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Reliable Protocols for Flow Cytometry Analysis of Intracellular Proteins in Pluripotent Stem Cell Derivatives: A Fit-For-Purpose Approach

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Abstract

Human pluripotent stem cell (hPSC) derivatives are valuable for a variety of research applications and have the potential to revolutionize approaches to personalized medicine. However, differentiation efficiency varies among cell lines and protocols. Therefore, methods to reliably determine cell type identity in cultures of hPSC derivatives in a manner that is consistent among laboratories are needed. While flow cytometry is apt for routine assessment of population heterogeneity, standardized protocols are not available for most cell types. This Unit describes a workflow for establishing a fit-for-purpose protocol for flow cytometric analysis of hPSC derivatives. Based on the application of this workflow, a standard operating procedure (SOP) was developed for the analysis of cardiac troponin in hPSC-derived cardiomyocytes (hPSC-CM). Throughout the Unit, important concepts related to antibody validation and gating strategies are presented to enable users to properly validate any antibody of interest and develop rigorous SOP for their experimental needs.

Keywords

flow cytometry; intracellular proteins; heterogeneity; quality control; hPSC-CM

INTRODUCTION

Human pluripotent stem cells (hPSC) are an inexhaustible source for the generation of a variety of cell types useful for the study of human development, disease modeling, drug testing, and personalized medicine (Braam et al., 2010; Carvajal-Vergara et al., 2010; Moretti et al., 2010). Over the past decade, significant advancements have been made in our ability to direct the differentiation of hPSC to a variety of cell types (*e.g.* hepatocytes,

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cardiomyocytes, retinal epithelial); however, the resulting cultures remain a heterogeneous mixture with regards to cell type and subtype, and this heterogeneity can be exacerbated by variations among cell lines and protocols (Ohno et al., 2013). Ultimately, this heterogeneity can pose challenges to interpreting functional data and determining, which cells are the most relevant models to use for specific applications. Importantly, the ability to accurately and precisely assess cell identity in differentiation cultures is a necessary aspect of conducting well-defined studies that can be replicated among laboratories.

Flow cytometry is a powerful analytical tool capable of single-cell based population studies that simultaneously measure physical properties such as size and internal complexity of a cell, and presence and quantity of cellular molecules such as DNA and proteins through the use of fluorescent-labeled molecules such as dyes and monoclonal antibodies (mAb). Flow cytometry has been extensively used for immunophenotyping hematopoietic cells to assess their identity, purity, and distinct antigen pattern during development and disease. It has also been used to assess heterogeneity and cell identity within hPSC and hPSC derivatives by using antibodies that recognize cell-type specific proteins.

There are numerous experimental parameters that are critical to consider for accurate interpretation of an antibody-based flow cytometry experiment. However, for many hPSC derivatives, these parameters have not been well-defined. In fact, for most hPSC derivatives, there is currently no consensus regarding which proteins should be used as markers or which sample preparation strategies and reagents are most apt for detecting those markers. To facilitate the development, validation, and application of fit-for-purpose protocols for flow cytometry assessment of hPSC derivatives, the Strategic Planning and Critical Parameters sections of this Unit outline a workflow and metrics to aid in the development of highly reliable and reproducible standard operating procedures (SOP) for any marker and any hPSC derivative. The Basic Protocols provide detailed instructions for the assessment of cardiac troponin in hPSC-derived cardiomyocytes (hPSC-CM) cultures and are examples of validated protocols that have been developed by applying the fit-for-purpose protocol development workflow. The Basic Protocols describe how to complete the protocol in a tube (Basic Protocol 1) and multi-well plate format (Basic Protocol 2). Each protocol includes detailed instructions regarding cell collection, cell labeling, and preparation for flow cytometry analysis. The Support Protocols provide instructions for antibody titration and validation of antibody specificity.

STRATEGIC PLANNING

The proper implementation of a flow cytometry experiment requires careful consideration of numerous specific details in the sample preparation, data acquisition, and data analysis stages as each will influence the results and interpretation. The **Basic Protocols** provided were developed based on the fit-for-purpose protocol workflow (Figure 1) and have been validated for specific mAb clones for assessing cardiac troponin (*i.e.* TNNT2 and TNNI3) in hPSC-CM using undifferentiated hPSC as a negative cell type control. Importantly, the specificity of an antibody is always context dependent. As such, defining the set of experiments required to validate the utility of an antibody for an application (*i.e.* fit-for-purpose) is entirely dependent on the intended application, and each application may require

its own set of experiments. Specifically, validation of a flow cytometry protocol requires an investigation into the relationship between sample preparation and obtained signal, which can only be defined by proper controls. Hence, should the reader wish to use alternative antibodies or cell types, experimental details specific to those parameters should be validated as described in Figure 1. The Critical Parameters section provides an overview of the critical steps to consider when establishing the fit-for-purpose protocol and is provided to enable users to properly validate any antibody of interest and individualize the SOP for their experimental needs.

BASIC PROTOCOL 1

PROTOCOL FOR ROUTINE ASSESSMENT OF FIXED hPSC/hPSC-CM: TUBE FORMAT

This SOP is the outcome of applying the fit-for-purpose development workflow for a flow cytometry protocol to assess TNNI3- and TNNT2-positivity within cultures of hPSC-CM. We provide stepwise instructions for cell collection, cell labeling, and preparation for flow cytometry to assess the cardiomyocyte content in hPSC-CM differentiation cultures.

Materials—HyPure WFI Quality Water (sterile water) (HyClone, #SH30221.17)

Dulbecco's phosphate buffered saline, Ca^{2+}/Mg^{2+} free (1xDPBS −/−) (Sigma-Aldrich, #D8537)

RPMI 1640 medium (Thermo Fisher Scientific, #11875–093)

Liberase-TH (Sigma-Aldrich, #5401135001)

DNase 1 (Sigma-Aldrich, #10104159001)

Liberase/DNase solution (see recipe)

TrypLE express enzyme (1X), phenol red (Thermo Fisher Scientific, #12605–010)

Trypan blue solution, 0.4% (Thermo Fisher Scientific, #15250–061)

Accutase (Innovative Cell Tech., #AT104)

Stem cell basal media (based on individual stem cell culture system)

16% Formaldehyde (w/v), methanol-free (Thermo Fisher Scientific, #28906)

Fixation solution (see recipe)

Bovine serum albumin (Sigma-Aldrich, #A7906)

Saponin (Sigma-Aldrich, #47036)

Flow buffer 1 (see recipe)

Flow buffer 2 (see recipe)

Round bottom tubes (5 mL) (Fisher Scientific, #14–961–10A)

Filter top round bottom tubes (Fisher Scientific, #352235)

Hemocytometer

Centrifuge with 5 mL round bottom tube compatible bucket

Flow cytometer

Protocol steps

1. Important Notes
   - All steps are performed at room temperature, unless otherwise specified.
   - Experimental details are for collection of cells from 1 well of a 6-well plate.
   - Wash steps are performed by addition of 3 mL DPBS−/−, centrifugation at 200 × *g* for 3 min, and removal of supernatant.
2. Cell Collection

   2.1 hPSC-CM

   1. Gently wash cells in plate with 2 mL of DPBS−/−.
   2. Aspirate DPBS−/−.
   3. Add 1 mL of *Liberase/DNase solution*.
   4. Incubate cells at 37°C for 30 min.

      Between 20 and 30 minutes the sheet of cells should detach from the well as a cohesive monolayer. If sheet does not detach with gentle tapping, extend incubation by another 10 min.
   5. Add 1 mL of TrypLE.
   6. Incubate at 37°C for 3 min.
   7. Remove plate from incubator and tap the side of the plate with palm to disrupt the integrity of the monolayer.
   8. Gently triturate the cell suspension no more than three times using a P1000 microliter pipet.
   9. Further incubate the plate at 37°C for 2 min.
   10. Remove plate from incubator and tap the side of the plate with palm to disrupt the integrity of any clusters of cells.
   11. Gently triturate the cell suspension no more than three times using a P1000 microliter pipet.

12. Collect cells into 8 mL of growth media in a 15 mL conical tube, centrifuge at 200 × *g* for 5 min.

13. Aspirate supernatant.

14. Resuspend the pellet by finger-flicking the tube, then slowly triturate in 6 mL of DPBS−/−, perform cell count with trypan blue exclusion.

    If low cell recovery: ensure slow, gentle trituration and collection. Under these conditions, viability should be >90%.

2.2 hPSC

1. Gently wash cells with 2 mL of DPBS−/−.

2. Aspirate DPBS−/−.

3. Add 1 mL of Accutase.

4. Incubate cells for 4 – 6 min.

5. Tap the side of the plate with palm to disrupt the integrity of the monolayer.

   If no holes form in the monolayer, extend incubation with Accutase another 2 min.

6. Collect cells by gentle trituration into 1 mL of stem cell basal media (*e.g.* DMEM/F12) in 15 mL conical tube, centrifuge at 200 × *g* for 5 min.

7. Aspirate supernatant.

8. Resuspend the pellet by finger-flicking the tube, then slowly triturate in 4 mL of DPBS−/−, perform cell count with trypan blue exclusion.

   If low cell recovery: ensure slow, gentle trituration and collection. Under these conditions, viability should be >90%.

3. Cell Labeling and Preparation for Flow Cytometry

3.1 Fixation and Permeabilization

1. Place 1×10^6 cells in each 5 mL round bottom tube.

2. Centrifuge at 200 × *g* for 5 min, remove supernatant, and resuspend the cell pellets in 100 μL of *fixation solution* with gentle vortexing to ensure solution and cells are well-mixed.

3. For gentle agitation, place tubes on rocker for 20 min.

4. Wash 2x.

5. Resuspend pellets in 100 μL of *flow buffer 1*, place tubes on rocker for 15 min to begin permeabilization and blocking.

3.2 Antibody Labeling

1. Add appropriate amount of primary antibody to each tube.

   All primary antibodies should be titrated prior to initiation of an experiment (See Support Protocol 1). Isotype controls should be added at an equal mass to primary antibody.

2. Place tubes on rocker for 45 min.

3. Wash 2x.

4. *If secondary antibody is required*, resuspend pellets in 100 μL of *flow buffer 1*, add secondary antibody. Place tubes on rocker for 30 min followed by wash 2x.

3.3 Resuspension of Cells for Cytometer

1. Resuspend pellets in 450 μL of *flow buffer 2*.

2. Wet 35 μm mesh filter cap on round bottom tube by placing 40 μL of *flow buffer 2* on top of the filter, then gently pass cell suspension through cap by holding the tip of the pipette ~2mm above the mesh and allowing gravity to pull through droplets.

3.4 Acquire data using a Flow Cytometer – Fixed cells

1. While collecting data, adjust forward and side scatter laser settings to manipulate the distribution of cells and debris within the scatterplot. Greater than 75% of events should be within cell gate (Figure 2A).

   If cytometer has an adjustable flow rate, perform this step on a slower setting to minimize sample consumption prior to data acquisition. As the ability to interpret collected data relies on the capacity to gate on single cells, care should be taken in selecting the appropriate laser settings.

2. Fluorophore laser settings should be based on the fluorophore signal from the cell gate of the isotype control sample – the signal should be centered within the log values of 1×10^2 to 1×10^3 (Figure 2B).

3. Acquire 10,000 events for population of interest per experimental sample.

ALTERNATE PROTOCOL 1

PROTOCOL FOR ROUTINE ASSESSMENT OF FIXED hPSC/hPSC-CM: MULTI-WELL PLATE FORMAT

This SOP is the outcome of applying the fit-for-purpose development workflow for a flow cytometry protocol to assess TNNI3- and TNNT2-positivity within cultures of hPSC-CM.

We provide stepwise instructions for cell collection, cell labeling, and preparation for flow cytometry to assess the cardiomyocyte content in hPSC-CM differentiation cultures. This protocol utilizes round bottom 96-well plates for cell labeling and preparation for flow cytometry, which is advantageous when titrating antibodies or analyzing multiple hPSC derivatives.

Materials—HyPure WFI Quality Water (sterile water) (HyClone, #SH30221.17)

Dulbecco's phosphate buffered saline, Ca^{2+}/Mg^{2+} free (1xDPBS −/−) (Sigma-Aldrich, #D8537)

RPMI 1640 medium (Thermo Fisher Scientific, #11875–093)

Liberase-TH (Sigma-Aldrich, #5401135001)

DNase 1 (Sigma-Aldrich, #10104159001)

Liberase/DNase solution (see recipe)

TrypLE express enzyme (1X), phenol red (Thermo Fisher Scientific, #12605–010)

Trypan blue solution, 0.4% (Thermo Fisher Scientific, #15250–061)

Accutase (Innovative Cell Tech., #AT104)

Stem cell basal media (based on individual stem cell culture system)

16% Formaldehyde (w/v), methanol-free (Thermo Fisher Scientific, #28906)

Fixation solution (see recipe)

Bovine serum albumin (Sigma-Aldrich, #A7906)

Saponin (Sigma-Aldrich, #47036)

Flow buffer 1 (see recipe)

Flow buffer 2 (see recipe)

96-well round bottom plate (Fisher Scientific, #07–200–95)

Filter top round bottom tubes (Fisher Scientific, #352235)

Hemocytometer

Centrifuge with plate adapter

Flow cytometer

Protocol steps

1. Important Notes

- All steps are performed at room temperature, unless otherwise specified.
- Wash steps are performed by addition of 200 μL DPBS−/−, centrifugation at 200 × *g* for 3 min, and removal of supernatant.

2. Cell Collection

2.1 hPSC-CM

1. Gently wash cells in plate with 2 mL of DPBS−/−.

2. Aspirate DPBS−/−.

3. Add 1 mL of Liberase/DNase solution.

4. Incubate cells at 37°C for 30 min.

Between 20 and 30 minutes the sheet of cells should detach from the well as a cohesive monolayer. If sheet does not detach with gentle tapping, extend incubation by another 10 min.

5. Add 1 mL of TrypLE.

6. Incubate at 37°C for 3 min.

7. Remove plate from incubator and tap the side of the plate with your palm to disrupt the integrity of the monolayer.

8. Gently triturate the cell suspension no more than three times using a P1000 microliter pipet.

9. Further incubate the plate at 37°C for 2 min.

10. Remove plate from incubator and tap the side of the plate with your palm to disrupt the integrity of any clusters of cells.

11. Gently triturate the cell suspension no more than three times using a P1000 microliter pipet.

12. Collect cells into 8 mL of growth media in a 15 mL conical tube, centrifuge at 200 × *g* for 5 min.

13. Aspirate supernatant.

14. Resuspend the pellet by finger-flicking the tube, then slowly triturate in 6 mL of DPBS−/−, perform cell count with trypan blue exclusion.

If low cell recovery: ensure slow, gentle trituration and collection. Under these conditions, viability should be >90%.

2.2 hPSC

1. Gently wash cells with 2 mL of DPBS−/−.

2. Aspirate DPBS−/−.

3. Add 1 mL of Accutase.
4. Incubate cells for 4 – 6 min.
5. Tap the side of the plate with palm to disrupt the integrity of the monolayer.

   If no holes form in the monolayer, extend incubation with Accutase another 2 min.
6. Collect cells by gentle trituration into 1 mL of stem cell basal media (*e.g.* DMEM/F12) in 15 mL conical tube, centrifuge at 200 × *g* for 5 min.
7. Aspirate supernatant.
8. Resuspend the pellet by finger-flicking the tube, then slowly triturate in 4 mL of DPBS−/−, perform cell count with trypan blue exclusion.

   If low cell recovery: ensure slow, gentle trituration and collection. Under these conditions, viability should be >90%.

3. Cell Labeling and Preparation for Flow Cytometry

3.1 Fixation and Permeabilization

1. Place 1×10^6 cells in each well of a 96-well plate.
2. Centrifuge at 200 × *g* for 5 min, remove supernatant, and resuspend the cell pellets in 100 μL of *fixation solution*.
3. For gentle agitation, place plate on rocker for 20 min.
4. Wash 3x.
5. Resuspend pellets in 100 μL of *flow buffer 1*, place plate on rocker for 15 min to begin permeabilization and blocking.

3.2 Antibody Labeling

1. Add appropriate amount of primary antibody to each well.
2. Place plate on rocker for 45 min.
3. Wash 3x.
4. If secondary antibody is required, resuspend pellets in 100 μL of flow buffer 1, add secondary antibody. Place tubes on rocker for 30 min, followed by wash 3x.

3.3 Resuspension of Cells for Cytometer

1. Resuspend pellets in 200 μL of *flow buffer 2*.
2. Wet 35 μm mesh filter cap on round bottom tube by placing 40 μL of *flow buffer 2* on top of the filter, then gently pass cell

suspension through cap by holding the tip of the pipette ~2mm above the mesh and allowing gravity to pull through droplets.

3. Add an additional 250 μL of *flow buffer 2* to each well to ensure all cells have been collected.

4. Then, gently pass cell suspension through cap by holding the tip of the pipette ~2mm above the mesh and allowing gravity to pull through droplets.

3.4 Acquire data using a Flow Cytometer – Fixed cells

1. While collecting data, adjust forward and side scatter laser settings to manipulate the distribution of cells and debris within the scatterplot. Greater than 75% of events should be within cell gate (Figure 2A).

   If cytometer has an adjustable flow rate, perform this step on a slower setting to avoid using up sample before data acquisition. As the ability to interpret collected data relies on the capacity to gate on single cells, much care should be taken in selecting the correct laser settings.

2. Fluorophore laser settings should be based on the fluorophore signal from the cell gate of the isotype control sample – the signal should be centered within the log values of 1×10^2 to 1×10^3 (Figure 2B).

3. Acquire 10,000 events for population of interest per experimental sample.

SUPPORT PROTOCOL 1

TITRATION PROTOCOL FOR FIXED hPSC/hPSC-CM

This antibody titration protocol is provided in support of **Basic Protocol 1** and **Alternate Protocol 1**. Titration is performed to determine the antibody-to-cell ratio that produces the maximum intensity difference between negative and positive cells. Stepwise instructions for cell collection, cell labeling, and preparation for flow cytometry are provided to assess the optimal amount of antibody to use. Titration should be performed on both hPSC and hPSC-CM for each antibody lot. Due to the large number of experimental conditions to be tested in parallel, the protocol describes how to perform these steps in a 96-well plate format. The experiment can be adapted for use in 5mL round bottom tubes.

Materials—HyPure WFI Quality Water (sterile water) (HyClone, #SH30221.17)

Dulbecco's phosphate buffered saline, Ca^{2+}/Mg^{2+} free (1xDPBS −/−) (Sigma-Aldrich, #D8537)

RPMI 1640 medium (Thermo Fisher Scientific, #11875–093)

Liberase-TH (Sigma-Aldrich, #5401135001)

DNase 1 (Sigma-Aldrich, #10104159001)

Liberase/DNase solution (see recipe)

TrypLE express enzyme (1X), phenol red (Thermo Fisher Scientific, #12605–010)

Trypan blue solution, 0.4% (Thermo Fisher Scientific, #15250–061)

Accutase (Innovative Cell Tech., #AT104)

Stem cell basal media (based on individual stem cell culture system)

16% Formaldehyde (w/v), methanol-free (Thermo Fisher Scientific, #28906)

Fixation solution (see recipe)

Bovine Serum Albumin (Sigma-Aldrich, #A7906)

Saponin (Sigma-Aldrich, #47036)

Flow buffer 1 (see recipe)

Flow buffer 2 (see recipe)

96-well round bottom plate (Fisher Scientific, #07–200–95)

Filter top round bottom tubes (Fisher Scientific, #352235)

Hemocytometer

Centrifuge with plate adapter

Flow cytometer

Protocol steps

1. Important Notes

   - All steps are performed at room temperature, unless otherwise specified.
   - Experimental details are for collection of cells from 1 well of 6-well plate.
   - Wash steps are performed by addition of 200 μL DPBS−/−, centrifugation at 200 × *g* for 3 min, and removal of supernatant.
   - At least 4 amounts of primary antibody should be tested on both hPSC and hPSC-CM with corresponding amounts of isotype control.

     This requires ≥ 8×10^6 hPSC and ≥ 8×10^6 hPSC-CM per antibody lot to be titrated. Multiple wells may need to be collected to obtain this

number of cells. Antibodies which share an isotype can share a set of isotype controls.

2. Cell Collection

2.1 hPSC-CM

1. Gently wash cells in plate with 2 mL of DPBS−/−.

2. Aspirate DPBS−/−.

3. Add 1 mL of *Liberase/DNase solution*.

4. Incubate cells at 37°C for 30 min.

Between 20 and 30 minutes the sheet of cells should detach from the well as a cohesive monolayer. If sheet does not detach with gentle tapping, extend incubation by another 10 min.

5. Add 1 mL of TrypLE.

6. Incubate at 37°C for 3 min.

7. Remove plate from incubator and tap the side of the plate with palm to disrupt the integrity of the monolayer.

8. Gently triturate the cell suspension no more than three times using a P1000 microliter pipet.

9. Further incubate the plate at 37°C for 2 min.

10. Remove plate from incubator and tap the side of the plate with palm to disrupt the integrity of any clusters of cells.

11. Gently triturate the cell suspension no more than three times using a P1000 microliter pipet.

12. Collect cells into 8 mL of growth media in 15 mL conical tube, centrifuge at 200 × *g* for 5 min.

13. Aspirate supernatant.

14. Resuspend the pellet by finger-flicking the tube, then slowly triturate in 6 mL of DPBS−/−, perform cell count with trypan blue exclusion.

If low cell recovery: ensure slow, gentle trituration and collection. Under these conditions, viability should be >90%.

2.2 hPSC

1. Gently wash cells with 2 mL of DPBS−/−.

2. Aspirate DPBS−/−.

3. Add 1 mL of Accutase.

4. Incubate cells for 4 – 6 min.

5. Tap the side of the plate with palm to disrupt the integrity of the monolayer.

   If no holes form in the monolayer, extend incubation with Accutase another 2 min.

6. Collect cells by gentle trituration into 1 mL of stem cell basal media (*e.g.* DMEM/F12) in 15 mL conical tube, centrifuge at 200 × *g* for 5 min.

7. Aspirate supernatant.

8. Resuspend the pellet by finger-flicking the tube, then slowly triturate in 4 mL of DPBS−/−, perform cell count with trypan blue exclusion.

   If low cell recovery: ensure slow, gentle trituration and collection. Under these conditions, viability should be >90%.

3. Cell Labeling and Preparation for Flow Cytometry

3.1 Fixation and Permeabilization

1. Place 1×10^6 cells in each well of a 96-well plate.

2. Centrifuge at 200 × *g* for 5 min, remove supernatant, and resuspend the cell pellets in 100 μL of *fixation solution* with gentle vortexing to ensure solution and cells are well-mixed.

3. For gentle agitation, place on rocker for 20 min.

4. Wash 3x.

5. Resuspend pellets in 100 μL of *flow buffer 1*, incubate for 15 min to begin permeabilization and blocking.

3.2 Antibody Labeling

1. Add a range of amount primary antibody amounts to each hPSC and hPSC-CM (at least 4 amounts).

   Ideal amounts of antibody can range drastically - use previous titration results, published literature, or manufacturer's recommendations as a starting point. It is recommended to test 0.25x, 0.5x, 1x, 1.5–2x of manufacturer's recommendations. Results are best when the total amount of antibody for all samples is prepared as a single master stock at a concentration such that no less than 1.5 μL is added to an individual sample. All conditions for titration samples should have matching isotype control samples.

2. Incubate for 45 min.

3. Wash 3x.

4. If secondary antibody is required, resuspend pellets in 100 μL of flow buffer 1, add secondary antibody. Place tubes on rocker for 30 min, followed by wash 3x.

3.3 Resuspension of Cells for Cytometer

1. Resuspend pellets in 200 μL of *flow buffer 2*.
2. Wet 35 μm mesh filter cap on round bottom tube by placing 40 μL of *flow buffer 2* on top of the filter, then gently pass cell suspension through cap by holding the tip of the pipette ~2mm above the mesh and allowing gravity to pull through droplets.
3. Add an additional 250 μL of *flow buffer 2* to each well to ensure all cells have been collected.
4. Then, gently pass cell suspension through cap by holding the tip of the pipette ~2mm above the mesh and allowing gravity to pull through droplets.

3.4 Acquire data using a Flow Cytometer – Fixed cells

1. While collecting data, adjust forward and side scatter laser settings to manipulate the distribution of cells and debris within the scatterplot. Greater than 75% of events should be within cell gate (Figure 2A).

   If cytometer has an adjustable flow rate, perform this step on a slower setting to avoid using up sample before data acquisition. As the ability to interpret collected data relies on the capacity to gate on single cells, much care should be taken in selecting the correct laser settings.
2. Fluorophore laser settings should be based on the fluorophore signal from the cell gate of the isotype control sample – the signal should be centered within the log values of 1×10^2 to 1×10^3 (Figure 2B).
3. Acquire 10,000 events for population of interest per experimental sample.

SUPPORT PROTOCOL 2

EPITOPE COMPETITION PROTOCOL FOR FIXED hPSC/hPSC-CM

This epitope competition protocol is provided in support of **Basic Protocol 1** and **Alternate Protocol 1**. A competition protocol tests the specificity of an antibody for its epitope (if the epitope is reported) by comparing signal from naïve antibody to antibody pre-incubated with peptide antigen. In this manner, a diminution or ablation of signal caused by incubation with the peptide antigen can be indicative of specificity for the reported epitope. This protocol should be performed using a titrated amount of antibody determined using **Support Protocol 1**. Epitope competition needs only to be performed on cell types for which positive

signal is observed. A caveat of this approach is that it uses peptides, which may lack the secondary or tertiary structure of the native epitope. Therefore, though successful blocking is indicative of specificity, the failure to block should not be considered strong evidence that an antibody lacks specificity. Due to the large number of experimental conditions to be tested in parallel, the protocol provided is in a 96-well plate. The experiment can be adapted for use in 5mL round bottom tubes.

Materials—HyPure WFI Quality Water (sterile water) (HyClone, #SH30221.17)

Dulbecco's phosphate buffered saline, Ca^{2+}/Mg^{2+} free (1xDPBS −/−) (Sigma-Aldrich, #D8537)

RPMI 1640 medium (Thermo Fisher Scientific, #11875–093)

Liberase-TH (Sigma-Aldrich, #5401135001)

DNase 1 (Sigma-Aldrich, #10104159001)

Liberase/DNase solution (see recipe)

TrypLE express enzyme (1X), phenol red (Thermo Fisher Scientific, #12605–010)

Trypan blue solution, 0.4% (Thermo Fisher Scientific, #15250–061)

Accutase (Innovative Cell Tech., #AT104)

Stem cell basal media (based on individual stem cell culture system)

16% Formaldehyde (w/v), methanol-free (Thermo Fisher Scientific, #28906)

Fixation solution (see recipe)

Bovine Serum Albumin (Sigma-Aldrich, #A7906)

Saponin (Sigma-Aldrich, #47036)

Flow buffer 1 (see recipe)

Flow buffer 2 (see recipe)

96-well round bottom plate (Fisher Scientific, #07–200–95)

Filter top round bottom tubes (Fisher Scientific, #352235)

Hemocytometer

Centrifuge with plate adapter

Flow cytometer

Protocol steps

1. Important Notes

- All steps are performed at room temperature, unless otherwise specified.
- Experimental details are for collection of cells from 1 well of 6-well plate.
- Wash steps are performed by addition of 200 μL DPBS−/−, centrifugation at 200 × *g* for 3 min, and removal of supernatant.
- At least 3 amounts of peptide-to-antibody should be tested on the positive control cell type. One well should contain a positive control (no antigen peptide) and one well a corresponding amount of isotype control. In addition, we recommend the use of a negative control (*i.e.* a different peptide sequence).

  The inclusion of additional peptide controls should be considered if the epitope is modified by post-translational modification or has high sequence conservation with other proteins that are present. This requires ≥ 6×10^6 cells per antibody to be tested. Multiple wells may need to be collected to obtain this number of cells. If testing multiple antibodies, the antigen for one antibody can be used as a negative control antigen for a different antibody, provided they target different epitopes.

2. Cell Collection

2.1 hPSC-CM

1. Gently wash cells in plate with 2 mL of DPBS−/−.

2. Aspirate DPBS−/−.

3. Add 1 mL of *Liberase/DNase solution*.

4. Incubate cells at 37°C for 30 min.

Between 20 and 30 minutes the sheet of cells should detach from the well as a cohesive monolayer. If sheet does not detach with gentle tapping, extend incubation by another 10 min.

5. Add 1 mL of TrypLE.

6. Incubate at 37°C for 3 min.

7. Remove plate from incubator and tap the side of the plate with palm to disrupt the integrity of the monolayer.

8. Gently triturate the cell suspension no more than three times using a P1000 microliter pipet.

9. Further incubate the plate at 37°C for 2 min.

10. Remove plate from incubator and tap the side of the plate with palm to disrupt the integrity of any clusters of cells.

11. Gently triturate the cell suspension no more than three times using a P1000 microliter pipet.

12. Collect cells into 8 mL of growth media in 15 mL conical tube, centrifuge at 200 × *g* for 5 min.

13. Aspirate supernatant.

14. Resuspend the pellet by finger-flicking the tube, then slowly triturate in 6 mL of DPBS−/−, perform cell count with trypan blue exclusion.

    If low cell recovery: ensure slow, gentle trituration and collection. Under these conditions, viability should be >90%.

2.2 hPSC

1. Gently wash cells with 2 mL of DPBS−/−.

2. Aspirate DPBS−/−.

3. Add 1 mL of Accutase.

4. Incubate cells for 4 – 6 min.

5. Tap the side of the plate with palm to disrupt the integrity of the monolayer.

   If no holes form in the monolayer, extend incubation with Accutase another 2 min.

6. Collect cells by gentle trituration into 1 mL of stem cell basal media (*e.g.* DMEM/F12) in 15 mL conical tube, centrifuge at 200 × *g* for 5 min.

7. Aspirate supernatant.

8. Resuspend the pellet by finger-flicking the tube, then slowly triturate in 4 mL of DPBS−/−, perform cell count with trypan blue exclusion.

   If low cell recovery: ensure slow, gentle trituration and collection. Under these conditions, viability should be >90%.

3. Cell Labeling and Preparation for Flow Cytometry

3.1 Pre-incubation of Antibody with Antigen Peptides

1. Prepare various molar ratios of peptide-to-antibody (*e.g.* 100:1, 1000:1, 10,000:1) in *flow buffer 1* maintaining the same total volume for all conditions.

For IgG antibodies, an approximate molecular mass of 150,000 Da can be used to calculate molar ratios. Treat isotype control and the no-peptide positive control identically to the other samples (i.e. dilute and pre-incubate albeit with no peptide).

2. Incubate samples for 45 minutes.

   This step should be performed while cells are being fixed and permeabilized.

3.2 Fixation and Permeabilization

1. Place 1×10^6 cells in each well of a 96-well plate.
2. Centrifuge at 200 × *g* for 5 min, remove supernatant, and resuspend the cell pellets in 100 μL of *fixation solution* with gentle vortexing to ensure solution and cells are well-mixed.
3. For gentle agitation, place on rocker for 20 min.
4. Wash 3x.
5. Resuspend pellets in 100 μL of *flow buffer 1*, incubate for 15 min to begin permeabilization and blocking.

3.3 Antibody Labeling

1. Add the pre-incubated antibody solutions to the pre-designated wells.
2. Incubate for 45 min.
3. Wash 3x.
4. If secondary antibody is required, resuspend pellets in 100 μL of flow buffer 1, add secondary antibody. Place tubes on rocker for 30 min, followed by wash 3x.

3.4 Resuspension of Cells for Cytometer

1. Resuspend pellets in 200 μL of *flow buffer 2*.
2. Wet 35 μm mesh filter cap on round bottom tube by placing 40 μL of *flow buffer 2* on top of the filter, then gently pass cell suspension through cap by holding the tip of the pipette ~2mm above the mesh and allowing gravity to pull through droplets.
3. Add an additional 250 μL of *flow buffer 2* to each well to ensure all cells have been collected.
4. Then, gently pass cell suspension through cap by holding the tip of the pipette ~2mm above the mesh and allowing gravity to pull through droplets.

3.5 Acquire data using a Flow Cytometer – Fixed cells

1. While collecting data, adjust forward and side scatter laser settings to manipulate the distribution of cells and debris within the scatterplot. Greater than 75% of events should be within cell gate (Figure 2A).

   If cytometer has an adjustable flow rate, perform this step on a slower setting to avoid using up sample before data acquisition. As the ability to interpret collected data relies on the capacity to gate on single cells, much care should be taken in selecting the correct laser settings.

2. Fluorophore laser settings should be based on the fluorophore signal from the cell gate of the isotype control sample – the signal should be centered within the log values of 1×10^2 to 1×10^3 (Figure 2B)

3. Acquire 10,000 events for population of interest per experimental sample.

SUPPORT PROTOCOL 3

PROTOCOL FOR DEMONSTRATING RANGE OF USE ON FIXED hPSC/hPSC-CM

Mixed population experiments demonstrate the capacity of the antibody and protocol to discriminate between positive and negative cells within the same experimental sample, which is the ultimate test of suitability for assessing population heterogeneity. To perform this experiment, populations of cells are mixed together in known ratios immediately following their collection.

Materials—HyPure WFI Quality Water (sterile water) (HyClone, #SH30221.17)

Dulbecco's phosphate buffered saline, Ca^{2+}/Mg^{2+} free (1xDPBS −/−) (Sigma-Aldrich, #D8537)

RPMI 1640 medium (Thermo Fisher Scientific, #11875–093)

Liberase-TH (Sigma-Aldrich, #5401135001)

DNase 1 (Sigma-Aldrich, #10104159001)

Liberase/DNase solution (see recipe)

TrypLE express enzyme (1X), phenol red (Thermo Fisher Scientific, #12605–010)

Trypan blue solution, 0.4% (Thermo Fisher Scientific, #15250–061)

Accutase (Innovative Cell Tech., #AT104)

Stem cell basal media (based on individual stem cell culture system)

16% Formaldehyde (w/v), methanol-free (Thermo Fisher Scientific, #28906)

Fixation solution (see recipe)

Bovine Serum Albumin (Sigma-Aldrich, #A7906)

Saponin (Sigma-Aldrich, #47036)

Flow buffer 1 (see recipe)

Flow buffer 2 (see recipe)

Round bottom tubes (5 mL) (Fisher Scientific, #14–961–10A)

Filter top round bottom tubes (Fisher Scientific, #352235)

Hemocytometer

Centrifuge with 5 mL round bottom tube compatible bucket

Flow cytometer

Protocol steps

1. Important Notes

- All steps are performed at room temperature, unless otherwise specified.
- Experimental details are for collection of cells from 1 well of 6-well plate.
- Wash steps are performed by addition of 3 mL DPBS−/−, centrifugation at 200 × *g* for 3 min, and removal of supernatant.

  At least 3×10^6 cells of hPSC and hPSC-CM are required per antibody to be tested for this experiment.

2. Cell Collection

2.1 hPSC-CM

1. Gently wash cells in plate with 2 mL of DPBS−/−.

2. Aspirate DPBS−/−.

3. Add 1 mL of *Liberase/DNase solution*.

4. Incubate cells at 37°C for 30 min.

Between 20 and 30 minutes the sheet of cells should detach from the well as a cohesive monolayer. If sheet does not detach with gentle tapping, extend incubation by another 10 min.

5. Add 1 mL of TrypLE.

6. Incubate at 37°C for 3 min.

7. Remove plate from incubator and tap the side of the plate with palm to disrupt the integrity of the monolayer.
8. Gently triturate the cell suspension no more than three times using a P1000 microliter pipet.
9. Further incubate the plate at 37°C for 2 min.
10. Remove plate from incubator and tap the side of the plate with palm to disrupt the integrity of any clusters of cells.
11. Gently triturate the cell suspension no more than three times using a P1000 microliter pipet.
12. Collect cells into 8 mL of growth media in 15 mL conical tube, centrifuge at 200 × *g* for 5 min.
13. Aspirate supernatant.
14. Resuspend the pellet by finger-flicking the tube, then slowly triturate in 6 mL of DPBS−/−, perform cell count with trypan blue exclusion.

    If low cell recovery: ensure slow, gentle trituration and collection. Under these conditions, viability should be >90%.

2.2 hPSC

1. Gently wash cells with 2 mL of DPBS−/−.
2. Aspirate DPBS−/−.
3. Add 1 mL of Accutase.
4. Incubate cells for 4 – 6 min.
5. Tap the side of the plate with palm to disrupt the integrity of the monolayer.

    If no holes form in the monolayer, extend incubation with Accutase another 2 min.
6. Collect cells by gentle trituration into 1 mL of stem cell basal media (*e.g.* DMEM/F12) in 15 mL conical tube, centrifuge at 200 × *g* for 5 min.
7. Aspirate supernatant.
8. Resuspend the pellet by finger-flicking the tube, then slowly triturate in 4 mL of DPBS−/−, perform cell count with trypan blue exclusion.

    If low cell recovery: ensure slow, gentle trituration and collection. Under these conditions, viability should be >90%.

3. Cell Labeling and Preparation for Flow Cytometry

3.1 Fixation and Permeabilization

1. Prepare the following six tubes for the mixed-population experiment.
   a. 1.00 ×10^6 hPSC-CM
   b. 0.75 ×10^6 hPSC-CM, 0.25 ×10^6 hPSC
   c. 0.50 ×10^6 hPSC-CM, 0.50 ×10^6 hPSC (for isotype control)
   d. 0.50 ×10^6 hPSC-CM, 0.50 ×10^6 hPSC
   e. 0.25 ×10^6 hPSC-CM, 0.75 ×10^6 hPSC
   f. 1.00×10^6 hPSC
2. Centrifuge at 200 × *g* for 5 min, remove supernatant, and resuspend the cell pellets in 100 μL of *fixation solution* with gentle vortexing to ensure solution and cells are well-mixed.
3. For gentle agitation, place on rocker for 20 min.
4. Wash 2x.
5. Resuspend pellets in 100 μL of *flow buffer 1*, incubate for 15 min to begin permeabilization and blocking.

3.2 Antibody Labeling

1. Add appropriate amount of primary antibody to each tube.

   All primary antibodies should be titrated prior to initiation of an experiment (See Support Protocol 1). Isotype controls should be added at an equal mass to primary antibody.
2. Incubate for 45 min.
3. Wash 2x.
4. If secondary antibody is required, resuspend pellets in 100 μL of flow buffer 1, add secondary antibody. Place tubes on rocker for 30 min, followed by wash 2x.

3.3 Resuspension of Cells for Cytometer

1. Resuspend pellets in 450 μL of *flow buffer 2*.
2. Wet 35 μm mesh filter cap on round bottom tube by placing 40 μL of *flow buffer 2* on top of the filter, then gently pass cell suspension through cap by holding the tip of the pipette ~2mm above the mesh and allowing gravity to pull through droplets.

3.4 Acquire data using a Flow Cytometer – Fixed cells

1. While collecting data, adjust forward and side scatter laser settings to manipulate the distribution of cells and debris within the

scatterplot. Greater than 75% of events should be within cell gate (Figure 2A).

If cytometer has an adjustable flow rate, perform this step on a slower setting to avoid using up sample before data acquisition. As the ability to interpret collected data relies on the capacity to gate on single cells, much care should be taken in selecting the correct laser settings.

2. Fluorophore laser settings should be based on the fluorophore signal from the cell gate of the isotype control sample – the signal should be centered within the log values of 1×10^2 to 1×10^3 (Figure 2B).

3. Acquire 10,000 events for population of interest per experimental sample.

REAGENTS AND SOLUTIONS

Liberase-TH stock solution – Store as 40 μL aliquots in −20°C freezer (shelf life of 3 months)

Sterile water containing:

13 Unit/mL

DNase I stock solution – Store as 10 μL aliquots in −20°C freezer

Sterile water containing:

50,000 Unit/mL

Liberase/DNase solution – make fresh from Liberase-TH and DNase aliquots

RPMI 1640 medium containing:

0.5U/mL Liberase-TH

50U/mL DNase I

Fixation solution – make fresh from 16% formaldehyde stock (Thermo Fisher Scientific, #28906)

DPBS −/− containing:

2% formaldehyde

Bovine serum albumin (BSA) stock solution – Store as 1 mL aliquots in −20°C freezer

DPBS −/− containing:

10% w/v Bovine serum albumin

Saponin stock solution – Store as 0.25 mL aliquots in −20°C freezer

DPBS −/− containing:

10% w/v Saponin

Flow buffer 1 – make fresh from BSA and saponin aliquots

DPBS −/− containing:

0.5% w/v BSA

0.5% w/v Saponin

Flow buffer 2 – make fresh from BSA aliquot

DPBS −/− containing:

0.5% w/v BSA

COMMENTARY

Critical Parameters

1. Implementation of the Validated Protocol

1.1 Timing of Experiments:

It is best to prepare all buffers and analyze cells on the day of experiment. However, in the event that this is not possible, some delay is tolerable between the fixation, staining, and analysis. For cells that are fixed and then stained/analyzed at a later date (up to one week), store cells at 4°C in DPBS −/− w/0.5% (w/v) BSA. Alternatively, some cell types tolerate being stored in 0.01% formaldehyde in DPBS−/−. Importantly, cells stained with a fluorophore should be stored in the dark. Wait until the day of analysis to process cells through a filter top round bottom tube.

1.2 Cell Collection:

Flow cytometry requires single cells suspensions. For cells that are adhered to a culture plate, this requirement can pose specific challenges. In general, it is recommended that cells are triturated minimally and as slowly as necessary to disperse the cells. Trituration with a serological pipet or tapping on plates are more tolerated albeit less effective means of cell dispersion than using a P1000 pipette tip. Tapping plates with your palm can assist dispersion without compromising viability. Routinely assess viability post-collection by trypan blue exclusion using a hemocytometer. Although we have found assessment by trypan blue to be sufficient for routine

analysis, flow cytometry-based assays provide a more rigorous assessment of cellular viability.

1.3 Gating Strategies and Number of Events to Collect:

While collecting data, forward and side scatter laser settings are adjusted to manipulate the distribution of cells and debris within the scatterplot. Greater than 75% of events should be within the cell gate (Figure 2A). It is recommended that if the cytometer has an adjustable flow rate to perform this step on a slower setting to avoid using up sample before data acquisition. As the ability to interpret collected data relies on the capacity to gate on single cells, much care should be taken in selecting the correct laser settings. Fluorophore laser settings should be based on the fluorophore signal from the cell gate of the isotype control sample – the signal should be centered within the log values of 1×10^2 to 1×10^3 (Figure 2B). Per experimental sample, 10,000 events should be collected.

1.4 Recording and Publishing Experimental Details:

Recording and reporting key sample processing and reagent details are critical to ensuring the experiments and interpretations can be replicated among laboratories. Good record keeping begins with recording details for each experiment. The information necessary to replicate a flow cytometry experiment are summarized in Table 1 and are provided to facilitate other laboratories to record and report a similar level of experimental detail. This table can be included as supplemental information for most scientific journals.

2. Establishing the Fitness-for-Purpose of a Protocol

2.1 Marker Selection:

When selecting a marker, it is critical to consider the purpose of the experiment. In the case where cell-type identity or population heterogeneity is being assessed, the marker must be capable of distinguishing between cell types of interest within the context of the experiment. Critically, a protein that serves as a marker of cell-type identity in one context may not be specific to that cell type in another context. It is essential that during marker selection, the presence of the marker among cell types is considered. Although literature review can be informative for this purpose, previous publications do not guarantee that a marker was fully justified or validated. Also, the user should consider how the experimental differences between published literature and their study may change the cell-type specificity. For these reasons, antibody-independent approaches are highly recommended (*e.g.* targeted mass spectrometry) to confirm the presence of the selected marker at the protein level.

2.2 Antibody Clone Selection:

For quantitative measurements, mAb are essential. Selecting an antibody clone deserves careful consideration of the identity of the reported epitope, as well as, how the antibody was generated. Testing more than one clone is advised, if available. Antibodies can be generated against peptides or proteins, and this can have implications for its use. Although some features of the epitope may not preclude its use for flow cytometry, they may affect interpretation of results. For example, if the epitope contains a site of post-translational modification (*e.g.* phosphorylation, acetylation), the biology of these modifications should be considered as their effect on epitope recognition by the antibody may not be predictable. The sequence identity or homology of an epitope to other proteins should be investigated. Often, the same antibody clone is available from different vendors. The format (*i.e.* conjugated, unconjugated to fluorophore), concentration, and purification scheme (*e.g.* Protein A/G versus affinity purification) can be important factors when selecting a particular formulation or vendor. Another important consideration is the isotype of the available antibodies, as this factor can influence the design of co-immunodetection experiments.

2.3 Antibody Screen and Protocol Selection:

Each antibody clone of interest should be tested with multiple sample preparation protocols using both positive and negative control cell types. Establishing how sample preparation reagents affect antibody binding is an important aspect of data interpretation. An **essential** control for antibody testing is the negative cell type control. This can be a cell type that does not contain the protein naturally, or a cell that has been manipulated (genetically or otherwise) to deplete the protein of interest. Using an antibody-independent method to confirm the absence of a protein in the negative cell type control is recommended. Though the application of an ideal amount of antibody (determined by titration) is necessary to fairly judge antibody performance, the results of the screen may indicate which combination of clones and/or sample preparation conditions are not worth pursuing (*e.g.* signal on the negative cell type control is stronger than signal on the positive control).

2.4 Antibody Titration:

Titration is a critical step in any flow cytometry protocol and is performed to determine the antibody-to-cell ratio that produces the maximum intensity difference between negative and positive cells. See Support Protocol 1 for detailed instructions.

2.5 Verifying Antibody Specificity:

To verify the specificity of an antibody, several types of experiments are possible to provide different types of evidence, each with their own advantages and limitations.

2.5.1 Epitope competition

See Support Protocol 2.

2.5.2 Immunofluorescent imaging

Immunofluorescent microscopy is another strategy for evaluating antibody specificity. Imaging offers the ability to visualize the cellular localization of the antibody. If the localization of a protein target is known, antibody binding in the expected localization pattern provides additional evidence of antibody specificity. Key considerations for this approach are that users should proceed with caution when relying on database annotations for subcellular localization information if they lack experimental verification. Users should recognize that subcellular localization of a protein can be cell-type or context-dependent. Quantitation of the percentage of a population that is positive for a protein by microscopy is challenging. Due to the high degree of cellular density in a well, cells may need to be passaged to obtain a density that enables delineation of discrete cells.

2.5.3 Co-immunodetection

Co-immunodetection experiments can be used to compare the subpopulations marked by two or more antibodies and the extent to which the marked populations overlap. Though a powerful technique to tease out the relationship between two clones or two subpopulations, co-immunodetection experiments are technically more challenging to plan and perform than routine single-color experiments. The antibodies included in a co-immunodetection experiment can be against different proteins or can be multiple clones against the same protein. Co-immunodetection experiments require careful consideration of the isotypes of antibodies and the secondary antibodies used for detection. The brighter fluorophore is typically used for detecting the protein that is less abundant. The use of conjugated primary antibodies can simplify experimental design. The use of multiple fluorophores in a flow cytometry experiment requires compensation controls to deal with the potential issue of overlapping fluorescent spectra.

2.5.4 Mixed population experiment

See Support Protocol 3.

Troubleshooting

Indentified Problem	Possible Cause	Suggested Remedy
Low percentage of events in SSC-A vs FSC-A gate	Poor cell viability (increased debris)	Cell dissociation solutions/enzymes (*e.g.* trypsin, collagenase, chelating agents), incubation times, and collection buffer may need to be altered for each cell type. In general, longer incubation in enzyme solutions and plate tapping is favorable to trituration for facilitating dispersion. Trituration can affect various cell types differently. We have found hPSC-CM to be very sensitive to trituration, whereas hPSC are more resilient.

Indentified Problem	Possible Cause	Suggested Remedy
	Suboptimal fixation or permeabilization conditions	Methanol-free formulations of formaldehyde (such as the product recommended here) demonstrate superior scatterplot characteristics. Fixation and permeabilization solutions and incubation times may need to be altered for each cell type, antibody, and target protein. Certain scenarios may require harsher fixation (*e.g.* methanol) and permeabilization (*e.g.* Triton X) than the conditions suggested here.
	Unsuitable laser setting	Increase FSC and SSC power. Appropriate laser settings should result in a high percentage of events that cluster away from debris and off the axes.
Incomplete separation of cells from debris	Old buffers	Make buffers fresh.
	Unsuitable laser setting	Increase FSC and SSC power. Appropriate laser settings should result in a high percentage of events that cluster away from debris and off the axes.

Understanding Results

An example of scatterplots and histograms that should be obtained by following **Basic Protocol 1** or **Alternate Protocol 1** are shown in Figure 3. The histograms from isotype controls on hPSC and hPSC-CM should line up with the anti-cardiac troponin hPSC histogram – these three samples are negative controls. The histograms from these three samples should be unimodal (*i.e.* a single, normal distribution) and should have the same median fluorescent intensity. The anti-cardiac troponin hPSC-CM histogram shows a rightward shift (*i.e.* an increase in median fluorescent intensity) relative to the other samples. The population to the right of the negative controls, denoted by the gate in Figure 3, are cardiac troponin-positive (*i.e.* hPSC-CM). Depending on the percent positivity and the antibody applied, there may not be a clear bimodal distribution.

Example data for multiple antibodies using **Support Protocol 1 – 3** can be found in Waas *et al.* (Waas et al., 2019).

For **Support Protocol 1**, incubation with increasing amount of antibodies should result in an increase in the median fluorescent intensity (*i.e.* a rightward shift) for the positive population. Results from **Support Protocol 1** should indicate that there is an amount of antibody for which there is a maximum difference in median fluorescent intensity between negative and positive cells. Therefore, titration must be performed on both hPSC and hPSC-CM.

For **Support Protocol 2**, incubation of antibody with the antigen peptide should result in a decrease in the median fluorescent intensity (*i.e.* a leftward shift) for the positive population. The diminution in signal may not be complete, but it should increase as the molar ratio of peptide to antibody increases. Results from **Support Protocol 2** are difficult to interpret, because a major caveat of this approach is that it uses peptides, which may lack the secondary or tertiary structure of the native epitope. Though successful blocking is indicative of specificity, the failure to block should not be considered strong evidence that an antibody lacks specificity.

For **Support Protocol 3**, the percentage of cells which are gated as positive should be proportional to the percentage of the mixed population that is hPSC-CM. The histograms for the mixed populations should be bimodal and the relative locations of the positive and negative populations should remain stable across the different samples. The percent positivity measured for each mixed population sample should be compared to the expected positivity, calculated based on the percentages of the pure, unmixed populations. An important metric to record for this experiment is the event rate on the flow cytometer, as it can help troubleshoot/interpret results. As the number of cells in each tube is equivalent, so should the flow rate. If there are differences in flow rate, this indicates that the cell counting was not accurate.

Time Considerations

Cell collection – The total time from plate to tube or plate is ~50 min for hPSC-CM. The total time to collect hPSC is ~20 min. With experience, and if comfortable, the hPSC can be collected during the 30 min Liberase-TH/DNase incubation.

Cell labeling – The time for cell labeling depends on whether a secondary antibody is required. Fixation through primary takes ~90 min, the requirement of a secondary adds ~45 min.

Sample preparation – Transferring samples to filter tubes takes around ~45 s/per sample.

Data acquisition – The set-up of the instrument and suitable laser settings takes ~5 min. Acquisition of data can take ~20 s/sample.

ACKNOWLEDGEMENT (mandatory for NIH, optional for all others)

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Significance Statement

Human pluripotent stem cell (hPSC) derivatives are valuable for a variety of applications, but reliable methods to determine cell-type identity in hPSC-derived cultures are lacking. Flow cytometry is apt for assessment of population heterogeneity, but standardized protocols are not yet available for most cell types. To facilitate the development of reliable and reproducible protocols, this Unit describes a workflow for establishing a 'fit-for-purpose' flow cytometry protocol for hPSC derivatives. Based on the application of this workflow, a validated protocol was developed for the assessment of cardiac troponin in hPSC-derived cardiomyocytes (hPSC-CM). Throughout the Unit, important concepts regarding antibody validation and data collection are discussed to enable users to develop rigorous protocols for any hPSC derivatives.

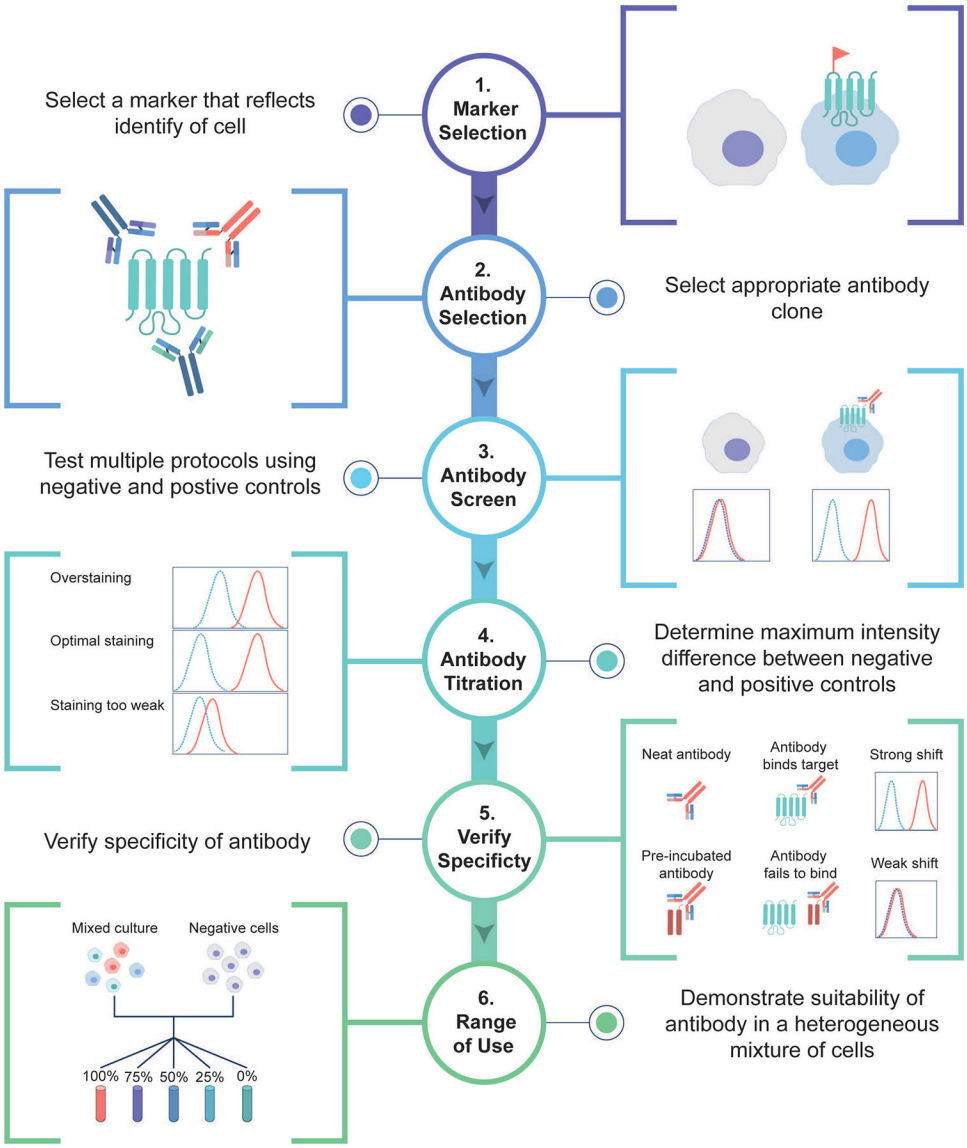

Figure 1.

Workflow for Establishing a Fit-for-Purpose Flow Cytometry Protocol. This workflow highlights critical steps when selecting and validating antibodies for the development of a flow cytometry assay to assess cells.

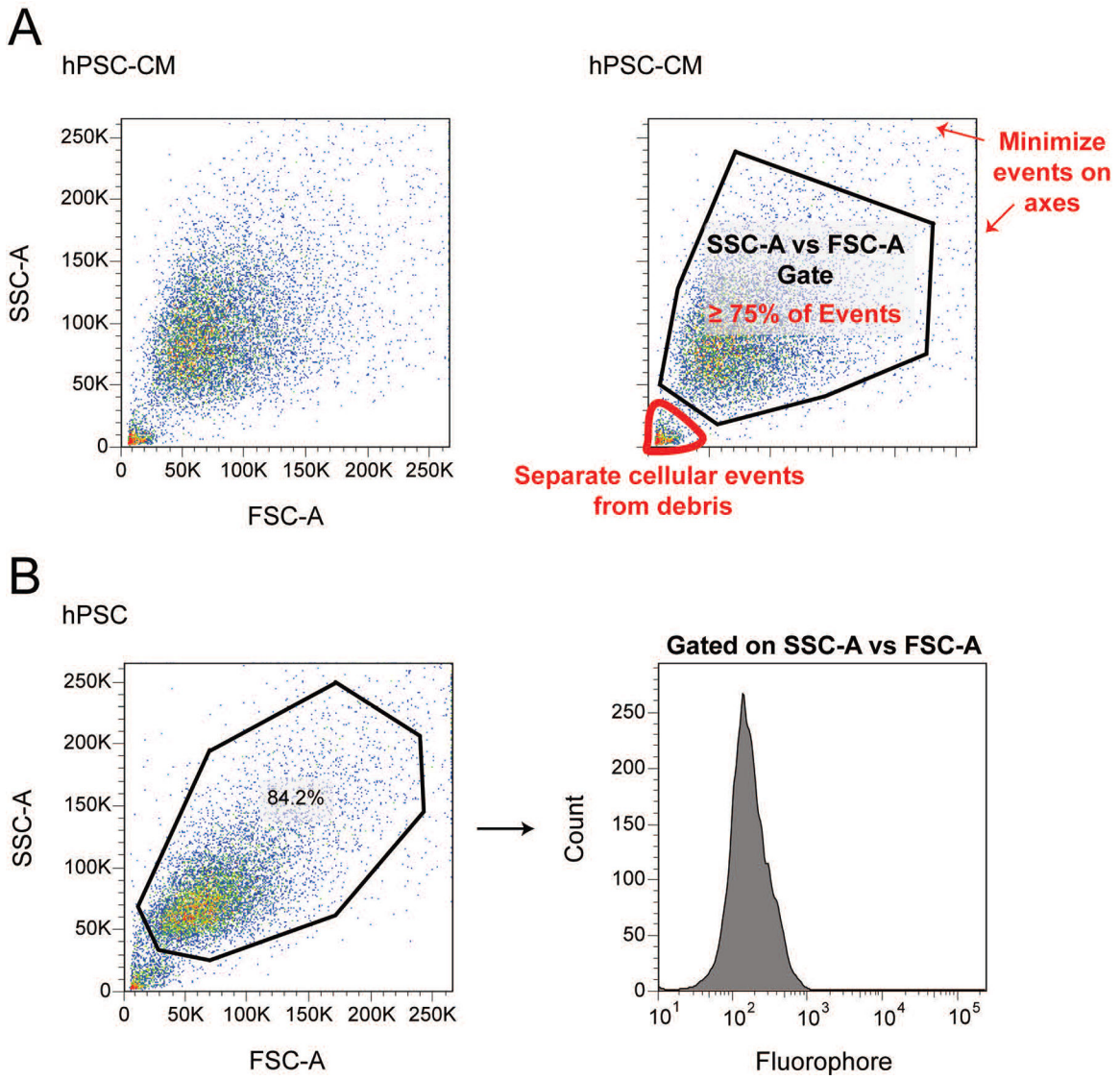

Figure 2.

Acquisition and Gating Strategies. (A) Forward (FSC-A) and side scatter (SSC-A) are adjusted to minimize events on the axes resulting in a single cell population including >75% of total cells. Cells should cluster away from debris when optimized conditions have been achieved. (B) Cells gated on FSC-A versus SSC-A result in the isotype and negative control histograms centered between 10^2 and 10^3 fluorescent intensity.

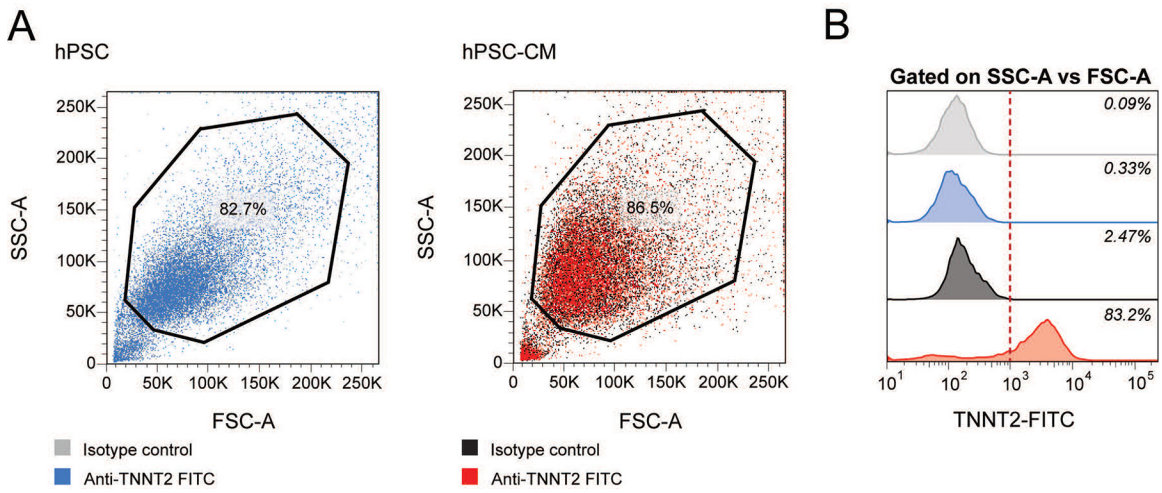

Figure 3.

Example Data for Flow Cytometric Analysis of hPSC and hPSC-CM using Anti-TNNT2 Antibody. (A) Forward versus side scatter of hPSC and hPSC-CM stained with isotype control FITC and anti-TNNT2 FITC. (B) Histograms for hPSC and hPSC-CM stained with isotype control FITC and anti-TNNT2 FITC are shown with gate drawn and the percentage of positive cells, gated off the negative control sample, in italics.

Table 1.

Minimum information to be recorded and reported for a flow cytometry experiment.

Sample Information							
Cell type(s)							
Cell line(s)							
Passage #							
Dissociation Conditions:							
Enzyme							
Solution							
Time, Temp							
Total Cell Counts							
# of Cells per Tube/Well							

Protocol Steps	**Time**	**Reagents**	**Recipe, Catalog #s**
1. Fixation		Fixation	
2. Wash		Permeabilization	
3. Permeabilization		Resuspension	
4. Wash		Block Solution	
5. Block		Wash Solution	
6. 1°Antibody			
7. Wash			
8. 2° Antibody (if applicable)			
9. Wash (if applicable)			
10. Resuspension			

Antibodies				
Target	**Clone**	**Vendor**	**Catalog #**	**Lot #**

Samples Analyzed							
Tube #	**Cell Type**	**1°Ab**	**1° Ab Quantity (µg)**	**1° Ab Volume (µl)**	**2° Ab**	**2° Ab Quantity (µg)**	**2° Ab Volume (µl)**

Instrument Configuration	
Instrument	
Laser line	
Emission filter	

Fluorochrome	