



# HuCAL® Antibodies Technical Manual

## Applications: ELISA

An ELISA (enzyme-linked immunosorbent assay) is used to detect antigens or antibodies in samples, either qualitatively or quantitatively.

There are several types of ELISA:

- **Indirect ELISA** is used to confirm binding of the antibody to its antigen, but not to unrelated antigens. It is performed using two antibodies for detection, a primary antibody specific to the antigen and a secondary antibody specific to the primary antibody. The secondary antibody is coupled to an enzyme that acts on a chromogenic or fluorogenic substrate to generate a signal. The enzymatic step amplifies the signal so that even low levels of protein can be detected. Since almost all HuCAL antibodies are tested for positive performance in ELISA, an indirect ELISA is a good control for testing all the reagents in the assay set-up
- **Direct ELISA** is very similar to indirect ELISA, but avoids the use of a secondary antibody by using a labeled primary antibody
- **Sandwich ELISA** is used to detect and quantify an antigen in a sample by the use of two specific primary antibodies binding to non-overlapping epitopes. One antibody captures the antigen on the plate and the second is used for detection
- **Bridging ELISA** is a special case of a sandwich ELISA in which a dimeric or oligomeric antigen (most often an antibody in a sample) is detected by a capture and detection antibody. The antigen bridges the two specific antibodies. Bridging ELISAs are most frequently used for the detection of IgG, e.g. in pharmacokinetic (PK) or anti-drug antibody (ADA) assays
- **Competition (or Inhibition) ELISA** detects antigens and monitors their concentration using competitive binding of an antibody to free versus immobilized antigen

### Use of HuCAL Antibodies in ELISA

ELISAs can be performed with HuCAL antibodies in the same protocols used for other polyclonal or monoclonal antibodies provided that a suitable secondary antibody is used. Since HuCAL antibodies in Fab format do not contain the Fc domain, an anti-human Fab secondary antibody is recommended for samples that do not contain human Ig. Monoclonal antibodies against an epitope tag (e.g. Strep-tag® or 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 and is similar to that of full IgGs. A list of recommended secondary antibodies is given in Table 1.

All HuCAL antibodies are routinely tested by indirect ELISA. The HuCAL custom services team is highly experienced and well equipped to run all types of ELISAs at high throughput, e.g. testing for sandwich pairs, inhibition ELISAs, etc. We offer additional ELISA services in conjunction with any custom HuCAL project.

**Table 1. Recommended secondary antibodies**

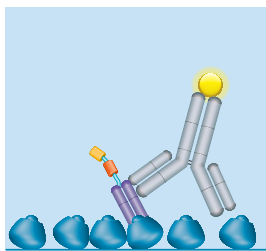
Secondary Antibody	Recommended Dilution	Catalog Number
Anti-human F(ab') <sub>2</sub> :AP	1:5000	STAR126A
Anti-human F(ab') <sub>2</sub> :HRP	1:5000	STAR126P
Anti-human F(ab') <sub>2</sub> :Biotin	1:5000	STAR126B
Anti-Strep-tag Classic:HRP	1:5000	MCA2489P
Anti-Strep-tag Immo (for Fab immobilization)		MCA2488
Anti-Penta Histidine tag:HRP	1:1000-1:2000	MCA5995P
Anti-V5-tag:HRP	1:1000	MCA1360P
Anti-DYKDDDDK-tag:HRP	1:10000-1:50000	AHP1074P
Anti-DYKDDDDK-tag:HRP	1:100-1:1000	MCA4764P

## General Points

- Sources for the reagents and antibodies recommended in the protocols are listed in Table 1 and the appendices
- 96-well plates can be used instead of 384-well plates. For the 96-well format, use 100  $\mu$ l (instead of 20  $\mu$ l) of antigen, antibodies or substrate and 300  $\mu$ l for the blocking step
- In the examples shown either an alkaline phosphatase (AP) conjugated antibody was used, together with the AttoPhos<sup>®</sup> fluorescence detection reagent (optimal excitation wavelength 430-440 nm, emission maximum 560 nm) or a horseradish peroxidase (HRP) conjugated antibody together with QuantaBlu<sup>™</sup> fluorescence detection reagent (excitation maximum 325 nm, range 315-345 nm; emission maximum 420 nm, range 370-460 nm). Use transparent plates if detection is performed using a chromogenic substrate. Example chromogenic substrates for HRP are TMB Core+ (BUF062) and TMB Sensitive (BUF066); example substrate for AP is pNPP (BUF044)
- If the antibody is to be immobilized as a capture antibody, this can be done either by direct coating to polystyrene plates or by using an anti-Fab or anti-tag antibody for capture. The anti-Strep-tag Immo antibody (MCA2488) offers a good affinity and is well-suited for this purpose
- HISPEC buffer (BUF049) is recommended for antibody dilution. It inhibits weak interactions resulting in lower background and higher reproducibility, especially for secondary and directly labeled primary antibodies

## Indirect ELISA

Indirect ELISA is often recommended as a control assay to test the performance of reagents. In an indirect ELISA the antigen is immobilized on a surface, such as a well of a microtiter plate. The plate is blocked to prevent the non-specific binding of detection antibodies, and then a specific primary 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 next, which is typically conjugated to an enzyme such as HRP or AP, and binds to the primary antibody. The plate is washed and the appropriate substrate is applied. Finally, the resulting chromogenic or fluorescent signal is analyzed using a spectrophotometer, Figure 1.



**Fig. 1. Indirect ELISA.** The antigen (blue) is coated to a surface. The primary antibody (purple monovalent Fab antibody) binds to the antigen and an enzyme-linked secondary anti-Fab antibody (gray full-size antibody) is used for detection.

## Protocol

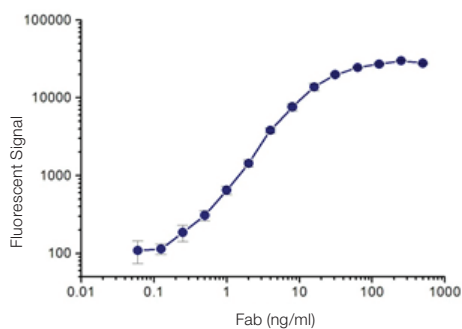
### Indirect ELISA with Fluorescence Readout

Buffer recipes and recommended sources for the reagents and antibodies are listed in Table 1 and the appendices.

1. Prepare the antigen at 5  $\mu$ g/ml in PBS. Coat the required number of wells of a 384-well microtiter plate with 20  $\mu$ l per well of the prepared capture antigen and incubate overnight at 4°C. Use a black, 384-well ELISA microtiter plate with square wells and flat-bottom.
2. Wash the microtiter plate five times with phosphate-buffered saline-Tween<sup>®</sup>-20 (PBST).
3. Block the microtiter plate by adding 100  $\mu$ l 5% non-fat dried milk in PBST to each well and then incubate for 1-2 hours at room temperature (RT).
4. Wash the microtiter plate five times with PBST.
5. Transfer 20  $\mu$ l HuCAL antibody to each well. A standard concentration of 2  $\mu$ g/ml in PBST or HISPEC buffer is recommended. Incubate for 1 hour at RT.  
**Note:** Optimize the concentrations of each HuCAL antibody by titration.
6. Wash the microtiter plate five times with PBST.
7. Add 20  $\mu$ l of the secondary antibody to each well and incubate for 1 hour at RT. Anti-human F(ab')<sub>2</sub>:AP (STAR126A), diluted 1:5000 in HISPEC buffer is recommended.
8. Wash the microtiter plate five times with PBST.
9. Add 20  $\mu$ l AttoPhos to each well. Measure fluorescence after 10 minutes.

## Example

The antigen adalimumab was coated on an ELISA plate and was detected with different concentrations of anti-adalimumab antibody (HuCAL monovalent Fab, HCA202), Figure 2. Since the antigen and the detection antibody are human antibodies, an HRP conjugated anti-His tag secondary antibody (MCA5995P) was used.

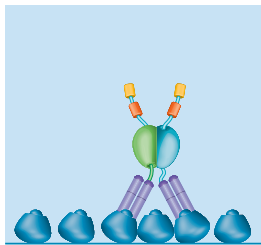


**Fig. 2. Indirect ELISA.** Immobilized adalimumab was detected with a HuCAL anti-adalimumab Fab-FH antibody and anti-His tag:HRP, with QuantaBlu detection. Data are mean of three measurements.

## Direct ELISA

In a direct ELISA, the primary antibody is labeled, for example, by genetic fusion with AP or using a LYNX Rapid Conjugation Kit<sup>®</sup>. This set-up saves time and money because no secondary antibody is required, which is valuable for frequently run assays. It also avoids the cross-reactivity and background issues sometimes introduced by secondary antibodies.

Antibodies directly conjugated with HRP show excellent sensitivity, often similar or even better than those achieved in indirect ELISAs. The AP genetic fusion system, e.g. Fab-A-FH is usually less sensitive when the AP activity is used directly for detection, Figure 3 and Figure 4. If it turns out that the sensitivity is too low, indirect detection via a secondary antibody can be used.



**Fig. 3. Direct ELISA.** The antigen (blue) is coated to a surface. A fusion protein consisting of the primary antibody and bacterial alkaline phosphatase (blue/green) is used in combination with an AP substrate for detection.

## Protocol

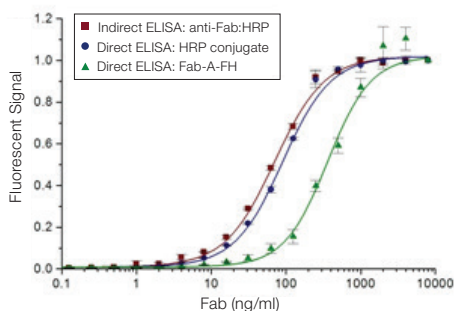
### Direct ELISA with an HRP Conjugated Primary Antibody or AP-Fusion Fab Antibody

Buffer recipes and recommended sources for the reagents and antibodies are listed in Table 1 and the appendices.

1. Prepare the antigen at 5 µg/ml in PBS. Coat the required number of wells of a 384-well microtiter plate with 20 µl per well of the prepared capture antigen and incubate overnight at 4°C. Use a black, 384-well ELISA microtiter plate with square wells and flat-bottom.
2. Wash the microtiter plate five times 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 RT.
4. Wash the microtiter plate five times with PBST.
5. Transfer 20 µl of HRP conjugated HuCAL antibody or AP-fusion Fab antibody to each well. Use a standard concentration of 2 µg/ml in PBST or HISPEC buffer. Incubate for 1 hour at RT.  
**Note:** Optimize the concentrations of each HuCAL antibody by titration.
6. Wash the microtiter plate five times using PBST.
7. For HRP conjugated antibodies: Add 20 µl QuantaBlu fluorescence detection reagent to each well and measure the fluorescence after 10 minutes;  
For AP-fusion antibodies: Add 20 µl AttoPhos to each well and measure the fluorescence after 10 minutes.

## Example

Human recombinant ST2 was detected by both direct and indirect ELISA. Direct ELISA was performed using a HuCAL anti-ST2 antibody fused with AP (Fab-A-FH format), or with an HRP conjugated anti-ST2 bivalent mini-antibody (same clone in Fab-dHLX-MH format). Indirect ELISA was performed using the same mini-antibody and anti-Fab:HRP detection (STAR126P), Figure 4.



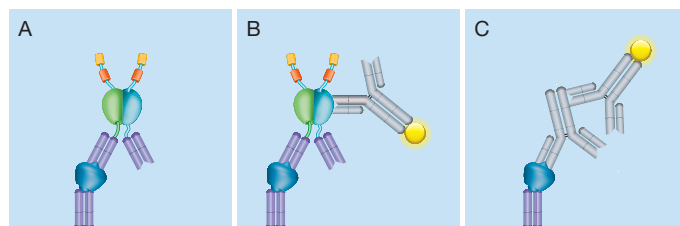
**Fig. 4. Comparison of Direct and Indirect ELISA.** Direct ELISA was performed using a HuCAL anti-ST2 antibody either in the Fab-A-FH format (green triangle) or in the bivalent mini-antibody Fab-dHLX-MH format conjugated to HRP (blue circle). For the indirect ELISA, the mini-antibody was detected with anti-Fab:HRP (red square). Note that the use of a secondary antibody or the use of antibody directly conjugated with HRP provides an amplification effect that increases the sensitivity of the assay.

## Sandwich ELISA

Sandwich ELISAs are a highly sensitive and specific method of detecting antigens in solution and provide fast and accurate determination of the antigen concentration in a given sample. The technique uses two primary antibodies, both of which are specific to the antigen of interest, but which bind the antigen at non-overlapping epitopes.

In a sandwich ELISA it is possible to use two different HuCAL antibodies as the sandwich pair, or to combine a HuCAL antibody as the capture or detection antibody together with a non-HuCAL antibody. When two HuCAL antibodies are used, the detection antibody is typically conjugated to HRP, e.g. with a LYNX Rapid Conjugation Kit (LNK001P), or biotinylated for detection via streptavidin. Alternatively, the two HuCAL antibodies can be equipped with different tags to allow detection via an anti-tag antibody that is specific for the detection antibody, e.g. Fab-dHLX-MSx2 capture antibody, Fab-A-V5Sx2 detection antibody and a suitable anti-tag antibody, Figure 5.

Best sensitivities are achieved with detection antibodies in IgG format closely followed by bivalent Fab-AP fusion antibodies (Fab-A formats). The format of the capture antibody is of lesser importance for the sensitivity of the assay.



**Fig. 5. Sandwich ELISA.** Sandwich ELISAs are carried out using three detection options. **A:** Two HuCAL antibodies with direct detection. **B:** Two HuCAL antibodies with indirect detection using HRP conjugated anti-tag antibody. **C:** A HuCAL capture antibody and an Ig detection antibody (monoclonal, polyclonal, HuCAL Ig format) plus an HRP conjugated anti-Ig secondary antibody. The capture antibody (purple monovalent Fab antibody) is immobilized and binds the antigen (blue). HRP represented by yellow circle.

## Protocol

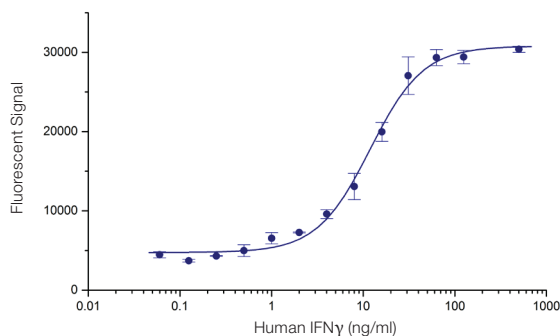
### Sandwich ELISA using Two HuCAL Antibodies

Buffer recipes and recommended sources for the reagents and antibodies are listed in Table 1 and the appendices.

1. Prepare the capture antibody at 5 µg/ml in PBS. Coat the required number of wells of a 384-well microtiter plate with 20 µl per well of the prepared capture antibody and incubate overnight at 4°C. Use a black, 384-well ELISA microtiter plate with square wells and flat-bottom.
2. Wash the microtiter plate five times with PBST.
3. Block the microtiter plate by adding 100 µl 5% BSA in PBST to each well and incubate for 1 hour at RT.
4. Wash the microtiter plate five times with PBST.
5. Add 20 µl of antigen to each well of the microtiter plate and incubate for 1 hour at RT. Use a range of antigen concentrations, diluted in PBST with 1% BSA or in HISPEC buffer.
6. Wash the microtiter plate five times with PBST.
7. Add 20 µl HuCAL detection antibody (1-2 µg/ml concentration in HISPEC buffer) to each well, and incubate for 1 hour at RT. Use one of the following options:
  - a. Use an HRP conjugated detection antibody, e.g. conjugated via LYNX Rapid Conjugation Kit. Move directly to step 10.
  - b. Use a biotinylated detection antibody.
  - c. Use a detection antibody carrying different tags to the capture antibody, e.g. His-6 tagged capture antibody and Strep-tag detection antibody.
8. Wash the microtiter plate five times with PBST.
9. Transfer 20 µl of secondary detection antibody to each well and incubate for 1 hour at RT. Choose option below to match step 7.
  - a. Skip this step.
  - b. Use either streptavidin:HRP (STAR5B) or streptavidin:AP (STAR6B) diluted 1:1000 in HISPEC buffer in combination with matching substrate.
  - c. Use matching epitope tag antibodies, e.g. anti-Strep-tag Classic:HRP (MCA2489P) diluted 1:5000 in HISPEC buffer.
10. Wash the microtiter plate ten times with PBST.
11. Add 20 µl QuantaBlu to each well for HRP conjugated secondary antibodies or AttoPhos for AP conjugated secondary antibodies and measure the fluorescence directly.

## Example

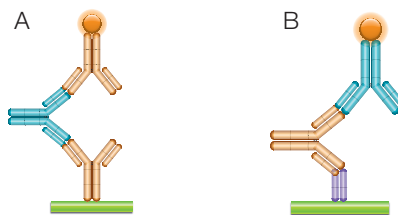
A sandwich ELISA was performed using a pair of HuCAL antibodies, Figure 6.



**Fig. 6. Sandwich ELISA for IFN $\gamma$ .** A bivalent mini-antibody directed against IFN $\gamma$  in the Fab-dHLX-MH format (HCA043) was used for capture. A serial dilution of the antigen IFN $\gamma$  (PHP050) was detected with an HRP conjugated bivalent Fab (Fab-A-FH format, HCA044) together with QuantaBlu HRP substrate for detection.

## Bridging ELISA

A bridging ELISA is a special case of a sandwich ELISA in which the antigen is a dimer or oligomer that bridges the capture and detection molecules. Most commonly it is used for the detection and quantification of IgG, i.e. therapeutic or anti-idiotypic IgG, in pharmacokinetic (PK) and anti-drug antibody (ADA) assays, Figure 7. The HuCAL technology has proven to be excellent for the generation of anti-idiotypic antibodies. Although in a bridging ELISA capture and detection antibody could be the same clone (the epitope is available twice or more often on the antigen), the use of different antibodies often leads to higher sensitivities (unpublished observations).



**Fig. 7. ADA and PK Bridging ELISA.** **A:** The ADA bridging ELISA is performed by immobilizing the therapeutic antibody (gold). An anti-idiotypic antibody (or anti-drug antibody, blue) is detected by forming a bridge to the conjugated therapeutic antibody (gold antibody with circle representing enzyme label). **B:** For a PK bridging ELISA, the anti-idiotypic antibody is immobilized (purple monovalent Fab antibody). The therapeutic antibody (gold) forms the bridge and is detected by a conjugated anti-idiotypic antibody (blue IgG).

In a standard PK bridging assay, a HuCAL capture antibody is coated to a plate. The therapeutic Ig binds with only one arm to the immobilized HuCAL antibody and uses the other arm to form the bridge to the HuCAL detection antibody. Therefore, it is very important to optimize the coating concentration to avoid bivalent binding of the IgG to the plate but coat as much capture antibody as possible to keep the signals as strong as possible. The detection antibody is usually conjugated to HRP to allow detection without the use of a tertiary antibody.



## Protocol

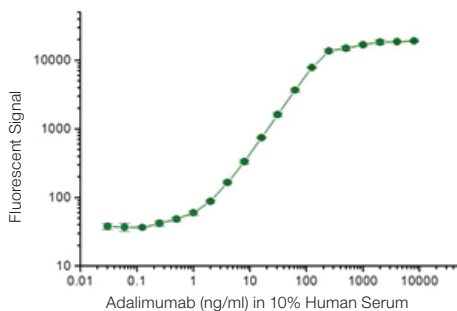
### PK Bridging ELISA for Quantification of Human IgG

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

1. Coat the capture antibody by adding 20  $\mu$ l to the required number of wells of a 384-well microtiter plate and incubate overnight at 4°C. Optimal concentration has to be determined for each antibody and usually is in the range of 0.5-1.5  $\mu$ g/ml in PBS. Use a black, 384-well ELISA microtiter plate with square wells and flat-bottom.
2. Wash the microtiter plate five times with PBST.
3. Block the microtiter plate by adding 100  $\mu$ l 5% BSA in PBST to each well and incubate for 1 hour at RT.
4. Wash the microtiter plate five times with PBST.
5. Add 20  $\mu$ l of IgG to each well of the microtiter plate and incubate for 1 hour at RT. Use a range of IgG concentrations diluted in PBST with or without addition of normal serum, e.g. 10% normal human serum (NHS).
6. Wash the microtiter plate five times with PBST.
7. Add 20  $\mu$ l HRP conjugated HuCAL detection antibody (1-2  $\mu$ g/ml concentration in HISPEC buffer) to each well and incubate for 1 hour at RT.
8. Wash the microtiter plate ten times with PBST.
9. Add 20  $\mu$ l QuantaBlu to each well for HRP conjugated secondary antibodies and measure the fluorescence after 30 minutes.

## Example

A PK Bridging ELISA was performed using two HuCAL anti-idiotypic antibodies against adalimumab. The capture antibody was a monovalent Fab (Fab-FH format, HCA202). A serial dilution of the drug adalimumab in 10% NHS was detected with HRP conjugated HuCAL IgG1 (HCA204P) and QuantaBlu substrate, Figure 8.



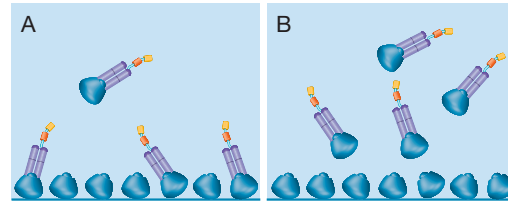
**Fig. 8. PK Bridging ELISA for Adalimumab.** In this ELISA a monovalent anti-adalimumab Fab was immobilized as the capture antibody. Adalimumab in 10% human serum was titrated. Detection was performed with HRP conjugated anti-adalimumab IgG and QuantaBlu fluorescence substrate.

## Competition (or Inhibition) ELISA

A 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 by two antibodies, 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 has bound to the immobilized antigen is detected using a secondary antibody conjugated to a detection label such as HRP. The appropriate substrate is applied and the resulting chromogenic or fluorescent signal is analyzed using a spectrophotometer. With higher amounts of free antigen in the sample, there are fewer antibody molecules available to bind the immobilized antigen, resulting in a weaker signal. Conversely, lower amounts of free antigen in solution generate stronger signals, Figure 9. Other variations of a competition ELISA are also possible.

Inhibition ELISAs are also frequently used to confirm binding of the antibody to the free, unmodified antigen, especially for hapten or peptide antigens. A monovalent antibody format is preferred for inhibition assays since one free antigen molecule is sufficient to inhibit binding of the antibody to the plate whereas two antigens are required for a bivalent IgG.



**Fig. 9. Examples of Competition ELISAs.** **A:** Competition ELISA with low concentration of free antigen (blue); HuCAL antibodies (purple) bind to the coated antigen. **B:** Competition ELISA with high concentration of free antigen. More HuCAL antibodies are kept in solution by binding to free antigen and are washed away before the addition of the secondary antibody.

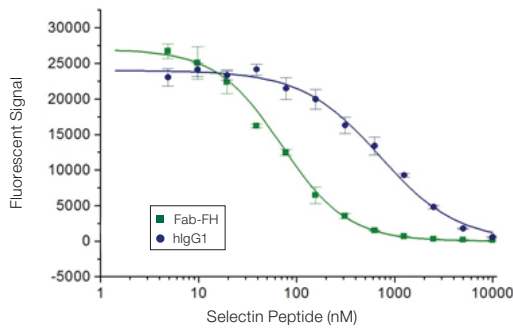
## Protocol

Buffer recipes and recommended sources for the reagents and antibodies are listed in Table 1 and the appendices.

1. Prepare the antigen at 5  $\mu$ g/ml in PBS. Coat the required number of wells of a 384-well microtiter plate with 20  $\mu$ l per well of the prepared capture antigen and incubate overnight at 4°C. Use a black, 384-well ELISA microtiter plate with square wells and flat-bottom.
2. Wash the microtiter plate five times with PBST.
3. Block the microtiter plate by adding 100  $\mu$ l of 5% non-fat dried milk in PBST to each well and incubate for 1-2 hours at RT.
4. Incubate 15  $\mu$ l of antigen solution with 15  $\mu$ l of HuCAL antibody (final concentration 2  $\mu$ g/ml) for 1 hour at RT. Use a range of antigen concentrations diluted in PBS or HISPEC buffer.
5. Wash the microtiter plate five times with PBST.
6. Transfer 20  $\mu$ l of HuCAL antibody/antigen mix to each well of the microtiter plate and incubate for 30 minutes at RT.
7. Wash the microtiter plate five times with PBST.
8. Transfer 20  $\mu$ l of the secondary antibody (anti-human F(ab')<sub>2</sub>:AP) diluted 1:5000 in HISPEC buffer to each well and incubate for 1 hour at RT.
9. Wash the microtiter plate ten times with PBST.
10. Add 20  $\mu$ l AttoPhos to each well. Incubate for 10 minutes at RT and measure fluorescence.

## Example

Binding of an antibody to a short peptide derived from human selectin was measured, Figure 10. Neutravidin was coated to a microtiter plate and biotinylated selectin peptide was captured. A mix of anti-selectin antibody and varying amounts of free peptide were transferred to the ELISA plate. The antibody as monovalent Fab was compared to the bivalent IgG format. Both antibodies were used at equimolar concentrations of the antigen binding sites. As expected, the monovalent antibody format showed better sensitivities for this assay set-up. For both formats detection was performed using anti-human F(ab')<sub>2</sub>:AP and AttoPhos substrate.



**Fig. 10. Inhibition ELISA.** Binding of an anti-selectin HuCAL antibody to biotinylated selectin immobilized via coated neutravidin is inhibited by addition of varying amounts of unbiotinylated selectin peptide. Comparison of the antibody in monovalent Fab (Fab-FH) and bivalent IgG1 format shows that the monovalent Fab format is preferred for inhibition assays. Detection was performed with an anti-F(ab')<sub>2</sub>:AP secondary antibody. Data are shown as mean of 3 experiments.

## Troubleshooting

Problem	Possible Cause and Course of Action
No signal in ELISA	<ol style="list-style-type: none"><li>1. Assay incorrectly set up or incorrect reagents used. Include a positive control.</li><li>2. Improper secondary antibody used. Use an anti-human Fab or a suitable epitope tag antibody.</li><li>3. Antibody stored at 4°C for several weeks or subjected to several freeze-thaw cycles. Use fresh aliquot of antibody that has been stored at -20°C or below or test antibody sample first on purified antigen.</li><li>4. Detection reagent contaminated. Use freshly prepared detection reagent.</li><li>5. Antigen not coated properly. Try longer coating times, different buffers, or use avidin plates with biotinylated antigen.</li><li>6. Incorrect settings on plate reader for this detection system. Check values (wavelength, filters, gain etc).</li></ol>

Problem	Possible Cause and Course of Action
Weak signal	<ol style="list-style-type: none"><li>1. Insufficient amount of antigen coated to microtiter plate. Use more antigen for coating step.</li><li>2. Concentration of primary or secondary antibody too low. Optimize the protocol for the reagents.</li><li>3. Detection reagent too old or contaminated. Use fresh detection reagent.</li><li>4. Detection reagent diluted. Use a higher concentration of detection reagent.</li><li>5. Incubation time with detection reagent too short. Allow longer incubation after addition of detection reagent before starting the measurement.</li><li>6. Incorrect settings on plate reader for this detection system. Check values (wavelength, filters, gain etc).</li></ol>
High background signal	<ol style="list-style-type: none"><li>1. Concentration of primary and/or secondary antibody too high. Optimize antibody concentrations.</li><li>2. Too much detection reagent used. Repeat with more dilute detection reagent.</li><li>3. Number of washing cycles too low. Increase number of washing cycles.</li><li>4. Contaminated blocking agent. Use fresh blocking agent.</li><li>5. Incorrect concentration of blocking agent. Make sure that the correct concentration is used, as described in the protocol.</li><li>6. Incubation time of detection reagent too long. Start measurement soon after addition of detection reagent.</li><li>7. Incorrect settings on the reader. Adjust the settings (e.g. gain).</li><li>8. Insufficient amount of Tween-20 in the buffers. Use PBS with 0.05% Tween-20.</li><li>9. Use HISPEC buffer for primary and secondary antibody dilution.</li></ol>

## Appendices

### Buffer composition

Buffer	Composition	Storage
PBS	136 mM NaCl 2.68 mM KCl 8.1 mM Na <sub>2</sub> HPO <sub>4</sub> 1.46 mM KH <sub>2</sub> PO <sub>4</sub>	Room temperature
PBST	PBS with 0.05% Tween-20	Room temperature

## Source of antibodies and reagents

Reagent	Supplier	Catalog Number
96-well, round bottom microtiter plates, sterile	Thermo Fisher Scientific	163320
96-well, flat-bottom, black MaxiSorp™ microtiter plates	Thermo Fisher Scientific	437111
384-well, flat-bottom, black MaxiSorp microtiter plates	Thermo Fisher Scientific	460518
Antibody diluent	Bio-Rad	BUF014
Anti-adalimumab	Bio-Rad	HCA202, Fab-FH
Anti-adamlumab: HRP	Bio-Rad	HCA204P, IgG1
Anti-human IFN $\gamma$	Bio-Rad	HCA043, Fab-dHLX-MH HCA044, Fab-A-FH
AttoPhos substrate for the detection of alkaline phosphatase	Roche	11681982001
BSA	Sigma	A7906
HISPEC Buffer	Bio-Rad	BUF049
Recombinant human IFN $\gamma$	Bio-Rad	PHP050
LYNX Rapid HRP Conjugation Kit	Bio-Rad	LNK001P
pNPP substrate for the detection of alkaline phosphatase	Bio-Rad	BUF044
QuantaBlu Fluorogenic Peroxidase Substrate Kit	Thermo Fisher Scientific	15169
Streptavidin:AP	Bio-Rad	STAR6B
Streptavidin:RPE	Bio-Rad	STAR4B
Streptavidin:HRP	Bio-Rad	STAR5B
TMB Core+ substrate for HRP	Bio-Rad	BUF062
TMB Sensitive substrate for HRP	Bio-Rad	BUF066

## 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](mailto:antibody_sales_muc@bio-rad.com) or visit [bio-rad-antibodies.com/HuCAL](http://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](mailto: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](http://bio-rad-antibodies.com) for more information.



**Bio-Rad**  
Laboratories, Inc.