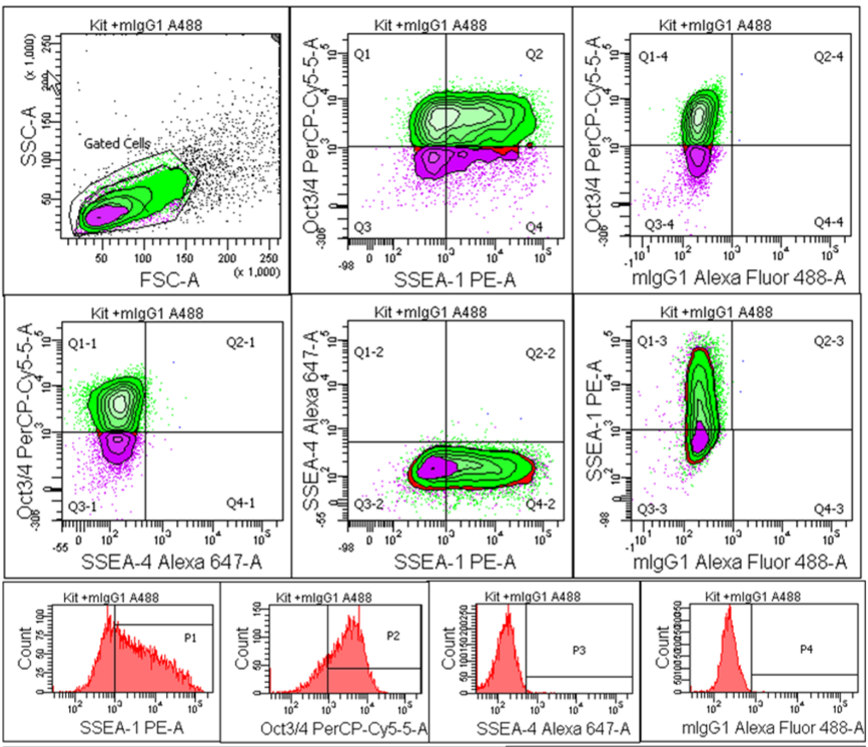
Population	Oct3/4 PerCP-Cy5-5-A Mean	SSEA-1 PE-A Mean	SSEA-4 Alexa 647-A Mean
Gated Cells	9,948	484	49,953
Oct3/4+ Sox2-	7,340	1,110	45,248
Oct3/4+ Sox2+	10,312	451	50,915
Oct3/4- Sox2-	361	280	18,183
Oct3/4- Sox2+	708	524	28,382
P1	12,009	2,587	71,580
P2	10,150	488	50,598
P3	9,975	484	50,092
P4	10,242	451	50,782

Tube: Kit +Sox2

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Gated Cells	8,207	82.1	82.1
Oct3/4+ Sox2-	450	5.5	4.5
Oct3/4+ Sox2+	7,591	92.5	75.9
Oct3/4- Sox2-	157	1.9	1.6
Oct3/4- Sox2+	9	0.1	0.1
P1	330	4.0	3.3
P2	8,037	97.9	80.4
P3	8,184	99.7	81.8
P4	7,777	94.8	77.8

Example with FMO control for Nanog 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 anti-Mouse Nanog).



Tube Name: Kit +mIgG1 A488

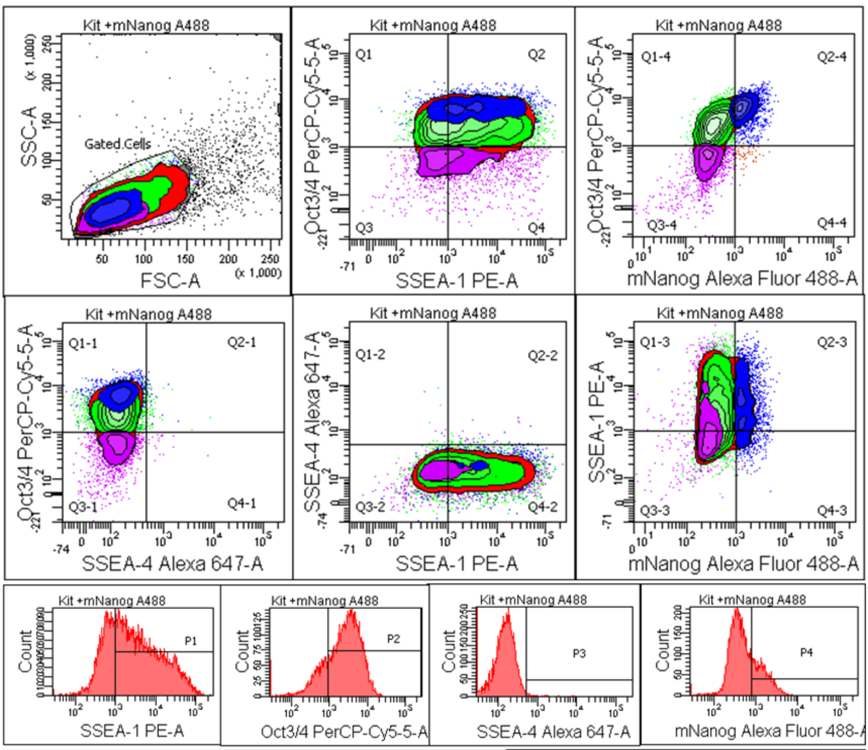
Population	Oct3/4 PerCP-... Mean	SSEA-1 PE-A Mean	mIgG1 Alexa Fl... Mean	SSEA-4 Alexa 6... Mean
Gated Cells	3,785	8,754	248	167
Q1-4	4,690	8,951	256	171
Q2-4	17,400	20,649	1,512	1,901
Q3-4	550	8,038	218	150
Q4-4	####	####	####	####
P1	4,121	12,405	256	170
P2	4,571	8,944	256	171
P3	6,036	5,512	533	772
P4	12,308	10,307	1,105	1,143

Tube: Kit +mIgG1 A488

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Gated Cells	8,576	85.8	85.8
Q1-4	6,694	78.1	66.9
Q2-4	2	0.0	0.0
Q3-4	1,880	21.9	18.8
Q4-4	0	0.0	0.0
P1	5,935	69.2	59.4
P2	6,924	80.7	69.2
P3	59	0.7	0.6
P4	6	0.1	0.1

Example with Nanog 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 anti-Mouse Nanog, a marker for pluripotent hESCs (Catalog No. 560278).



Tube Name: Kit +mNanog A488

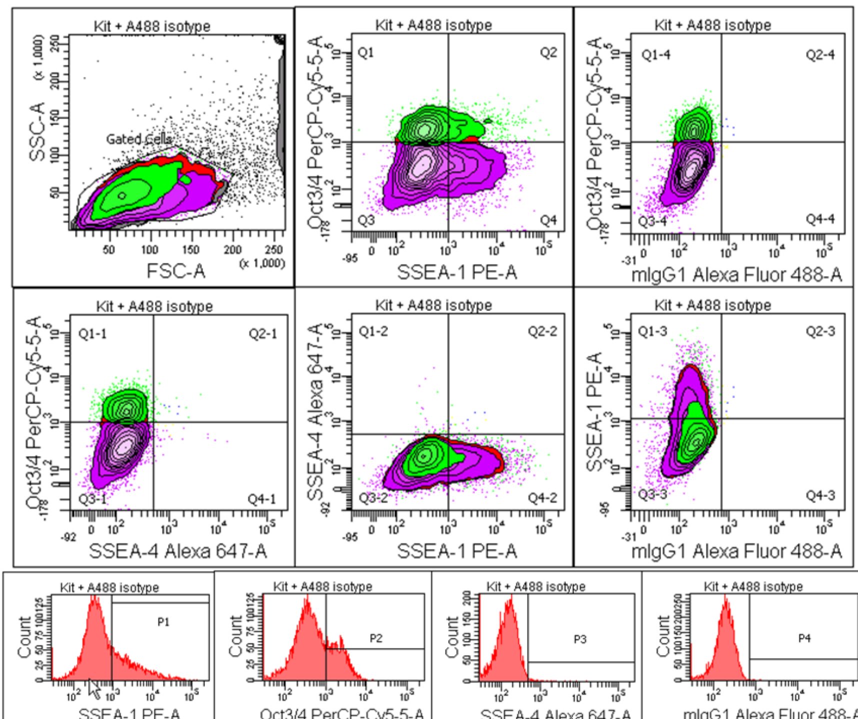
Population	Oct3/4 PerCP-... Mean	SSEA-1 PE-A Mean	mNanog Alexa ... Mean	SSEA-4 Alexa 6... Mean
Gated Cells	3,432	8,450	734	164
Q1-4	3,334	7,958	464	163
Q2-4	6,851	10,703	1,869	175
Q3-4	540	7,668	312	153
Q4-4	678	4,633	1,884	168
P1	3,733	11,760	793	166
P2	4,179	8,701	833	166
P3	3,322	7,116	790	1,024
P4	6,196	10,283	1,635	176

Tube: Kit +mNanog A488

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Gated Cells	8,615	86.2	86.2
Q1-4	4,824	56.0	48.2
Q2-4	1,810	21.0	18.1
Q3-4	1,928	22.4	19.3
Q4-4	53	0.6	0.5
P1	6,071	70.5	60.7
P2	6,871	79.8	68.7
P3	62	0.7	0.6
P4	2,437	28.3	24.4

Example with 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 4 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 Name: Kit + A488 isotype

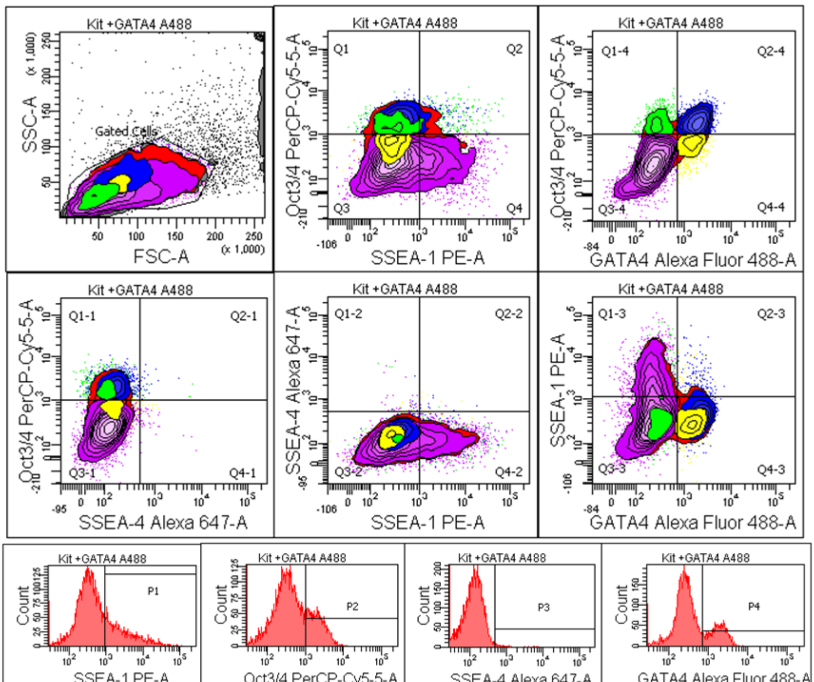
Population	mIgG1 Alexa Fl... Mean	SSEA-1 PE-A Mean	Oct3/4 PerCP-C... Mean	SSEA-4 Alexa 6... Mean
Gated Cells	194	1,833	890	163
Q1-4	227	2,012	2,416	187
Q2-4	980	2,812	2,299	1,047
Q3-4	182	1,771	372	153
Q4-4	840	2,156	789	702
P1	203	5,469	935	167
P2	229	2,043	2,308	188
P3	428	1,154	1,359	1,341
P4	886	2,325	1,491	868

Tube: Kit + A488 isotype

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Gated Cells	7,586	75.9	75.9
Q1-4	1,917	25.3	19.2
Q2-4	5	0.1	0.0
Q3-4	5,658	74.6	56.6
Q4-4	6	0.1	0.1
P1	2,188	28.8	21.9
P2	2,084	27.5	20.8
P3	66	0.9	0.7
P4	12	0.2	0.1

Example with 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 4 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 Name: Kit +GATA4 A488

Population	GATA4 Alexa Fl... Mean	SSEA-1 PE-A Mean	Oct3/4 PerCP-C... Mean	SSEA-4 Alexa 6... Mean
Gated Cells	651	1,684	818	145
Q1-4	337	2,613	2,325	150
Q2-4	2,130	979	2,409	189
Q3-4	249	1,855	308	130
Q4-4	1,564	656	633	176
P1	505	5,285	837	146
P2	1,498	1,523	2,278	177
P3	1,171	1,771	1,235	1,202
P4	1,929	872	1,799	185

Tube: Kit +GATA4 A488

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Gated Cells	7,661	76.6	76.6
Q1-4	604	7.9	6.0
Q2-4	1,184	15.5	11.8
Q3-4	5,263	68.7	52.6
Q4-4	610	8.0	6.1
P1	2,078	27.1	20.8
P2	1,934	25.2	19.3
P3	74	1.0	0.7
P4	1,806	23.6	18.1

5

Cytometer procedures

This section provides guidelines for application setup and data acquisition for analysis of cells stained with the BD Stemflow Human and Mouse Pluripotent Stem Cell 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 hESCs, 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.

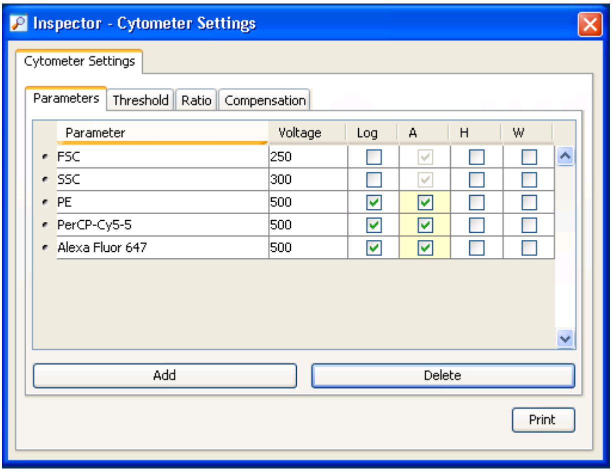
Complete the steps in Preparation of cells and beads (page 21).

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 and proceed to step 4.

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.



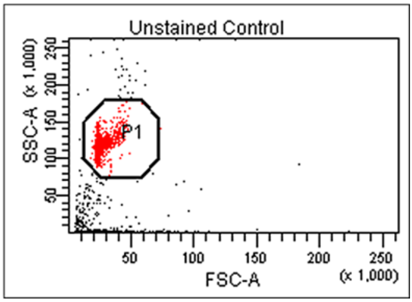
Note: The voltage settings that appear in this window will vary for each instrument.

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

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

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

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



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

Cell type	Parameter	Mean of bead population
hESCs	FSC	10,000 to 20,000
hESCs	SSC	90,000 to 115,000
mESCs	FSC	20,000 to 30,000
mESCs	SSC	130,000 to 155,000

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

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

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

11. Place each of the stained compensation control tubes on the cytometer in turn, and adjust the 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	At 10^4

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

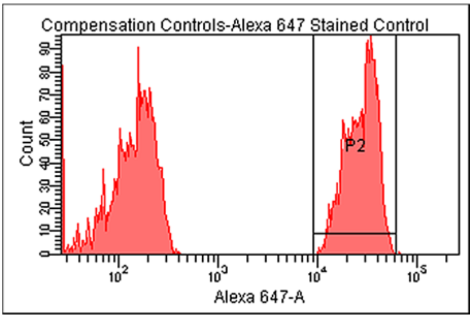
    a. In the **Browser**, right-click **Cytometer Settings** and select **Application Settings** > **Save**.

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

13. Return to the compensation worksheets.

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

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



16. Calculate compensation.

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

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

Next step

Proceed to Setting up the workspace for running cells (page 44).

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 40).

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 46).
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 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 46).

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 44).

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.

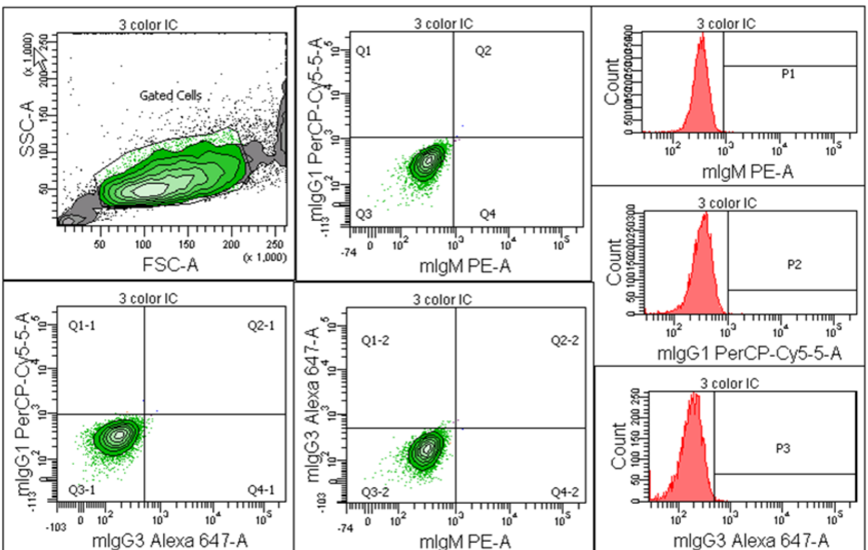
   Do not adjust the fluorescence settings at this stage. Adjusting the fluorescence settings now will invalidate the compensation calculations.
3. Create a P1 gate on the population in the FSC-A vs SSC-A plot.

   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 hESCs

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



Tube Name: 3 color IC

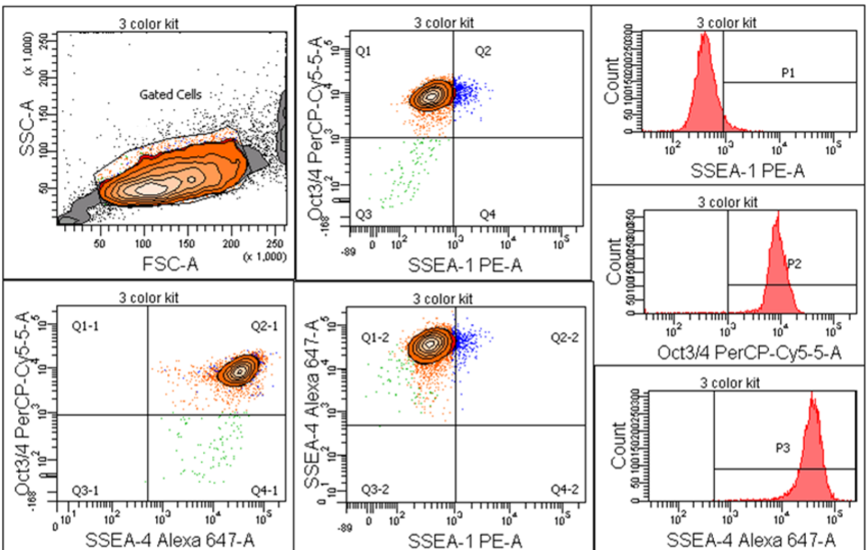
Population	mIgM PE-A Mean	mIgG1 PerCP-C... Mean	mIgG3 Alexa 64... Mean
Gated Cells	340	342	189
Q1	803	1,059	249
Q2	1,153	1,475	642
Q3	339	341	188
Q4	982	898	628
P1	1,050	1,129	634
P2	1,036	1,336	511
P3	597	563	566

Tube: 3 color IC

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Gated Cells	7,721	77.2	77.2
Q1	1	0.0	0.0
Q2	2	0.0	0.0
Q3	7,715	99.9	77.1
Q4	3	0.0	0.0
P1	5	0.1	0.0
P2	3	0.0	0.0
P3	28	0.4	0.3

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.



Tube Name: 3 color kit

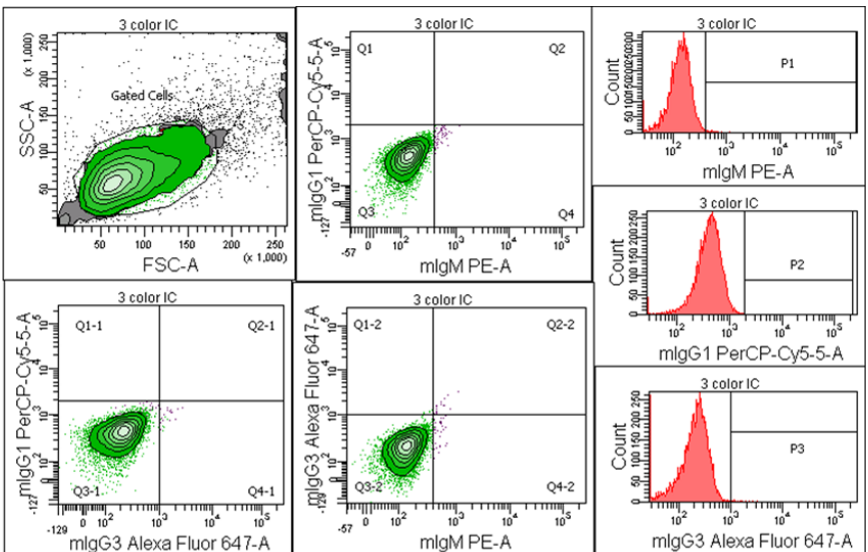
Population	SSEA-1 PE-A Mean	Oct3/4 PerCP-Cy... Mean	SSEA-4 Alexa 64... Mean
Gated Cells	470	9,135	38,950
Q1	423	9,162	38,905
Q2	1,259	10,518	44,646
Q3	201	377	14,695
Q4	898	1,027	30,125
P1	1,244	10,474	44,579
P2	473	9,237	39,240
P3	470	9,136	38,955

Tube: 3 color kit

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Gated Cells	7,915	79.1	79.1
Q1	7,349	92.8	73.5
Q2	469	5.9	4.7
Q3	96	1.2	1.0
Q4	1	0.0	0.0
P1	488	6.2	4.9
P2	7,824	98.9	78.2
P3	7,914	100.0	79.1

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 Name: 3 color IC

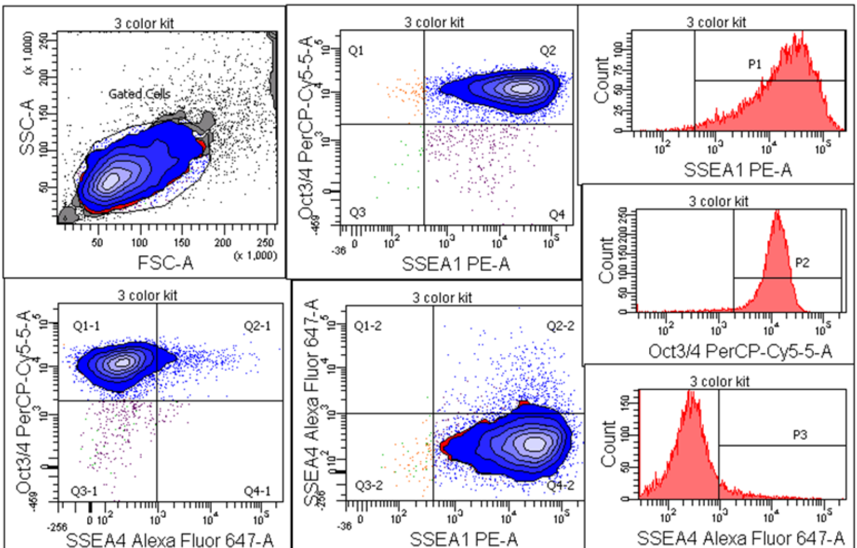
Population	mIgM PE-A Mean	mIgG1 PerCP-C... Mean	mIgG3 Alexa Flu... Mean
Gated Cells	146	446	224
Q1	####	####	####
Q2	####	####	####
Q3	143	442	220
Q4	518	1,189	885
P1	516	1,176	882
P2	1,094	1,907	3,217
P3	493	959	1,465

Tube: 3 color IC

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Gated Cells	8,298	83.0	83.0
Q1	0	0.0	0.0
Q2	0	0.0	0.0
Q3	8,250	99.4	82.5
Q4	48	0.6	0.5
P1	49	0.6	0.5
P2	1	0.0	0.0
P3	23	0.3	0.2

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 Name: 3 color kit

Population	SSEA1 PE-A Mean	Oct3/4 PerCP-Cy... Mean	SSEA4 Alexa Flu... Mean
Gated Cells	31,556	13,100	721
Q1	247	11,185	127
Q2	32,645	13,543	736
Q3	233	556	269
Q4	8,061	859	437
P1	31,809	13,142	727
P2	32,361	13,510	732
P3	33,800	15,222	5,432

Tube: 3 color kit

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Gated Cells	8,268	82.7	82.7
Q1	63	0.8	0.6
Q2	7,928	95.9	79.3
Q3	20	0.2	0.2
Q4	257	3.1	2.6
P1	8,185	99.0	81.8
P2	8,001	96.8	80.0
P3	723	8.7	7.2

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.

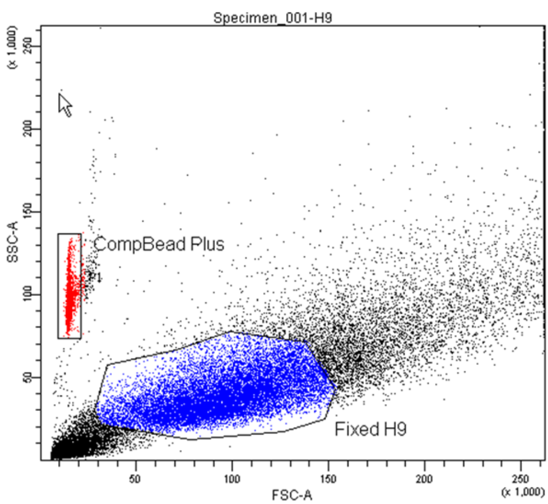
Problem	Recommended actions
Too few events during acquisition	Try one or more of the following: • Start with 1 x 10^6 cells per tube (some cell loss is expected during washes). • Centrifuge at a higher speed. • Centrifuge for a longer period of time. • Aspirate gently after centrifugation to avoid disturbing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

Problem	Recommended actions
High background staining	Decrease the amount of antibody used. **Note:** This kit has been tested on human (H9, H7) and mouse (ATCC CRL-1821, ES-E14TG2a) embryonic stem cell lines, and no problematic background staining has been observed.
Insoluble precipitate observed in 10X BD 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

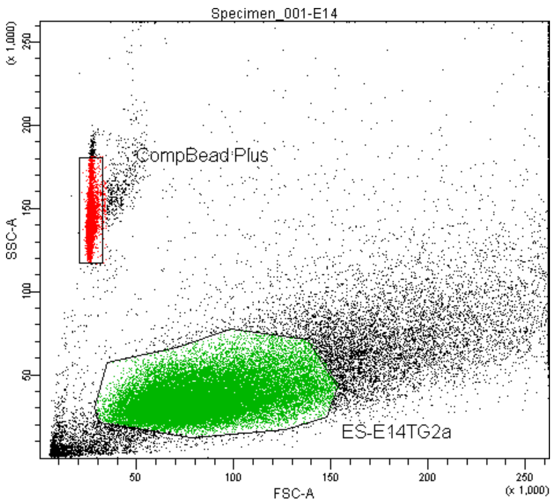
Example with hESCs

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



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

1. Ginis I, Luo Y, Miura T, et al. Differences between human and mouse embryonic stem cells. *Dev Biol.* 2004;269:360–380.

2. Xu C. Characterization and evaluation of human embryonic stem cells. *Methods Enzymol.* 2006;420:18–37.

3. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol.* 2000;18:399–404.

4. 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.

5. 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.

6. Solter D, Shevinsky L, Knowles BB, Strickland S. The induction of antigenic changes in a teratocarcinoma stem cell line (F9) by retinoic acid. *Dev Biol*. 1979;70:515–521.

7. Henderson JK, Draper JS, Baillie HS, et al. Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. *Stem Cells*. 2002;20:329–337.

8. 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.

9. 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.

10. Park I-H, Zhao R, West JA, et al. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature*. 2008;451:141–146.

Notes

Notes

United States
877.232.8995

Canada
866.979.9408

Europe
32.2.400.98.95

Japan
0120.8555.90

Asia/Pacific
65.6861.0633

Latin America/Caribbean
55.11.5185.9995

Becton, Dickinson and Company
BD Biosciences
San Jose, CA 95131
Toll free: 877.232.8995 (US)
Tel: 408.432.9475
Fax: 408.954.2347
bdbiosciences.com

Rev# 2