HuCAL® Antibodies Technical Manual
Application: Western Blotting

Western blotting is a widely used analytical method for the detection and identification of protein antigens in complex samples such as cell lysates, cell culture supernatants, and tissue homogenates. The procedure involves separation of proteins by size using gel electrophoresis, a subsequent transfer of the proteins onto a solid support membrane, followed by detection with antigen-specific antibodies.

Prior to gel loading and separation, samples for western blotting are denatured by boiling in a buffer containing SDS and a reducing agent to unfold the proteins and disrupt disulfide bonds. It is customary to measure the protein content of each sample before denaturation to ensure the loading of equivalent amounts in each lane. After gel electrophoresis, the proteins are transferred to a blotting membrane using an electric field. The membrane is then blocked to prevent non-specific binding, before incubation with a primary antibody that recognizes the antigen of interest. Typically, a labeled secondary antibody is then applied that binds to the primary antibody. The most common labeling system utilizes the enzyme HRP and its substrate, which generate a chemiluminescent product detectible by an imager or on X-ray film.

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 a secondary antibody cannot be directed against the Fc domain, which is absent in HuCAL Fab format antibodies. Instead an anti-human Fab secondary antibody is recommended, to achieve signal amplification.

Alternatively, secondary antibodies against the epitope tags (typically part of the HuCAL Fab antibody), such as Strep-tag, His-6, V5, DYKDDDDK and c-myc are suitable. If the Fab antibody contains a bacterial alkaline phosphatase (BAP) domain, an anti-BAP antibody can also be used for detection. If a primary antibody is used regularly, or if the secondary antibody causes background, then the primary antibody can be labeled directly, for instance with a LYNX Rapid Conjugation Kit® (see Antibody Conjugation chapter). Primary antibodies conjugated with LYNX Rapid Conjugation Kits achieve comparable sensitivity to polyclonal secondary antibody detection, with the advantage of a significantly reduced assay time.

The sensitivity of a primary antibody in a given application largely depends on its affinity or avidity towards the antigen of interest. However, the structural conformation of the antigen in the application of choice also plays a role in determining the binding strength of the antibody. The affinities of HuCAL antibodies are comparable to those of other monoclonal antibodies. Bivalent antibodies have higher sensitivity than monovalent antibodies due to avidity effects, therefore the bivalent format of HuCAL antibodies is recommended for most applications, including western blotting.

Bio-Rad is equipped to run a large number of western blots in parallel, allowing us to offer the testing of purified HuCAL antibodies resulting from an antibody generation project as an additional service. Besides the classical western blot testing of purified antibodies, we also offer a screening of crude *E. coli* lysates expressing HuCAL Fab antibodies with an increased throughput. This service allows the identification of western blot positive antibodies early in the antibody generation process.

General Tips

We strongly recommend sonication of lysates using ultrasound. This serves to disrupt genomic DNA and should reduce non-specific background signals.

For membrane blocking, we recommend either Block ACE (catalog #BUF029) dissolved in water or 5% non-fat dried milk dissolved in tris-buffered saline-Tween 20 (TBST). Phosphate-buffered saline-Tween 20 (PBST) can be used instead of TBST but may lead to increased background during the detection of phosphoproteins. For western blotting of phosphoproteins, do not use milk as blocking agent, as it contains casein, a phosphoprotein itself; use Block ACE or 5% BSA instead.

It is recommended that both a positive control (antigen used for the antibody generation) and negative control (for instance, lysate not containing the antigen of interest) should be included to confirm the specificity of results.

It is also important to use an appropriate secondary antibody for the HuCAL monoclonal antibody being tested. It is best to refer to the HuCAL antibody product sheet for the antibody format and tags. A list of recommended HRP conjugated secondary antibodies is given in Table 1.

When working with antibodies that have been labeled with a LYNX Rapid Conjugation Kit, we strongly recommend HISPEC Assay Diluent (catalog #BUF049) for antibody dilution.

It is necessary to test for optimal antibody concentration to achieve sensitivities and background levels equivalent to detection with the most favorable secondary antibodies. Colorimetric detection substrates can also be used instead of chemiluminescent detection.

Bio-Rad's Stain-Free Technology offers a fast and elegant way to detect proteins in a gel both before and after transfer, as well as total protein content on a blotting membrane without the need of additional protein staining for instance, Coomassie Brilliant Blue, Ponceau S.

Table 1. Recommended HRP Conjugated Secondary Antibodies.

Secondary Antibody	Recommended Diluton	Catalog #
Goat Anti-Human F(ab')$_2$:HRP	1:5,000	STAR126P
Mouse Anti-Strep-Tag Classic:HRP	1:2,000-1:10,000	MCA2489P
Mouse Anti-Histidine Tag:HRP	1:1,000-1:5,000	MCA1396P
Mouse Anti-Penta Histidine Tag:HRP	1:2,500-1:10,000	MCA5995P
Mouse Anti-V5-Tag:HRP	1:500-1:1,000	MCA1360P
Human Anti-Bacterial Alkaline Phosphatase:HRP	1:1,000-1:10,000	HCA275P
Rat Anti-DYKDDDDK Tag:HRP	1:100-1:1,000	MCA4764P
Mouse Anti-c-Myc Tag:HRP	1:100-1:500	MCA2200P
TidyBlot™ Western Blot Detection Reagent:HRP	1:40-1:400	STAR209P

For other conjugates visit
bio-rad-antibodies.com/hucal-technical

Protocol

1. Run the samples on SDS-PAGE and transfer proteins onto a blotting membrane (polyvinylidene fluoride (PVDF) or nitrocellulose). For initial testing, use 40 µg of cell lysate proteins or 100 ng of pure antigen.
2. Optional: Check protein transfer by staining the membrane with Ponceau S for 1 min. Completely destain the membrane by washing with distilled water.
3. Block the membrane with Block ACE (#BUF029) or 5% non-fat dried milk in TBST for 1 hr on a shaker at RT, or overnight at 4˚C.
4. Rinse the membrane with TBST.
5. Dilute the primary HuCAL antibody in TBST with 1% non-fat dried milk and add to the membrane. The optimal concentration of HuCAL antibodies is typically between 1 and 10 µg/ml, but this must be determined empirically for each antibody. A concentration of 5 µg/ml is recommended as a starting point.
6. Incubate for 1 hr at RT on a shaker. Make sure that the membrane is completely immersed in the liquid.
7. Wash the membrane three times (3x) for 5 min per wash with a generous amount of TBST.
8. Add an appropriate enzyme-conjugated secondary antibody to the membrane. Goat Anti-Human F(ab')$_2$:HRP Antibody (#STAR126P) is recommended at a 1:5,000 dilution, either in TBST with 1% non-fat dried milk or in HISPEC Assay Diluent (cat. #BUF049).
9. Shake for 1 hr at RT.
10. Wash the membrane 3x for 5 min per wash with a generous amount of TBST.
11. Develop the membrane using Clarity™ Western ECL Blotting Substrate (Bio-Rad) according to the protocol. Capture the image with an imager such as ChemiDoc™ MP Imaging System or X-ray film.

Examples

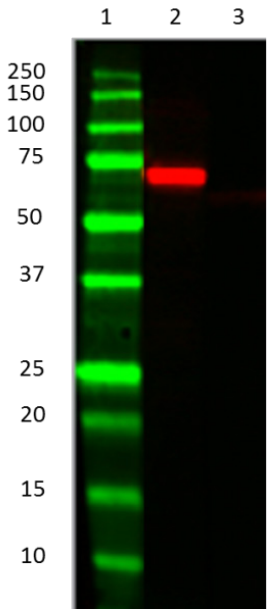

Fig. 1. Western blot analysis of HeLa cell lysate with Human Anti-Heat Shock Protein 8 (HSPA8) Antibody. Western blot analysis was performed by loading 25 µl HeLa lysate (lanes 2 and 3) and Precision Plus Protein™ Pre-stained Standards (lane 1) onto a AnykD™ Criterion™ TGX Stain-Free™ Gel. The gel was run under reducing conditions and proteins were transferred to a nitrocellulose membrane. Lane 2 was probed with Human Anti-HSPA8 Antibody, clone AbD28028 Fab-A-FH format, at a concentration of 10 µg/ml. Lanes 2 and 3 were then probed with secondary antibody Human Anti-Bacterial Alkaline Phosphatase:HRP Antibody, clone AbD25296_hIgG2 (#HCA275P) at a dilution of 1:1,000. Visualization was carried out using the ChemiDoc™ MP Imaging System with an auto exposure. The western blot bands (red) and protein standards (green) have been pseudocolored. Fab-A-FH format is a bivalent bacterial alkaline phosphatase fusion antibody with DYKDDDDK- and His-6-tags.

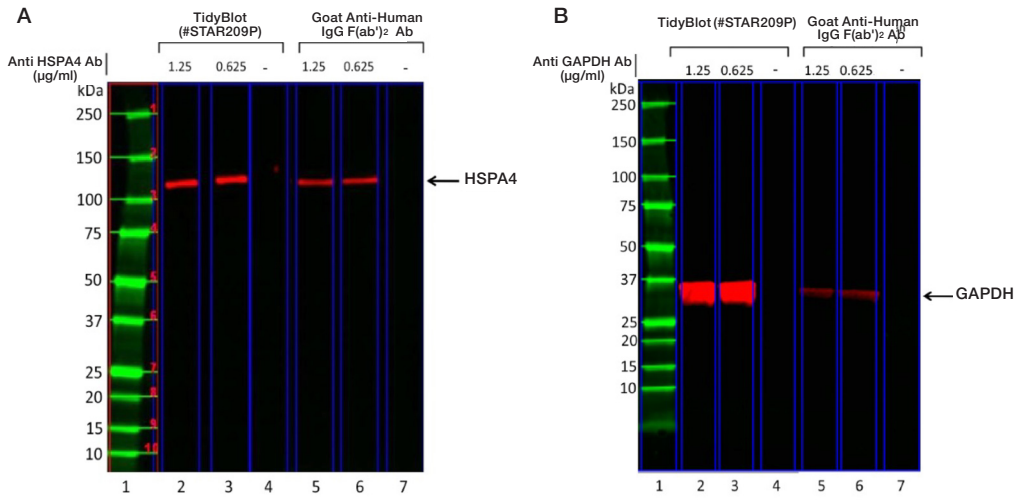

Fig. 2. Western blot analysis of two housekeeping proteins with different secondary antibodies. A, western blot analysis was performed by loading 25 µl MCF7 lysate (lanes 2-7) onto a AnykD Criterion TGX Stain-Free Gel. Precision Plus Protein Prestained Standards were run in lane 1. Lanes 2 and 3, and 5 and 6 were probed with Human Anti-HSPA4 Antibody, clone AbD23625_hIgG1 (#HCA273) at 1.25 µg/ml (lanes 2 and 5) and 0.625 µg/ml (lanes 3 and 6). Lanes 2-4 were probed with TidyBlot Western Blot Detection Reagent:HRP (#STAR209P) at 1:200. Lanes 5-7 were probed with Goat Anti-Human IgG F(ab')$_2$:HRP Antibody (#STAR126P) at 1:2,500. Visualization was carried out using the ChemiDoc MP Imaging System with an auto exposure. **B**, western blot analysis was performed by loading 25 µl Jurkat lysate (lanes 2-7) onto a AnykD Criterion TGX Stain-Free Gel. Precision Plus Protein Prestained Standards were run in lane 1. Lanes 2 and 3, and 5 and 6 were probed with Human Anti-GAPDH Antibody, clone AbD22549_hIgG1 (#HCA272) at 1.25 µg/ml (lanes 2 and 5) and 0.625 µg/ml (lanes 3 and 6). Lanes 2-4 were probed with TidyBlot Western Blot Detection Reagent:HRP (#STAR209P) at 1:200. Lanes 5-7 were probed with Goat Anti-Human IgG F(ab')$_2$:HRP Antibody (#STAR126P) at 1:2,500. Visualization was carried out using the ChemiDoc MP Imaging System with an auto exposure.

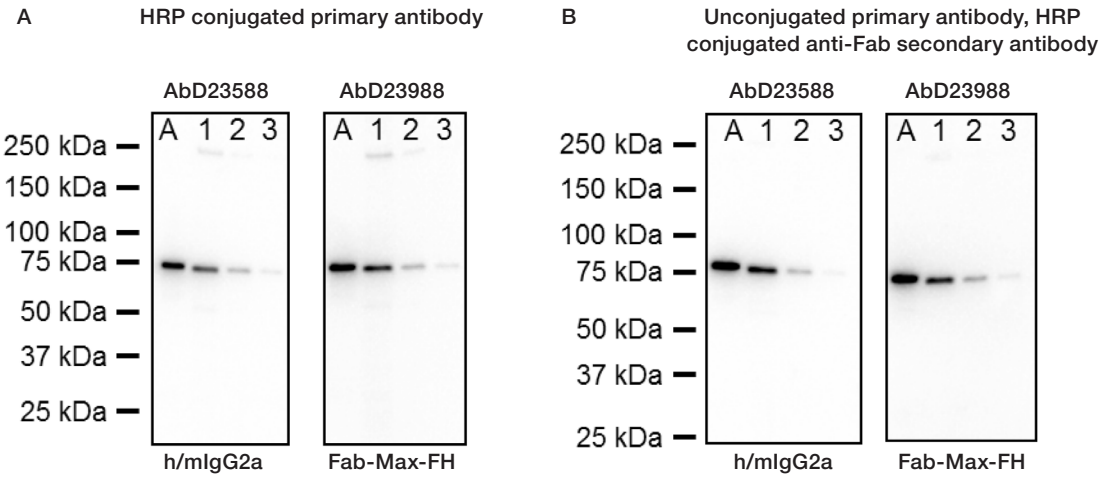

Fig. 3. Western blot analysis of HKB11 cell lysate with different detection methods. Western blot analysis was performed by loading 50 ng purified protein (lane A) or decreasing amounts (3-0.3 µg) of HKB11 lysate (lanes 1-3) and Precision Plus Protein Prestained Standards onto a AnykD Criterion TGX Stain-Free Gel. The gel was run under reducing conditions and proteins were transferred to a nitrocellulose membrane. **A**, blots were probed with HRP conjugated Human Anti-HSPA5 Antibody, clones AbD23588 h/mIgG2a format or AbD23988 Fab-Max-FH format, at a concentration of 10 nM. Visualization was carried out using the ChemiDoc MP Imaging System with an auto exposure. **B**, blots were probed with unconjugated Human Anti-HSPA5 Antibody, clones AbD23588 h/mIgG2a format or AbD23988 Fab-A-FH format, at a concentration of 10 nM. Blots were then probed with secondary Goat Anti-Human F(ab')$_2$:HRP Antibody (#STAR126P) at a dilution of 1:5,000. Visualization was carried out using the ChemiDoc MP Imaging System with an auto exposure.

Troubleshooting

Problem	Possible Cause and Course of Action
No bands on the western blot	1. Transfer did not work. Check protein by staining the gel and/or the membrane. 2. Incorrect secondary antibody used. Try an anti-human Fab or a suitable epitope tag antibody. 3. Antibody stored at 4˚C for several weeks or subjected to several freeze-thaw cycles. Use a fresh aliquot of antibody that has been stored at -20˚C or below. 4. Detection reagents are contaminated. Use fresh detection reagents. 5. Antigen not expressed in the source used or insufficient antigen loaded on the gel. Check source is appropriate or increase amount of source material. 6. Antibody not suitable for western blotting. Run a western blot with sufficient amounts of pure antigen.
Weak signal	1. Low transfer efficiency. Perform transfer according to the manufacturer's protocol. 2. Insufficient sample loaded on the gel. Load more sample. 3. Primary or secondary antibody concentration was too low. Optimize the protocol for the reagents. 4. Exposure time (for ECL detection) was too short. Re-expose the blot for a longer time.
White (negative) bands after detection with ECL system	1. Too much protein loaded or antibody concentrations too high, leading to depletion of substrate. Load less sample and optimize antibody concentrations.
High background signal	1. Concentration of primary and/or secondary antibody too high. Optimize antibody concentrations. 2. Transfer and incubation buffers are contaminated. Use fresh buffers. 3. Contaminated blocking agent. Use fresh blocking agent. 4. Incorrect concentration of blocking agent. Make sure the correct concentration is used, as described in the general tips/protocol section. 5. Membrane dried out during one or more steps of the procedure. Repeat, making sure that the membrane is not allowed to dry at any time during the procedure. 6. Washing time too short or insufficient buffer volume used for washes. Increase length of washing steps and/or volume of wash buffer. 7. Insufficient amount of Tween 20 in the buffers. Use TBS with 0.05-0.1% Tween 20. 8. Film overexposed or blot developed for too long. Reduce exposure and/or development times.
High background signal continued	9. Antibodies stored at 4°C for several weeks or subjected to several freeze-thaw cycles. Use a fresh aliquot of antibody that has been stored at -20 °C or below. 10. Non-specific interaction with genomic DNA. Sonicate the lysates with ultrasound or treat with DNases to disrupt/digest genomic DNA. 11. Secondary antibody is cross-reactive. Perform western blot without primary antibody to confirm cross-reactivity of secondary. Replace cross-reactive secondary with substitute that is suitable for the primary HuCAL antibody format in use.

Technical Assistance

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 specific 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** for more information or visit **bio-rad-antibodies.com/HuCAL**. 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.

Visit **bio-rad-antibodies.com/western-blotting** for a detailed guide dedicated to western blotting.

Visit **bio-rad-antibodies.com** for more information.

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