HuCAL® Antibodies Technical Manual
Applications: Immunofluorescence

Immunofluorescence (IF) is a technique frequently used to provide information about the localization of a protein of interest in cells (Immunocytochemistry/ICC) or tissues (Immunohistochemistry/IHC). A primary antibody binds specifically to the protein and is then visualized using a fluorescent detection system with a fluorescence or confocal microscope. There are two types of IF staining methods: direct staining using primary antibodies conjugated to fluorophores; indirect staining using secondary antibodies conjugated to fluorophores.

Whereas indirect staining protocols result in signal amplification, as secondary antibodies carrying multiple labels bind to the primary antibody, there is no signal amplification using a directly conjugated primary antibody. This might result in weak staining or no staining at all if the target protein is present at low levels. Therefore, the use of directly conjugated antibodies in IF is only recommended for the detection of very abundant target proteins.

HuCAL antibodies can be used in immunofluorescence assays in the same manner as conventional antibodies with appropriate secondary reagents. Their high specificity reduces background caused by non-specific binding and therefore makes them ideal reagents for immunofluorescence analysis. The use of bivalent Fab formats further improves target recognition due to avidity effects. Recommended fluorescent labeled secondary antibodies and unconjugated anti-tag antibodies to be used in conjunction with fluorescent labeled antibodies are listed in Tables 1 and 2.

Table 1. Recommended fluorescent labeled secondary antibodies

Secondary Antibody	Emission Color	Recommended Dilution	Catalog Number
Anti-human F(ab')$_2$: FITC	Green	1:50-1:200	STAR126F
Anti-His-6-tag: Alexa Fluor® 488	Green	Neat-1:100	MCA1396A488
Anti-V5-tag:Alexa Fluor 488	Green	1:100	MCA1360A488
Anti-His-6-tag:DyLight® 549	Orange	1:50-1:200	MCA1396D549
Anti-His-6-tag: Alexa Fluor 647	Red	Neat-1:100	MCA1396A647
Anti-V5-tag:Alexa Fluor 647	Red	1:100	MCA1360A647
Anti-mouse IgG (H + L):DyLight 649	Red	1:500-1:1000	STAR117D649

Table 2. Recommended unconjugated anti-tag antibodies

Secondary Antibody	Recommended Dilution	Catalog Number
Anti-Strep-tag®	1:50-1:200	MCA2489
Anti-His-6-tag	1:50-1:200	MCA1396
Anti-V5-tag	Not determined	MCA1360

To find options for fluorescent labeled antibodies for use with the unconjugated anti-tag antibodies in Table 2, enter the catalog number (e.g. MCA2489) in the search tool at bio-rad-antibodies.com and check the 'Secondary Antibodies' section on the product page.

Protocol

Part 1. Sample Preparation

Buffer recipes and recommended sources for the reagents and antibodies are listed in the appendices.

There are three ways to prepare samples, depending on whether cells or tissue are to be stained.

A. Cultured cells (ICC)
B. Paraffin sections (IHC)
C. Frozen sections (IHC)

A. Cultured cells (ICC)

1. Grow cells in mutli-well plates.
2. Rinse cells briefly with sterile PBS.
3. Aspirate PBS and fix cells with either:
   a. 2-4% paraformaldehyde (PFA) for 10 minutes at 37°C.
   b. 100% methanol for 10 minutes at -20°C.
   Note: use sufficient solution to cover cells completely.

4. Aspirate fixative and wash cells with PBS, three times for 5 minutes per wash.
5. Optional: permeabilization; choose either option a or b:
   a. Add 0.2-0.5% Triton X-100 for 5 minutes at 37°C, rinse with PBS for 5 minutes.
   b. Remove PBS and add ice-cold methanol to cells. Use a sufficient amount to cover cells completely. Cells must not dry out. Incubate cells for 10 minutes at -20°C. Rinse with PBS for 5 minutes.

B. Paraffin sections (IHC)

B.1. Procedure for deparaffinization 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.
   Important: use adhesive slides.
2. Place the slides in a cuvette containing sufficient xylene to cover the tissue completely and incubate for 5 minutes with gentle shaking.
3. Transfer the slides to a cuvette containing fresh xylene and repeat step 2 for a further three washes, to give a total of four washes, each lasting 5 minutes.
   Important: use fresh xylene for each wash; use a fume food as xylene vapors are toxic.
4. Wash the slides twice in 100% ethanol (2 minutes per wash).
5. Wash the slides twice in 90% ethanol (2 minutes per wash).
6. Wash the slides twice in 80% ethanol (2 minutes per wash).
7. Wash the slides twice in 70% ethanol (2 minutes per wash).
8. Wash the slides twice in 60% ethanol (2 minutes per wash).
9. Wash the slides twice in 50% ethanol (2 minutes per wash).
10. Wash the slides twice in TBST (2 minutes per wash).

B.2. Procedure for antigen retrieval (heat mediated)

1. Deparaffinize and rehydrate the slides (Procedure B1).
2. Wash the slides a further three times (2 minutes per wash) in TBST.
3. Submerge the slides in TBST, 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 three times (2 minutes per wash) in TBST.

C. Frozen sections (IHC)

Procedure for fixation

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

1. Submerge slides in one of the following:
   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 to ensure that the fixative is completely removed from the sample.

Part 2. Immunostaining (ICC, IHC)

1. Block sample with 3% bovine serum albumin (BSA) or fetal bovine serum (FBS) in PBS for 1 hour. Alternatively, use 3-5% serum from the same species as the secondary antibody in PBS.
2. Remove blocking solution and apply HuCAL antibody at 1-10 µg/ml in PBS. Incubate overnight at 4°C.
3. Wash sample three times with PBS (5 minutes per wash).
4. Apply secondary antibody in PBS with 1% BSA or FBS and incubate for 1-2 hours in the dark.
5. Wash sample three times with PBS (5 minutes per wash).
6. Mount and seal the slides. For best results examine samples immediately. Store slides at 4°C in the dark.

Examples

MCF7 breast carcinoma cells were stained for Ki-67, a proliferation marker localized in the nucleus, with a Ki-67 specific HuCAL Fab bivalent antibody (HCA006). Detection was performed with an Alexa Fluor 488 conjugated anti-His-6-tag secondary antibody (MCA1396A488). Cells were counterstained with Phalloidin (F-actin, red) and Hoechst 33342 (nucleus, blue). The green Ki-67 foci are indicative of cell proliferation, Figure 1. Images were taken on an IN Cell Analyzer 1000 (GE Healthcare) at 40x magnification.

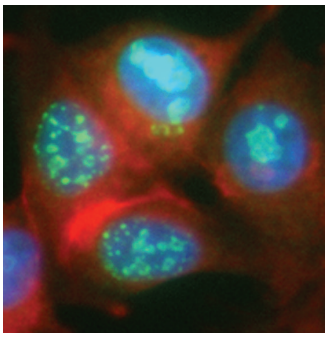

Fig. 1. Immunofluorescence Analysis of Ki-67 in MCF-7 Breast Cancer Cells.

Contactin-associated protein (Caspr) was examined in mouse nerve cells using a HuCAL anti-Caspr mini-antibody in combination with an Alexa Fluor 647 conjugated anti-human IgG secondary antibody. Caspr is found at the flanking paranodes (red). Nerve cells were stained with a mouse anti-ankyrin G antibody to mark the nodes of Ranvier (green), Figure 2. Images were captured with an Olympus FluoView 1000 laserpoint scanning confocal microscope.

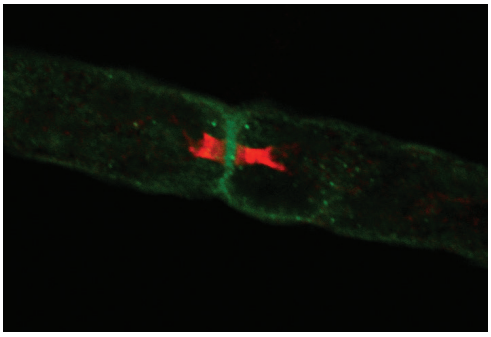

Fig. 2. Immunofluorescence Analysis of Caspr (red) and Ankyrin G (green) in Mouse Cerebellum Sections.

Data courtesy of B. Ranscht, Burnham Institute for Medical Research, La Jolla, CA, USA.

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
TBST	50 mM Tris-HCl, pH 7.4 0.15 M NaCl 0.1% Tween-20	Room temperature

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
Alexa Fluor 600 Phalloidin	Thermo Fisher Scientific	A22285
Anti-Ki67	Bio-Rad	HCA006 (Fab-dHLX-MH)
BSA	Sigma	A7906
FBS	PAN-Biotech	P30-3302
Hoechst 33342	Thermo Fisher Scientific	H1399
TritonX-100 detergent	Bio-Rad	1610407

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.

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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