HuCAL® Antibodies Technical Manual
Applications: Flow cytometry

Flow cytometry is a widely used scientific method for characterizing optical and fluorescence parameters of suspended cells or particles as they pass through one or multiple lasers. In flow cytometry, cells are fluorescently labeled using antibodies conjugated to fluorophores, which emit light of different wavelengths upon excitation by a specific laser. Common characteristics measured in a single flow cytometry experiment are cell size, relative granularity and relative fluorescence, allowing a simultaneous multi-parameter analysis of single cells. Standard procedures performed are cell counting, cell sorting and biomarker detection. In diagnostics, flow cytometry is routinely used for immunophenotyping of cells, e.g. it allows the detection and distinction between benign and malignant hematopoietic disorders such as various leukemia and lymphoma types.

As with many other immunoassays, cells are incubated with an antigen-specific antibody and a fluorescence-labeled secondary antibody. Detection is performed by passing the cell suspension through a flow cytometer or FACS™ instrument. The suspension is then scanned by a laser and the light-scattering characteristics and fluorescence are measured, allowing detection of cells carrying fluorescence-labeled antibodies. If required, cells expressing a particular antigen can also be separated from those that do not by applying an appropriate electric charge which deflects the cells into different sample tubes.

A detailed flow cytometry application guide can be accessed at bio-rad-antibodies.com/flow.

Use of HuCAL® Antibodies in Flow Cytometry

HuCAL antibodies are well suited for use in flow cytometry. Their small size and lack of an Fc region result in low background and avoids potential interactions between the primary antibody and Fc receptors on cells. In addition, HuCAL antibodies routinely carry epitope tags that allow them to be detected on antibody-expressing cells, such as B cells. While the bivalent mini-antibody format (Fab-dHLX) is recommended, since it has higher avidity, the monovalent format can also be used. Table 1 lists the recommended secondary antibodies.

Table 1. Recommended secondary antibodies

Secondary Antibody	Recommended Dilution	Catalog Number
Anti-human IgG (H+L): RPE F(ab')$_2$ fragment	Neat-1:10	STAR97PE
Anti-Strep-tag® Immo		MCA2488
Anti-Strep-tag Classic		MCA2489
Anti-V5-tag:FITC	Neat-1:5	MCA2894F
Anti-DYKDDDDK-tag:FITC		MCA4764F
Anti-DYKDDDDK-tag	1:2000	Sigma, F3165
Anti-His-6-tag:FITC	1:10	MCA1396F
Anti-mouse IgG (H+L):RPE F(ab')$_2$ fragment	1:5	STAR105PE
Anti-mouse IgG:FITC F(ab')$_2$ fragment	1:25-1:100	STAR9B

For the detection of HuCAL antibodies with cells that do not produce human IgG, we recommend the use of an anti-human IgG conjugate that is specific for heavy and light chains (STAR97PE), allowing detection of any Fab format, regardless of the tags present.

For cells which inherently display human IgG, detection via the tag on the HuCAL antibody is used. Check the format of the HuCAL antibody and use an appropriate secondary antibody from the list in Table 1. A conjugated anti-tag antibody or an unconjugated secondary antibody followed by a conjugated anti-mouse antibody can be used. Use of an anti-c-myc antibody is not recommended, since it can give higher background due to endogenous c-myc in dead cells.

Protocols

Cells expressing the target antigen and, where available, a negative control cell line, are required. All buffers used during the staining procedure must contain the appropriate supplements e.g. azide and ions, where necessary.

Recommended Controls

- **Background control:** Use PBS instead of the HuCAL antibody, with flow buffer and detection antibody. Use two control samples if a tertiary antibody is required (Sample 1: anti-tag + anti-mouse antibodies; Sample 2: anti-mouse antibody only)
- **Negative control:** Use unrelated negative control HuCAL antibody, with flow buffer and detection antibody. Bio-Rad recommends testing several dilutions of purified Fab for flow cytometry and using a non-related Fab in identical dilutions as a negative control

- **Positive control:** (if available) Include with each flow cytometry experiment to check whether the cells are expressing the selection antigen

Preparation of Cells

1. **Adherent cells:** Detach cells from their support using Trypsin-EDTA or Accutase® cell detachment solution, and collect in a 50 ml sterile plastic tube.
   Suspension cells: Collect in a 50 ml sterile plastic tube.
2. Wash the cells once in ice-cold flow buffer, add buffer and invert to mix, then centrifuge at 190 g for 5 minutes at 4°C. Preparation of flow buffer is described in the appendix.
3. Discard the supernatant and resuspend the cell pellet in the remaining drop. Add 5 ml ice-cold flow buffer and mix by inverting the tube. Centrifuge at 190 g for 5 minutes at 4°C.
4. Discard the supernatant and gently resuspend the cell pellet in the remaining drop. Add a small volume of ice-cold flow buffer.
5. Stain cells with Trypan Blue and count the cells. Add flow buffer and adjust the density of live cells to 0.5 x 10^6 cells/100 µl.
6. Add 100 µl of cell suspension (0.5 x 10^6 cells) to each well of a 96-well round bottom tissue culture plate, and centrifuge the plate at 716 g for 2 minutes at 4°C.
7. Carefully remove the supernatant and gently vortex the plate to bring the cells into suspension in the remaining liquid. When using only a few wells, aspirate the supernatant with a pipette. If using an entire 96-well plate, invert the plate quickly but carefully over a tray to remove the supernatant. Then, do not tap the plate on a paper towel; instead turn plate upwards and blot surface moisture from the top with a paper towel.

Procedure for Cell Staining

1. Prepare serial dilutions of HuCAL antibodies, between 0.1 and 100 µg/ml in flow buffer; (10 µg/ml is a standard concentration).
2. Add 100 µl of diluted antibody and control solutions to the wells containing antigen-expressing cells and negative control cells. Incubate for 1 hour at 4°C with gentle agitation.
3. Centrifuge at 716 g for 2 minutes at 4°C. Remove the supernatant and wash each well 2-3 times with 200 µl flow buffer. When using only a few wells, aspirate the supernatant with a pipette. If using an entire 96-well plate, invert the plate quickly but carefully over a tray to remove the supernatant. Then, do not tap the plate on a paper towel; instead blot surface moisture from the top of the plate with a paper towel.
4. Add one of the following secondary antibodies:
   a. Add 100 µl PE-conjugated goat anti-human IgG (H+L) specific F(ab')$_2$ fragment (STAR97PE) diluted 1:5 in flow buffer to the wells containing antigen-expressing cells and negative control cells.
   b. Add 100 µl conjugated anti-tag secondary antibody (e.g. MCA1396F) diluted in flow buffer to the wells containing antigen-expressing cells and negative control cells.
   c. Add 100 µl mouse anti-tag secondary antibody diluted in flow buffer to the wells containing antigen-expressing cells and negative control cells.
5. Incubate for 1 hour at 4°C with gentle agitation.
6. Centrifuge at 716 g for 2 minutes at 4°C. Remove the supernatant and wash 2-3 times with 200 µl flow buffer, as described in step 3.
7. Tertiary antibody incubation (only for unconjugated secondary antibodies, step 4C). Add 100 µl of PE-conjugated goat anti-mouse IgG antibody (STAR105PE) to each well, diluted 1:5 in flow buffer. Incubate for 1 hour at 4°C with gentle agitation followed by centrifugation and wash, as described in step 3.
8. After the final washing step, centrifuge at 716 g for 2 minutes at 4°C. Resuspend cells in 100-200 µl flow buffer and transfer to flow cytometry tubes. For short-term storage (up to 4 days), stained cells can be resuspended in 100 µl of 4% paraformaldehyde in PBS after the final washing step. Cover cells with aluminum foil and store at 4°C. Immediately before flow cytometry analysis, add flow buffer to the required final volume.

Examples

Transiently transfected HEK293 cells were analyzed by flow cytometry using a monovalent HuCAL antibody in Fab-FH format (with DYKDDDDK and His-6 epitope tags) against a human trans-membrane protein. Detection was performed either with a PE-conjugated anti-human IgG heavy and light chain specific secondary antibody, or with an anti-DYKDDDDK or an anti-His-6-tag antibody in combination with a PE-conjugated anti-mouse antibody. A clear shift of the transfected cells is visible for all three detection systems, Figure 1.

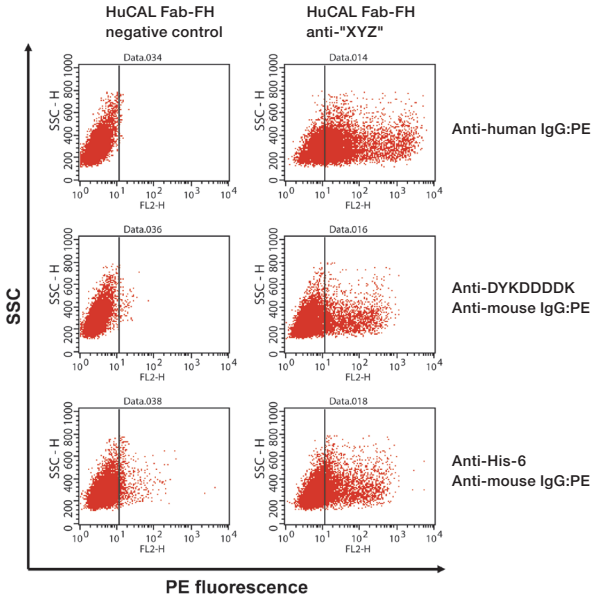

Fig. 1. Flow Cytometry Analysis of Transiently Transfected HEK293 Cells. Left column: unrelated antibody (control). **Right column:** antigen-specific antibody. **Row 1:** detection with a PE-conjugated anti-human IgG antibody. **Row 2:** detection with a mouse anti-DYKDDDDK antibody and a PE-conjugated anti-mouse IgG. **Row 3:** detection with a mouse anti-His-6-tag antibody and a PE-conjugated anti-mouse IgG.

In another example CD81-positive cells were detected by flow cytometry analysis from washed human blood using an anti-CD81 HuCAL antibody (HCA086 and HCA087 in Fab-dHLX-MH format or HCA112 and HCA113 in Fab-dHLX-MSx2 format) together with anti-Strep-tag Classic (MCA2489) and FITC-conjugated anti-mouse IgG antibody (STAR9B) detection. A clear shift of the peak can be observed when compared to the negative control sample.

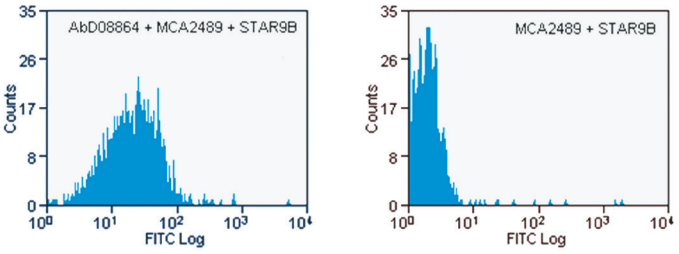

Fig. 2. Flow Cytometry of CD81-positive Cells from Washed Human Blood. **Left:** Anti-CD81 HuCAL antibody clone AbD08864 (HCA113) and detection with anti-Strep-tag Classic (MCA2489) and anti-mouse IgG FITC (STAR9B). **Right:** Negative control without HuCAL antibody.

Troubleshooting

Problem	Possible Cause and Course of Action
No staining	1. Antibody stored at 4°C for several weeks or subjected to several freeze-thaw cycles. Repeat the experiment using a fresh aliquot of antibody that has been stored at -20°C or below. 2. Incorrect secondary antibody used. Use a conjugated anti-human Fab specific, IgG (H+L), or a suitable anti-tag antibody with a matching tertiary antibody. 3. Failure of conjugation reaction (e.g. primary antibody to PE). Perform a positive control for the conjugated antibody and/or make a new preparation of conjugated antibody, taking care to follow the conjugation protocol accurately.
Weak shift	1. Antibodies not stored properly. Repeat the experiment using fresh antibodies. 2. Affinity of secondary antibody for the primary antibody too low. Try a different secondary antibody. If the recommended anti-human IgG (H+L) was used, try the anti-human Fab. 3. Antibody concentrations too low. Use a higher concentration of primary and/or secondary antibody.

Appendices

Buffer composition

Buffer	Composition	Storage
PBS	136 mM NaCl 2.68 mM KCl 8.1 mM Na_2HPO_4 1.46 mM KH_2PO_4	Room temperature
PBST	PBS with 0.05% Tween®-20	Room temperature

Flow buffer preparation

1. Mix ice-cold PBS, pH 7.4, 3% FCS, and 0.02% sodium azide.
   Note: Avoid azide if subsequent cell function is required.
2. Centrifuge at high speed until the suspended matter is pelleted (~ 30 min at 17700 x *g*) at 4°C.
3. Transfer supernatant to a new tube.
4. Store at 4°C for up to one week.

Source of antibodies and reagents

Reagent	Supplier	Catalog Number
4% Paraformaldehyde in PBS	Sigma	P6148
96-well round bottom microtiter plates, sterile	Thermo Fisher Scientific	163320
Anti-CD81	Bio-Rad	HCA086
	Bio-Rad	HCA087
	Bio-Rad	HCA112
	Bio-Rad	HCA113
FBS	Thermo Fisher Scientific	16250-078
Streptavidin:RPE	Bio-Rad	STAR4B
Trypan Blue	Bio-Rad	1450021
Trypsin-EDTA (0.05%), phenol red	Thermo Fisher Scientific	25300-054

Negative control antibodies

Refer to 'Reagents to Support HuCAL Assay Development' for a list of negative control antibodies corresponding to each of the available Fab and full immunoglobulin formats. Negative controls are specific for green fluorescent protein (GFP) that does not exist in mammalian cells.

Technical Assistance

A group of experienced technical advisors are available to help with any questions regarding HuCAL antibodies and their applications, both before project start and after antibody delivery. Since HuCAL technology provides many opportunities for optimizing the antibody generation process according to customer needs, we encourage the discussion of your antibody project with one of our scientists at an early stage. They can help with project design, antigen preparation, and provide information about the many options that are simply not available with typical animal-based technologies. Once the antibodies are delivered, they can also provide valuable advice about protocols and detection systems.

Contact us at antibody_sales_muc@bio-rad.com or visit bio-rad-antibodies.com/HuCAL for more information. General technical support requests related to catalog antibodies and accessory products can also be addressed to antibody_tech_uk@bio-rad.com.

Confidentiality

We treat all data and material provided to us by customers as completely confidential, including customer name and affiliation. Information from customers is used only as necessary for the successful and safe performance of the antibody generation project. We are pleased to provide a signed Confidential Disclosure Agreement (CDA) upon request.

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