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Fab-dHLX-MH (Negative Control)

Catalog No.: AM05839PU-N

Quantity: 0.1 mg

Concentration: 0.1mg/ml (after reconstitution)

Immunogen: Green fluorescent protein

Format: **State:** Lyophilised bivalent human recombinant Fab selected from the HuCAL® GOLD phage display library. Expressed in E. coli and purified using NiNTA affinity chromatography. This Fab fragment is dimerized via a helix-turn-helix motif. The antibody is tagged with a myc-tag (EQKLISEEDL) and a his-tag (HHHHHH) at the C-terminus of the antibody heavy chain.
Purification: Metal chelate affinity chromatography
Buffer System: PBS containing 0.09%Sodium Azide (NaN3) and 1% Bovine Serum Albumin
Reconstitution: Restore with 1.0ml distilled water

Applications: ELISA.
Flow Cytometry.
Western Blot.
Immunohistochemistry on frozen and paraffin sections.
Other applications not tested. Optimal dilutions are dependent on conditions and should be determined by the user.

Specificity: This antibody is a recombinant antibody with specificity for Green Fluorescent Protein. It has no known reactivity with mammalian proteins or other antigens. It is therefore recommended as a negative control reagent when using other HuCAL antibodies of the same format.
It is recommended that this reagent is used at the same concentration as the test reagent.

Add. Information: **Recommended Secondary Antibodies:**
Mouse Anti Human C-MYC (SM1863P) HRP
Goat Anti Human IgG F(ab')2 (AP05000HR-N) HRP
Mouse Anti Synthetic Peptide HISTIDINE TAG (SM1693P) HRP

Human **C**ombinatorial **A**ntibody **L**ibrary (HuCAL®) technology is a unique and innovative concept for in vitro generation of highly-specific and completely human antibodies - for a schematic drawing of available antibody formats see "Pictures" below.

Please Note:
HuCAL® antibodies are sold under license of U.S. Patents 6,300,064; 6,696,248; 6,708,484;
6,753,136; and European Patent 0,859,841 and corresponding patents.

Storage:

Prior to and following reconstitution store the antibody at -20°C.
Avoid repeated freezing and thawing.
Shelf life: 6 month from despatch.

Protocols:

Cited from:
HuCAL® Antibodies Technical Manual
First Edition
By Francisco Ylera

Use of HuCAL® antibodies in ELISA

ELISA can be performed using HuCAL® antibodies in protocols used for other poly- or monoclonal antibodies, provided a suitable secondary antibody is used. As HuCAL® antibodies do not contain the Fc domain, we recommend using an anti-human Fab secondary antibody (use of polyclonal antiserum amplifies the signal). Monoclonal antibodies against the tag (e.g. anti-Myc, anti-His-6) can also be used. The bivalent format of the HuCAL® mini-antibodies (Fab-dHLX or Fab-A) is recommended for ELISA assays with immobilized antigen because their avidity is higher, similar to that of full IgGs.

(A) Indirect ELISA

Indirect ELISA is recommended as a control assay to test the performance of reagents. In indirect ELISA, the antigen is immobilized on a surface, such as a well of a microtiter plate. The plate is blocked to prevent non-specific binding of antibodies, and a specific antibody is added to each well. The plate is washed to remove unbound antibody, leaving only the specific antigen-antibody complexes of interest. A secondary antibody is added. This is conjugated to an enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase, and binds the antibody-antigen complexes. The plate is washed and the appropriate substrate is applied. The resulting chromogenic or fluorescent signal is viewed using a spectrophotometer.

Protocol

Indirect ELISA with fluorescence readout

1. Coat the antigen by adding 20 µl of a solution of 5 µg/ml antigen in PBS to several wells of a 384-well Maxisorp(TM) microtiter plate, and incubate overnight at 4°C.
Use a black, 384-well ELISA microtiter plate: square well, flat bottom MaxiSorp(TM) PS (NUNC, Cat. No. 460518).
2. Wash the microtiter plate twice with PBST.
3. Block the microtiter plate by adding 100 µl of 5% non-fat dried milk in PBST to each well, and incubate for 1–2 hours at room temperature.
4. Wash the microtiter plate twice with PBST.
5. Transfer 20 µl of HuCAL® antibody to each well. We recommend a standard concentration of 2 µg/ml in PBST or HiSpec buffer BUF049). Incubate for 1 hour at room temperature.
Titrate using different concentrations of HuCAL® antibody.
6. Wash the microtiter plate 5 times with PBST.
7. Add 20 µl of secondary antibody to each well and incubate for 1 hour at room temperature.
We recommend using anti-human Fab:AP .
8. Wash the microtiter plate 5 times with PBST.
9. Add 20 µl of AttoPhos® (Roche) to each well and measure fluorescence after 10 minutes (excitation: 440±25 nm, emission: 550±35 nm).
Note: Instead of the 384-well format, a 96-well plate can be used (e.g. black, flat bottom MaxiSorp(TM)PS, NUNC, Cat. No. 437111). For the 96-well format use 100 µl instead of 20 µl of antigen, antibodies or substrate and 300 µl for the blocking step. Instead of an alkaline phosphatase-conjugated secondary antibody, an
HRP-conjugated anti-Fab can be used in combination with QuantaBlu® reagent (Pierce Cat.

No. 15169, excitation: 320±25 nm, emission: 430±35 nm).
If detection is done with a chromagenic substrate (e.g. BM Blue Soluble Peroxide Substrate, Roche, Cat. No. 1484281), transparent plates must be used.

(B) Direct ELISA

In direct ELISA, the primary antibody is labeled, for example, by genetic fusion with alkaline phosphatase. This avoids use of a secondary antibody, saving time and money, which is valuable for assays that are frequently run. However, the system lacks the amplification effect generated by the secondary antibody in indirect ELISA and is therefore less sensitive.

Protocol

Direct ELISA with alkaline phosphatase-conjugated primary antibody and fluorescence readout

1. Coat the antigen by adding 20 µl of a solution of 5 µg/ml antigen in PBS to several wells of a 384-well Maxisorp(TM) microtiter plate, and incubate overnight at 4°C.
Use a black, 384-well ELISA microtiter plate: square well, flat bottom, MaxiSorp(TM) PS (NUNC, Cat. No. 460518).
2. Wash the microtiter plate twice with PBST.
3. Block the microtiter plate by adding 100 µl of 5% non-fat dried milk in PBST to each well, and incubate for 1-2 hours at room temperature.
4. Wash the microtiter plate twice with PBST.
5. Transfer 20 µl of labeled HuCAL® antibody to each well. We recommend a standard concentration of 2 µg/ml in PBST. Incubate for 1 hour at room temperature. Titrate using different concentrations of HuCAL® antibody.
6. Wash the microtiter plate 5 times with PBST.
7. Add 20 µl of AttoPhos® (Roche) to each well and measure the fluorescence after 10 minutes (excitation: 440±25 nm, emission: 550±35 nm).

(C) Sandwich ELISA

Sandwich ELISA is a very sensitive and specific method of detecting antigens, and provides fast and accurate determination of the concentration of antigen in a sample. If a purified antigen standard is available, the method can also measure the absolute concentration of antigen in a given sample. The technique uses two antibodies, both of which are specific to the antigen of interest, and which bind the antigen at non-overlapping epitopes. In sandwich ELISA, it is possible to use a HuCAL® antibody as the capture or detection antibody together with an existing commercial antibody, or to use two different HuCAL® antibodies as the sandwich pair. The bivalent format of HuCAL® mini-antibodies is highly suitable for both capture and detection. When two HuCAL® antibodies are used, detection is best performed using a labeled anti-tag tertiary antibody (e.g. Fab-A-FH HuCAL® antibody with HRP-conjugated rabbit anti-bacterial alkaline phosphatase). Alternatively, the detection antibody can be biotinylated and visualized with streptavidin-AP.

Protocols

Sandwich ELISA using two HuCAL® antibodies

1. Coat the capture antibody by adding 20 µl of a solution of 5 µg/ml in PBS to several wells of a 384-well Maxisorp(TM) microtiter plate, and incubate overnight at 4°C.
Use a black, 384-well ELISA microtiter plate: square well, flat bottom, MaxiSorp(TM) PS (NUNC, Cat. No. 460518).
2. Wash the microtiter plate twice with PBST.
3. Block the microtiter plate by adding 100 µl of 5% BSA in PBST to each well, and incubate for 1 hour at room temperature.
4. Wash the microtiter plate twice with PBST.
5. Add 20 µl of antigen to each well of the microtiter plate, and incubate for 1 hour at room temperature.
Use a concentration range of the antigen in PBS.
6. Wash the microtiter plate 5 times with PBST.

7. Add 20 µl of HuCAL® detection antibody in Fab-A-FH format (2 µg/ml concentration in PBST or HiSpec buffer) to each well, and incubate for 1 hour at room temperature.
8. Wash the microtiter plate 5 times with PBST.
9. Transfer 20 µl of HRP-conjugated rabbit anti-bacterial alkaline phosphatase antibody and incubate for 1 hour at room temperature.
Use rabbit anti-bacterial alkaline phosphatase.
10. Wash the microtiter plate 5 times with PBST.
Add 20 µl of QuantaBlu® (Pierce) to each well and measure the fluorescence directly (excitation: 320±25 nm, emission: 420±35 nm).
Sandwich ELISA with polyclonal mouse and HuCAL® antibody
1. Coat the HuCAL® capture antibody by adding 20 µl of a solution of 5 µg/ml in PBS to several wells of a 384-well Maxisorp(TM) microtiter plate, and incubate overnight at 4°C.
2. Wash the microtiter plate twice with PBST.
3. Block the microtiter plate by adding 100 µl of 5% BSA in PBST to each well, and incubate for 1 hour at room temperature.
4. Wash the microtiter plate twice with PBST.
5. Add 20 µl of antigen to each well of the microtiter plate, and incubate for 1 hour at room temperature.
Use a concentration range of the antigen in PBS .
6. Wash the microtiter plate 5 times with PBST.
7. Add 20 µl of biotinylated polyclonal detection antibody (concentration as specified by the manufacturer) in HiSpec buffer to each well, and incubate for 1 hour at room temperature.
8. Wash the microtiter plate 5 times with PBST.
9.Transfer 20 µl alkaline phosphatase conjugated streptavidin and incubate for 1 hour at room temperature.

(D) Competition (or inhibition) ELISA

Competition (or inhibition) ELISA is ideal when only one suitable antibody is available for the target of interest or when the antigen is too small to be detected e.g. a hapten. The technique measures the concentration of a substance by its ability to interfere with an established pre-titrated system. The primary antibody is first incubated with the free antigen. It is then added to an antigen-coated well, and the plate is washed to remove unbound antibody. The amount of antibody that binds the immobilized antigen is detected using a secondary antibody linked to a detection system such as horseradish peroxidase. The appropriate substrate is applied and the resulting chromogenic or fluorescent signal is viewed using a spectrophotometer. The higher the concentration of free antigen in the sample, the less antibody is available to bind the immobilized antigen, and, therefore, the weaker the signal; conversely, lower amounts of free antigen in solution generate stronger signals. Other variations of competition ELISA are of course possible.

Protocol

1. Coat the antigen by adding 50 µl of a solution of 5 µg/ml antigen in PBS to several wells of a 384-well Maxisorp(TM) microtiter plate, and incubate overnight at 4°C.
2. Wash the microtiter plate twice with PBST.
3. Block the microtiter plate by adding 100 µl of 5% non-fat dried milk in PBST to each well, and incubate for 1-2 hours at room temperature.
4. Incubate 25 µl of antigen solution with 25 µl of HuCAL® antibody (final concentration 2 µg/ml) for 1 hour at room temperature. Use a range of concentrations of the antigen in solution, in PBS
5. Wash the microtiter plate twice with PBST.
6. Transfer 50 µl of the HuCAL® antibody/antigen mix to each well of the microtiter plate, and incubate for 1 hour at room temperature.
7. Wash the microtiter plate 5 times with PBST.
8. Transfer 50 µl of secondary antibody (anti-human Fab:AP and incubate for 1 hour at room

temperature.
9. Wash the microtiter plate 5 times with PBST.
10. Add 20 µl of AttoPhos® (Roche) to each well, incubate for 10 minutes at room temperature, and measure fluorescence (excitation: 440±25nm, emission: 550±35nm).

Use of HuCAL® antibodies in Western blotting
HuCAL® antibodies can be used for Western blotting following the standard protocols developed for conventional antibodies. The only difference is that the secondary antibody cannot be directed against the Fc domain, as this is lacking in HuCAL® Fab antibodies. Therefore, we suggest using an anti-human Fab secondary antibody due to the signal amplification with polyclonal secondaries or an antibody against the tag, for instance, anti-Myc. The sensitivity of an antibody in a given application depends on its affinity and avidity. Affinity is the strength with which the antibody binds its antigen in a 1:1 interaction. Avidity is the strength with which an antibody with multiple binding sites binds its antigens. Antibodies with multiple binding sites have better binding properties than monovalent antibodies when multiple epitopes are presented on a solid support. The affinities of HuCAL® antibodies are comparable to those of other monoclonal antibodies. Bivalent antibodies have higher sensitivity than monovalent antibodies as their avidity is greater. The bivalent format of HuCAL® antibodies, is therefore, recommended for most applications, including Western blotting.
Protocol
1. Run the sample on SDS-PAGE and transfer onto PVDF membrane. For initial testing, use 200 µg of cell lysate proteins or 300 ng of pure antigen.
2. Block the membrane with 5% non-fat dried milk in TBST for 1 hour on a shaker or overnight at 4°C.
3. Rinse the membrane with TBST.
4. Add HuCAL® antibody (primary antibody) to the membrane in TBST with 1% non-fat dried milk.
The optimal amount of HuCAL® antibody is typically between 1 and 10 µg/ml, but must be determined for each antibody. We recommend starting with a concentration of 5 µg/ml.
5. Incubate for 1 hour at room temperature on a shaker.
Ensure that the membrane is completely immersed in the buffer.
6. Wash the membrane 3 times, each for 5 minutes with TBST.
Use a generous amount of TBST.
7. Add secondary antibody to the membrane.
We recommend using anti-human Fab:HRP
8. Shake for 1 hour at room temperature.
9. Wash the membrane 3 times, each for 5 minutes with TBST.
Use a generous amount of TBST.
10. Develop the membrane using ECL Plus(TM) or ECL Advance(TM) (GE Healthcare) according to the manufacturer's instructions, and use an imager or X-ray film for detection.
Note: We strongly recommend sonication of lysates with ultrasound to disrupt genomic DNA in order to reduce non-specific background signals. Depending on the format of the HuCAL® Fab, an anti-tag secondary antibody can be used.

Use of HuCAL® antibodies in immunohistochemistry
Monoclonal antibodies are usually preferred over polyclonal antibodies, as they usually have higher specificity. HuCAL® antibodies offer a number of advantages over conventional monoclonal antibodies, including rapid generation and pre-existing epitope tags for detection.
Paraffinized sections
Paraffinized slides must be de-paraffinized and rehydrated before use, to ensure that the HuCAL® antibodies have full access to the tissue antigen. Samples then frequently require

pretreatment to unmask the antigen. This is because treatment with formaldehyde induces intra- and inter-molecular cross-linking in proteins, which can cause conformational changes, masking the domains of the antigen that are bound by the antibody and leading to non-specific staining. Procedures for de-paraffinization are described below.

Procedure for de-paraffinization and rehydration

1. Dry tissue sections at 60°C for 1 hour. Drying increases adhesion of the tissue sections to the surface of the glass slide.
2. Place the slides in a cuvette containing sufficient xylene to cover the tissue completely and incubate for 5 minutes with gentle shaking.
Important: Use adhesive slides.
3. Transfer the slides to a cuvette containing fresh xylene and repeat step 2 for a further 3 washes, to give a total of 4 washes each lasting 5 minutes.
Important: Use fresh xylene for each wash.
4. Wash the slides twice in 100% ethanol (for 2 minutes each time).
5. Wash the slides twice in 90% ethanol (for 2 minutes each time).
6. Wash the slides twice in 80% ethanol (for 2 minutes each time).
7. Wash the slides twice in 70% ethanol (for 2 minutes each time).
8. Wash the slides twice in 60% ethanol (for 2 minutes each time).
9. Wash the slides twice in 50% ethanol (for 2 minutes each time).
10. Wash the slides twice in TBST (for 2 minutes each time).

Cryosections

There are two types of cryostat sections or cryosections:

1. Fresh, or unfixed sections: Quickly frozen tissues are first cut, then airdried and/or fixed before staining.

2. Fixed frozen tissue: The tissue is first fixed, then cryoprotected with sucrose or another stabilizer, before freezing and sectioning. Frozen sections offer a number of advantages: they allow excellent antigen preservation; they are typically faster to perform; and they allow optimization of the fixative for each antigen, since any fixative can be used.

Important notes

- Avoid thawing the tissue sample, as this can destroy the antigen.
- Use adhesive slides.
- Air-dry sections completely before fixation.

Fixation

Methanol, acetone, and formaldehyde can be used for fixation. Methanol and acetone precipitate proteins, while formaldehyde cross-links them. The choice of fixation reagent is likely to affect the staining result, and the most suitable reagent should be determined by experimentation for each set of conditions.

Procedure

1. Submerge in A. Methanol for 10 minutes.
B. Acetone for 10 minutes.
C. 1:1 mixture of methanol and acetone for 10 minutes.
D. 4% formaldehyde for 2 minutes.
2. Wash sections several times in freshly made TBST.
This ensures that the fixative is completely removed from the sample.
3. Optional: Block endogenous peroxidases by treating samples with 0.3% H2O2 in ddH2O or methanol for 5 to 10 minutes.
Using concentrations of H2O2 greater than 0.3% results in a very strong reaction that will destroy the sample.

Mounting

Either aqueous or non-aqueous mounting media is suitable, depending on the chromogenic substrate used to detect the antigen. We recommend a nonaqueous medium for permanent mounting of DAB slides, although the faster procedure using an aqueous medium can also be used. Slides containing ethanol-soluble chromogens must be

mounted in aqueous medium.

Non-aqueous mounting

1. Transfer slides to 50% ethanol and agitate gently for a few seconds.
2. Transfer slides to 70% ethanol and agitate gently for a few seconds.
3. Transfer slides to 80% ethanol and agitate gently for a few seconds.
4. Transfer slides to 90% ethanol and agitate gently for a few seconds.
5. Transfer slides to 100% ethanol and agitate gently for a few seconds.
6. Transfer slides to xylene for 2 minutes.
7. To each slide, apply one or two drops of non-aqueous mounting medium to the uncovered tissue and apply the coverslip.

Apply the coverslip at an inclined angle to the slide and lower it gently to avoid trapping air bubbles.

8. Leave the slides to air-dry overnight, or dry in an incubator at 60°C for 15 minutes.
9. Apply the coverslip at an inclined angle to the slide and lower it gently to avoid trapping air bubbles.

Aqueous mounting

1. Add one or two drops of aqueous mounting medium to the stained tissue and apply the coverslip.
2. Apply the coverslip at an inclined angle to the slide and lower it gently to avoid trapping air bubbles.

Protocols

Cryosections and paraffin-embedded sections were stained with a HuCAL® bivalent mini-antibody (Fab-dHLX-MH) against recombinant human desmin produced by E. coli. Desmin belongs to class III of intermediate filaments, constituting part of the cytoskeleton, and is the characteristic intermediate filament of all three types of muscle cell (skeletal, cardiac, and smooth muscle). It is a 52 kDa protein encoded by nine exons of a gene located on chromosome 2q35. It forms cytoskeletal networks of muscle fibres between the plasma and nuclear membranes and is found in the subplasmalemmal region and the Z-band.
This section describes five alternative staining protocols using a HuCAL® antibody together with different amplification and detection systems.

(a) Staining of tagged HuCAL® Fabs with HRP-labeled anti-His-6 antibody

This is a fast but relatively insensitive method. It requires high concentrations of primary antibody (10–25 µg/ml), which can lead to high background signals. The antigen is detected by a direct-labeled anti-tag antibody.

1. De-paraffinize the slides.
2. Wash the slides a further 3 times (for 2 minutes each time) in TBST.
3. Submerge the slides in the buffer, place in a microwave oven (700–900 W), and heat until the buffer reaches boiling point.
4. Reduce the power to 250 W and boil the slides for 30 minutes.
5. Replace water evaporated during boiling with ddH2O and allow the sections to cool in the buffer for 15 minutes.
6. Wash the slides 3 times (for 2 minutes each time) in TBST.
7. Incubate in 3% H2O2 in methanol for 10 minutes to quench peroxidase in the tissue.
8. Wash the slides 3 times (for 2 minutes each time) in TBST.
9. Transfer slides to a solution of 10% BSA in PBS and incubate for 15 minutes at room temperature to block proteins. All subsequent steps in the protocol are performed at room temperature.
10. Tip excess blocking solutions off the sections, apply 10–25 µg/ml of primary HuCAL® antibody in antibody-diluent and incubate for 1 hour.

The concentration of antibody must be determined empirically for each antigen, staining system, and laboratory.

11. Wash the slides 3 times (for 2 minutes each time) in TBST.
12. Apply mouse anti-His-6 tag antibody conjugated to HRP (e.g. Acris Antibodies cat.no.

SM1693HRP), diluted 1:50-1:200 in antibody-diluent and incubate for 30 minutes.
13. Wash the slides 3 times (for 2 minutes each time) in TBST.
14. Add 1 drop (approximately 40 µl) of DAB chromogen per 1 ml of DAB substrate buffer, mix by swirling, and apply to tissue. Incubate for 10 minutes. 15. Wash the slides 3 times (for 2 minutes each time) in TBST.
16. Counterstain using hematoxylin for 1 minute.
17. Apply tap water to the tissue to enhance color development.
18. Rinse slides with ddH2O.
19. Dehydrate with graded alcohol and apply a permanent coverslip to the slides.

(b) Staining of tagged HuCAL® Fabs without amplification (ABC or LSAB method)

This straightforward procedure is suitable for tissues that do not contain endogenous biotin. A major advantage is the low concentration of primary antibody necessary (usually only 1–5 µg/ml), which reduces the risk of background staining. It is the most common method found in commercial detection kits.
The antigen is visualized using a primary antibody, an anti-tag secondary antibody, and a biotinylated anti-species antibody, followed by an enzymelabeled streptavidin complex.
1. De-paraffinize the slides.
2. Wash the slides 3 times (for 2 minutes each time) in TBST.
3. Submerge the slides in the buffer, place in a microwave oven (700–900 W), and heat until the buffer reaches boiling point.
4. Reduce the power to 250 W and boil the slides for 30 minutes.
5. Replace water evaporated during boiling with ddH2O and allow the sections to cool in the buffer for 15 minutes.
6. Wash the slides 3 times (for 2 minutes each time) in TBST.
7. Incubate in 3% H2O2 in methanol for 10 minutes to quench peroxidase in the tissue.
8. Wash the slides 3 times (for 2 minutes each time) in TBST.
9. Transfer slides to a solution of 10% BSA in PBS and incubate for 15 minutes to block proteins.
All subsequent steps in the protocol are performed at room temperature.
10.Tip excess blocking solution off the sections, apply 1–5 µg/ml of primary HuCAL® antibody in antibody-diluent and incubate for 1 hour.
The concentration of antibody must be determined empirically for each antigen, staining system, and laboratory.
11. Wash the slides 3 times (for 2 minutes each time) in TBST.
12. Apply mouse anti-His-6 tag antibody (e.g. Acris Antibodies cat.no. SM1693P), diluted 1:50–1:200 in antibody-diluent and incubate for 30 minutes.
13. Wash the slides 3 times (for 2 minutes each time) in TBST.
14. Apply biotinylated anti-mouse antibody, diluted to 1:50–1:200 in antibody diluent and incubate for 15 minutes.
15. Wash the slides 3 times (for 2 minutes each time) in TBST.
16. Apply streptavidin-peroxidase complex, and incubate for 15 minutes.
17. Wash the slides 3 times (for 2 minutes each time) in TBST.
18. Add 1 drop (approximately 40 µl) of DAB Chromogen per 1 ml of DAB substrate, mix by swirling, and apply to tissue. Incubate for 10 minutes.
19. Wash with ddH2O.
20. Counterstain using hematoxylin for 1 minute.
21. Apply tap water to the tissue to enhance color development.
22. Rinse slides with ddH2O.
23. Dehydrate with graded alcohol and apply a permanent coverslip to the slides.

(c) Staining of tagged HuCAL® Fabs with amplification using the EnVision(TM) system

The EnVision(TM) system is a fast and simple three-step amplification procedure that does not use streptavidin. The antigen is detected using a HuCAL® antibody, followed by a

mouse anti-His-6 and an anti-mouse antibody bound to a HRP-labeled dextran backbone. This system also uses low concentrations of primary antibody, which reduces background staining.
1. De-paraffinize the slides.
2. Wash the slides twice (for 2 minutes each time) in TBST.
3. Submerge the slides in the buffer, place in a microwave oven (700–900 W), and heat until the buffer reaches boiling point.
4. Reduce the power to 250 W and boil the slides for 30 minutes.
5. Replace water evaporated during boiling with ddH2O and allow the sections to cool in the buffer for 15 minutes.
6. Wash the slides 3 times (for 2 minutes each time) in TBST.
7. Incubate in 3% H2O2 in methanol for 10 minutes to quench peroxidase in the tissue.
8. Wash the slides twice (for 2 minutes each time) in TBST.
9. Transfer slides to a solution of 10% BSA in PBS and incubate for 15 minutes to block proteins.
All subsequent steps in the protocol are performed at room temperature.
10. Tip excess blocking solution off the sections, apply 1–5 µg/ml of primary HuCAL® antibody in antibody-diluent and incubate for 1 hour.
The concentration of antibody must be determined empirically for each antigen, staining system, and laboratory.
11. Wash the slides 3 times (for 2 minutes each time) in TBST.
12. Apply mouse anti-His-6 tag antibody (e.g. Acris Antibodies cat.no. SM1693P), diluted 1:50-1:200 in antibody-diluent and incubate for 30 minutes.
13. Wash the slides 3 times (for 2 minutes each time) in TBST.
14. Apply ready-to-use EnVision(TM) anti-mouse HRP, and incubate for 15 minutes.
15. Wash the slides 3 times (for 2 minutes each time) in TBST.
16. Add 1 drop (approximately 40 µl) of DAB Chromogen per 1 ml of DAB substrate, mix by swirling, and apply to tissue. Incubate for 5 minutes.
17. Wash with ddH2O.
18. Counterstain using hematoxylin for 1 minute.
19. Apply tap water to the tissue to enhance color development.
20. Rinse slides with ddH2O.
21. Dehydrate with graded alcohol and apply a permanent coverslip to the slides.

(d) Staining of tagged HuCAL® Fabs with tyramide amplification

This procedure avoids using tertiary antibodies and is suitable for murine tissues. It uses biotinylated tyramide to amplify the signal, resulting in improved detection sensitivity, and requires only very low concentrations of primary antibody (0.1–1 µg/ml). Antigens are detected using an HRP-conjugated secondary antibody against the tag of the HuCAL® Fab. Biotinylated tyramide is activated by the peroxidase of the secondary antibody to form highly-reactive, short-lived tyramide radicals, which react with tyrosine residues of proteins in the vicinity of the HRP target. The biotin on the bound tyramide is easily visualized using standard ABC techniques.
1. De-paraffinize the slides.
2. Wash the slides 3 times (for 2 minutes each time) in TBST.
3. Submerge the slides in the buffer, place in a microwave oven (700–900 W), and heat until the buffer reaches boiling point.
4. Reduce the power to 250 W and boil the slides for 30 minutes.
5. Replace water evaporated during boiling with ddH2O and allow the sections to cool in the buffer for 15 minutes.
6. Wash the slides 3 times (for 2 minutes each time) in TBST.
7. Incubate in 3% H2O2 in methanol for 10 minutes to quench peroxidase in the tissue.
8. Wash the slides 3 times (for 2 minutes each time) in TBST.
9. Transfer slides to a solution of 10% BSA in PBS and incubate for 15 minutes to block

proteins.
All subsequent steps in the protocol are performed at room temperature.
10. Tip excess blocking solution off the sections, apply 1–25 µg/ml of primary HuCAL® antibody in antibody-diluent and incubate for 1 hour.
11. Wash the slides 3 times (for 2 minutes each time) in TBST.
12. Apply HRP-labeled mouse anti-His-6 tag antibody, (e.g. Acris Antibodies cat.no. SM1693HRP), diluted 1:50-1:200 in antibody-diluent and incubate for 30 minutes.
13. Wash the slides 3 times (for 2 minutes each time) in TBST.
14. Prepare a working solution of activated biotinylated tyramide by adding 5 µl of tyramide stock solution (freshly thawed) and 2 µl of 30% H2O2 per 1 ml of TBST.
15. Apply the activated biotinylated tyramide to the slides for 15 minutes.
16. Wash the slides 3 times (for 2 minutes each time) in TBST.
17. Apply streptavidin-peroxidase complex and incubate for 15 minutes.
18. Wash the slides 3 times (for 2 minutes each time) in TBST.
19. Add 1 drop (approximately 40 µl) of DAB Chromogen per 1 ml of DAB substrate, mix by swirling, and apply to tissue. Incubate for 10 minutes.
20. Wash with ddH2O.
21. Counterstain using hematoxylin for 1 minute.
22. Apply tap water to the tissue to enhance color development.
23. Rinse slides with ddH2O.
24. Dehydrate with graded alcohol and apply a permanent coverslip to the slides.

(e) Staining of tagged HuCAL® Fabs using a mouse-on-mouse system

The mouse-on-mouse system is a simple method for staining of mouse tissues. A special blocking reagent eliminates background from endogenous mouse IgGs. Antigens are detected using a mouse anti-tag antibody and a biotinylated antimouse antibody, followed by an enzyme-labeled streptavidin complex. The protocol is performed using a customized detection kit from Vector Laboratories (Cat. No. PK-2200).

Important note

Before starting the protocol, prepare working solutions according to the manufacturer's instructions.
1. De-paraffinize the slides.
2. Wash the slides 3 times (for 2 minutes each time) in TBST. 3. Submerge the slides in the buffer, place in a microwave oven (700–900 W), and heat until the buffer reaches boiling point.
4. Reduce the power to 250 W and boil the slides for 30 minutes.
5. Replace water evaporated during boiling with ddH2O and allow the sections to cool in the buffer for 15 minutes.
6. Wash the slides 3 times (for 2 minutes each time) in TBST.
7. Incubate in 3% H2O2 in methanol for 10 minutes to quench peroxidase in the tissue.
All subsequent steps in the protocol are performed at room temperature.
8. Wash the slides 3 times (for 2 minutes each time) in TBST.
9. Incubate the tissue sections for 1 hour in a working solution of mouse-on-mouse IgG blocking reagent, prepared according to the manufacturer's instructions.
10. Wash the slides 3 times (for 2 minutes each time) in TBST.
11. Incubate tissue sections for 5 minutes in a working solution of Mouse-on-Mouse (M.O.M) (TM)Kit diluent, prepared according to the manufacturer's instructions.
12. Tip excess M.O.M. (TM) diluent off slides. Dilute the primary antibody in M.O.M. (TM) diluent to the appropriate concentration, and incubate the section with the diluted primary antibody for 1 hours.
13. Wash the slides 3 times (for 2 minutes each time) in TBST.
14. Incubate the slides for 30 minutes in a working solution of mouse anti-His-6 antibody (Product Code SM1693P), diluted 1:50-1:200 in M.O.M. (TM) diluent.

15. Wash the slides 3 times (for 2 minutes each time) in TBST.
16. Apply a working solution of M.O.M. (TM) biotinylated anti-mouse IgG reagent, prepared as described in the manufacturer's instructions. Incubate the sections for 10 minutes.
17. Wash the slides 3 times (for 2 minutes each time) in TBST.
18. Apply Vectastain ABC Reagent, prepared according to the manufacturer's instructions, and incubate the sections for 5 minutes.
Note that the reagent must be prepared 30 minutes before use.
19. Wash the slides 3 times (for 2 minutes each time) in TBST.
20. Add 1 drop (approximately 40 µl) of DAB chromogen per 1 ml of DAB substrate, mix by swirling, and apply to tissue. Incubate for 10 minutes.
21. Wash the slides 3 times (for 2 minutes each time) in TBST.
22. Counterstain using hematoxylin for 1 minute.
23. Apply tap water to the tissue to enhance color development.
24. Rinse slides with ddH2O.
25. Dehydrate with graded alcohol and apply a permanent coverslip to the slides.

Use of HuCAL® antibodies in flow cytometry

HuCAL® antibodies are well suited for use in flow cytometry. Their small size and the absence of the Fc region usually results in low background and avoids potential interactions of the primary antibody with Fc receptors on cells. In addition, HuCAL® antibodies carry tags that allow them to be detected on antibody-expressing cells, such as B cells. The bivalent mini-antibody format (Fab-dHLX) is recommended, as it has higher avidity, but the monovalent format can also be used. For detection via the tag, we recommend a format with a FLAG® tag (e.g. Fab-dHLX-FH or Fab-FH), as the anti-FLAG® M2 secondary antibody has a higher affinity than the anti-His-6 tag antibody.

Protocols

(a) For HuCAL® antibodies and cells that do not produce human IgG

This protocol describes the use of HuCAL® antibodies in combination with an anti-human IgG conjugate that is specific for heavy and light chains, allowing detection of any Fab format, regardless of its tags. This method can be used to analyze antigen-positive cells that do not themselves display human IgG. 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 HuCAL® antibody, with FACS buffer and detection antibody.
- Negative control: Use unrelated negative control HuCAL® antibody, with FACS buffer and detection antibody.
- We recommend testing several dilutions of purified Fab for FACS and using a non-related Fab in identical dilutions as a negative control.
- Positive control (if available): Include with each FACS experiment to check whether the cells are expressing the selection antigen.

Preparation of FACS buffer

To prepare FACS buffer, mix ice-cold PBS, pH 7.4, 3% FCS, and 0.02% sodium azide. Centrifuge at high speed until the suspended matter is pelleted (approximately 30 minutes at 17,700 x g), at 4°C, and transfer supernatant to a new tube. Store at 4°C for up to one week.

Preparation of cells

1.
A. Adherent cells: Detach cells from their support using Trypsin/EDTA (Versene), and collect in a 50 ml sterile plastic tube.
B. Cells grown in suspension: Collect in a 50 ml sterile plastic tube.

2. Wash cells once in ice-cold FACS buffer and centrifuge at 190 x g for 5 minutes at 4°C. Wash cells by adding ice-cold FACS buffer to the tube, closing the tube, and inverting to mix.
3. Discard the supernatant, resuspend the cell pellet in the remaining drop, add 5 ml of ice-cold FACS buffer, and mix by inverting the tube.
4. Wash cells once in ice-cold FACS buffer and centrifuge at 190 x g for 5 minutes at 4°C.
5. Discard the supernatant, gently resuspend the cell pellet in the remaining drop, and add a small volume of ice-cold FACS buffer.
6. Stain cells using Trypan Blue and count the cells. Add FACS buffer to adjust the number of living cells to 0.5 x 10e6 cells/100 µl.
7. Add 100 µl of cell suspension (0.5 x 10e6 cells) to each well of a 96-well round bottom tissue culture plate, and centrifuge the plate at 716 x g for 2 minutes at 4°C.
8. 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. Do not tap the plate on a paper towel, but place the plate on the bench 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 FACS buffer. 10 µg/ml is a standard concentration.
2. Add 100 µl of diluted antibody and controls to the wells containing antigen-expressing cells and the negative control cells. Incubate for 1 hour at 4°C with gentle agitation.
3. Centrifuge at 716 x g for 2 minutes at 4°C, remove the supernatant, and wash 2–3 times with 200 µl of FACS 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. Do not tap the plate on a paper towel, but place the plate on the bench and blot surface moisture from the top with a paper towel.
4. Add 100 µl of R-Phycoerythrin-conjugated goat anti-human IgG (H+L) specific F(ab')2 fragment secondary antibody to the wells (samples and controls) containing antigen-expressing cells and the negative control cells. Incubate for 1 hour at 4°C with gentle agitation.
5. Centrifuge at 716 x g for 2 minutes at 4°C, remove the supernatant, and wash 2–3 times with 200 µl of FACS 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. Do not tap the plate on a paper towel, but place the plate on the bench and blot surface moisture from the top with a paper towel.
6. After the last washing step, centrifuge again for 2 minutes at 4°C, resuspend cells in 100–200 µl of FACS buffer, and transfer to FACS 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 aluminium foil and store at 4°C. Immediately before FACS analysis, add FACS buffer to give the required volume.

(b) For cells that produce human IgG

This protocol describes the detection of HuCAL® antibodies with anti-tag antibodies. It is well suited for staining cells that produce and expose human IgG on their surface and, therefore, cannot be analyzed using an anti-human IgG specific antibody. Depending upon the tags on the HuCAL® antibodies, different detection antibodies can be used (anti-FLAG® M2 antibody, anti-c-Myc antibody, or anti-His-6 antibody), followed by a conjugated anti-mouse IgG antibody. Cells expressing the target antigen, and, where available, a negative control cell line, are required. All buffers used during the staining procedure must be supplemented with azide, appropriate ions etc.

Recommended controls
- Two background controls: Use PBS instead of HuCAL® antibody, with FACS buffer, with and without an anti-tag antibody and detection antibody.
- Negative control: Use unrelated negative control HuCAL® antibody, with FACS buffer and detection antibody.
- Positive control (if available): Include a positive control with each FACS experiment wherever possible, to check whether the cells are expressing the selected antigen.
- We recommend testing several dilutions of purified Fab for FACS and using a non-related Fab in identical dilutions as negative control.

Preparation of cells
1. Follow the protocol described before.

Procedure for cell staining
1. Prepare serial dilutions of HuCAL® antibodies, between 0.1-100 µg/ml in FACS buffer. 10 µg/ml is a standard concentration.
2. Add 100 µl of diluted antibody and controls to the wells containing antigen-expressing cells and the negative control cells. Incubate for 1 hour at 4°C with gentle agitation.
3. Centrifuge at 716 x g for 2 minutes at 4°C, remove the supernatant, and wash 2–3 times with 200 µl of FACS 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. Do not tap the plate on a paper towel, but place the plate on the bench and blot surface moisture from the top with a paper towel.
4. Add the secondary antibody.
A. For HuCAL® antibodies with a FLAG® tag (e.g. Fab-dHLX-FH or Fab-FH), add 100 µl of anti-FLAG® M2 antibody (Sigma, Cat. No. F3165) to each well, diluted 1:2000 in FACS buffer.
B. For HuCAL® antibodies with a c-Myc tag (e.g. Fab-dHLX-MH or Fab-MH), add 100 µl of anti-c-Myc antibody to each well, diluted 1:2000 in FACS buffer.
C. For HuCAL® antibodies with a His-6 tag (e.g. Fab-dHLX-MH or Fab-MH), add 100 µl of anti-His antibody to each well (e.g. Acris Antibodies cat.no. SM1693P) diluted 1:500 in FACS buffer.
Anti-c-Myc antibody can give higher background with endogenous c-Myc of dead cells. We therefore recommend the use of the anti-FLAG® M2 antibody where possible.
5. Add FACS buffer to one background control well, and anti-tag antibody to the second background control well.
6. Incubate the plates for 1 hour at 4°C, with gentle agitation on an ELISA shaker.
7. Centrifuge at 716 x g for 2 minutes at 4°C, remove the supernatant, and wash 2–3 times with 200 µl of FACS 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. Do not tap the plate on a paper towel, but place the plate on the bench and blot surface moisture from the top with a paper towel.
8. Add the tertiary antibody: 100 µl of R-Phycoerythrin-conjugated goat anti-mouse IgG antibody to each well, diluted 1:500 in FACS buffer.
9. Incubate the plates for 1 hour at 4°C, with gentle agitation on an ELISA shaker.
10. Centrifuge for 2 minutes at 4°C, remove the supernatant, and wash 2–3 times with 200 µl of FACS 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. Do not tap the plate on a paper towel, but place the plate on the bench and blot surface moisture from the top with a paper towel.
11. After the last washing step, centrifuge again for 2 minutes at 4°C, resuspend cells in 100–200 µl of FACS buffer, and transfer to FACS 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 aluminium foil and store at 4°C. Immediately before FACS analysis, add FACS flow buffer to give the required volume.

Pictures:

Schematic drawing of the main antibody formats that are available: Monovalent Fab with two tags (left); Bivalent Fab with two tags, functionally equivalent to a F(ab')2 fragment (middle); Full IgG (right). Monovalent and bivalent Fab antibodies are purified after bacterial expression via an epitope tag (red - orange).