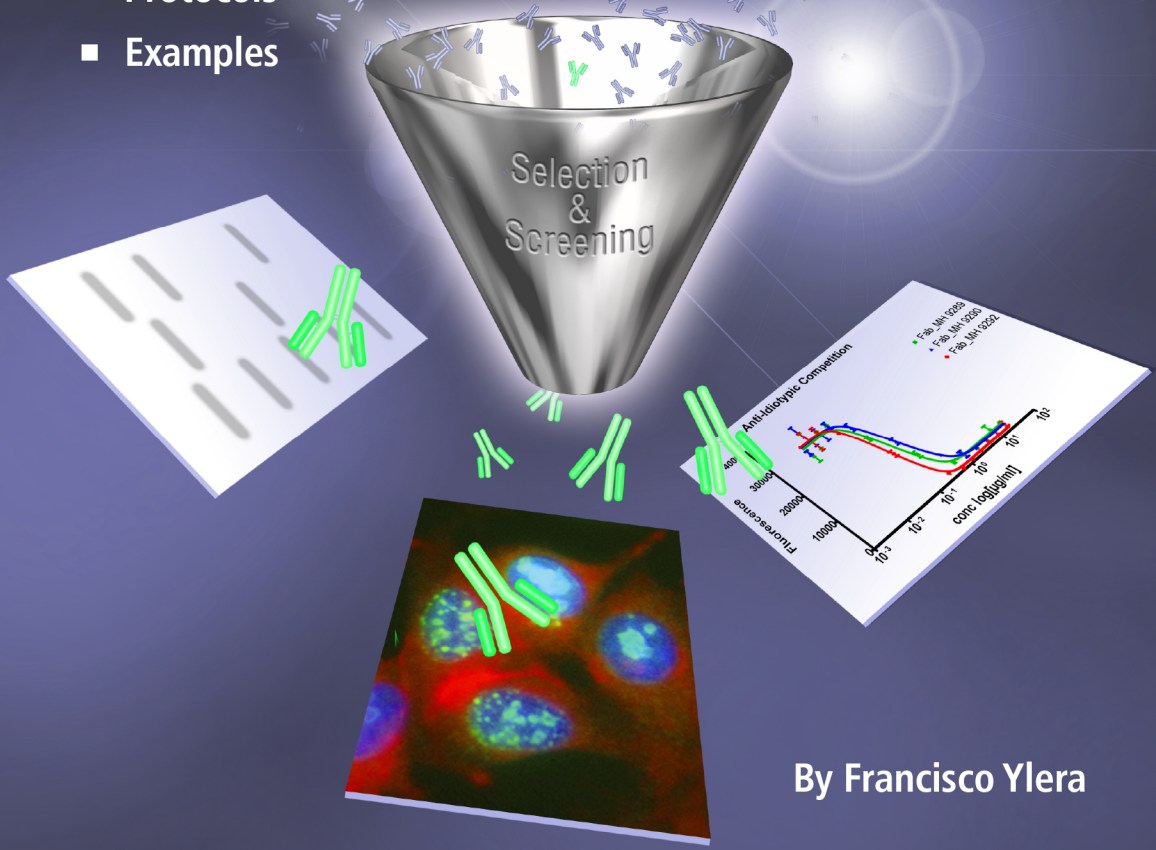
HuCAL® Antibodies Technical Manual

Second Edition

- Technology
- Protocols
- Examples



By Francisco Ylera

Your first choice for antibodies!

AbD serotec
A Division of MorphoSys

AbD serotec

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Abbreviations

ABC	Avidin biotin complex
ADA	Anti-drug antibody assay
AgX®	AbD Serotec antigen expression service
AP	Alkaline phosphatase
BCIP	5-Bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BSA	Bovine serum albumin
CDR	Complementary determining region
CH1, 2, 3	Constant region 1, 2, or 3 of the antibody heavy chain
CL	Constant region of the antibody light chain
c-myc	c-myc epitope tag with the sequence: AEEQKLISEEDLL
CRA	Closely related antigen
DAB	3,3' Diaminobenzidine
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence (Western blotting)
ECL	Electrochemiluminescence (SET)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS™	Fluorescence activated cell sorter
FDPS	Farnesyl diphosphate synthase
FITC	Fluorescein isothiocyanate
FLAG®	Epitope tag with the sequence: DYKDDDDK
FLISA	Fluorescence-linked immunosorbent assay
FMAT®	Fluorometric microvolume assay technology
HBS	Hepes-buffered saline
His-6	Epitope tag with the sequence: HHHHHH
HRP	Horseradish peroxidase
HuCAL®	Human Combinatorial Antibody Library
IHC	Immunohistochemistry
k_{on}	Association rate, on rate
k_{off}	Dissociation rate, off rate
K_D	Dissociation constant
LOD	Limit of detection

LSAB	Labeled streptavidin biotin
MWCO	Molecular weight cut-off
MSD™	Meso Scale Discovery™
NBT	Nitro-blue tetrazolium chloride
Ni-NTA	Nickel-nitrilotriacetic acid
ORI	Origin of replication
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline-Tween® 20
PCR	Polymerase chain reaction
PE (or RPE)	R-Phycoerythrin
PK	Pharmacokinetics
PVDF	Polyvinylidene fluoride
QC	Quality control
QCM	Quartz crystal microbalance
RPE (or PE)	R-Phycoerythrin
RT	Room temperature
scFv	Single-chain variable fragment
SDS	Sodium dodecyl sulfate
SET	Solution equilibrium titration
SPR	Surface plasmon resonance
ssDNA	Single stranded DNA
Strep-tag®	Epitope tag with the sequence: WSHPQFEK
Sx	Extended Strep-tag® with the sequence: SA-WSHPQFEK
TBST	Tris-buffered saline-Tween® 20
Trf	Human transferrin
TRIM	Trinucleotide mutagenesis
VH	Variable region of the antibody heavy chain
VL	Variable region of the antibody light chain

Introduction

Over the last three decades, the use of antibodies has increased greatly, both as tools for basic research and diagnostics, and as therapeutic agents. This has been driven in part by advances in recombinant antibody technology. At the forefront of these advances is The **Human Combinatorial Antibody Library (HuCAL®)**, one of the most powerful synthetic antibody libraries ever created.

At a glance, the HuCAL® library is simply a unique collection of billions of human antibodies. However, a more detailed examination shows that it is a highly sophisticated tool with features that make it the system of choice for basic research, target validation, drug screening, and to study novel proteins created by the proteomics revolution. It has proved to be a direct source of antibodies for diagnostic and therapeutic applications, and has been integrated into the research and development processes of many leading pharmaceutical and biotechnology companies.

With the production of more than 13,000 novel antibody specificities in recent years, and the rapid 8-week production turnaround, this technology has also succeeded in accelerating our clients´ research.

AbD Serotec is a Division of MorphoSys AG which provides for the custom development of novel antibody specificities using HuCAL® technology on a project by project basis for research, diagnostic, and other non-therapeutic commercial applications as a service to individual customers.

Up-to-date information on AbD Serotec's antibody generation services, and their catalog of over 14,000 antibodies and immunoreagents, can be found online at **www.abdserotec.com.**

For more information about MorphoSys AG, the development of therapeutic antibodies, and access to MorphoSys' HuCAL® technology, please visit **www.morphosys.com.**

We cordially invite you to learn more about the benefits of our sophisticated *in vitro* technology in this manual, which is divided into easy-to-use chapters full of key technical and application information.

The first two chapters outline the concept of the HuCAL® technology platform, and describe its many advantages over antibodies generated using traditional animal-based technologies.

The next seven chapters explain the use of HuCAL® antibodies in applications such as Western blotting, enzyme-linked immunosorbant assay (ELISA), immunoprecipitation, immunohistochemistry (IHC), flow cytometry, immunofluorescence, and bead-based assays. These sections feature standard protocols which have been optimized for HuCAL® monoclonals, as well as actual examples, hints, and troubleshooting tips.

The manual also includes procedures for affinity determination and for labeling antibodies with biotin, different fluorescent dyes, and enzymes, plus several other useful appendices, making this publication a comprehensive technical resource for HuCAL® recombinant antibody technology.

Introduction to Recombinant Antibodies

Chapter 1

What are Antibodies?

Antibodies are glycoproteins that are naturally produced in response to invading foreign particles (antigens) such as microorganisms and viruses. They play a critical role in the immune system's defense against infection and disease.

Ideally, every antibody recognizes and binds to just one antigen[1]. Antigens can be proteins (for instance, receptors expressed on cancerous cells), sugars on bacterial and viral cell surfaces, hormones, chemical compounds, nucleic acid structures, and so on. The region of an antigen that interacts with an antibody is termed the epitope. In the case of protein antigens, epitopes can be linear or three dimensional configurations of amino acids, or individual amino acids that have been modified (for example, by phosphorylation or oxidation). Typically, only foreign substances elicit an immune response.

Normally, an animal's immune system will generate a large group of antibodies that recognize several epitopes from a particular antigen. Each antibody is secreted by a different antibody-producing plasma cell, and may be specific for a different epitope. Since the antibodies found in serum are collectively produced by many plasma cells (clones), they are described as polyclonal. While this is an advantage for fighting infections in nature, the heterogeneity of polyclonal antibodies limits their use as research tools.

A major breakthrough in the development of antibodies for research applications was the production of monoclonal antibodies in 1975 by Köhler and Milstein[2]. Their technique involved removing B-cells (plasma cell precursors) from the spleen of an animal that had been challenged with an antigen, and subsequent fusing with an immortal myeloma tumor cell line. This resulted in a single-cell hybrid known as a hybridoma. The B-cells confer antibody production capability, while the myeloma cells enable hybridomas

[1] In reality, most antibodies are not fully monospecific and will also bind to other substances, albeit with lower affinity.

[2] Köhler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**:495–497

to divide indefinitely and to grow well in cell culture. Hybridomas secrete only one antibody type, effectively ensuring an infinite supply of antibodies selective for a single epitope, which are also known as monoclonals.

Basic Antibody Structure

Many of the key structural features of antibodies can be considered using immunoglobulin G (IgG) as a model, since IgG is the most abundant antibody in serum.

The classical representation of an antibody is as a Y-shaped molecule composed of four polypeptide subunits with two identical heavy and light chains (Figure 1). The N-terminus of each heavy chain associates with one of the light chains to create two antigen-binding regions; these are the arms of the Y shape and are termed Fab domains. The C-termini of the two heavy chains combine to form the Fc domain; this is the tail of the Y. The Fc domain is important for the antibody's interaction with macrophages and for activation of the complement cascade. The four polypeptide chains are held together by covalent disulfide bridges and non-covalent bonds.

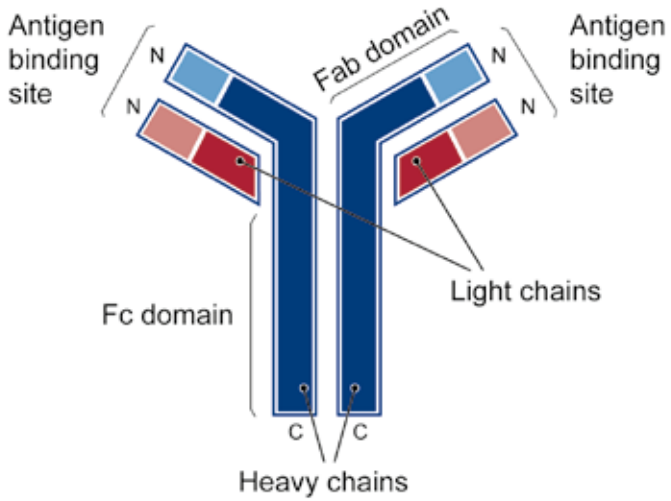

FIGURE 1 **Schematic Structure of an IgG Molecule.**

Sequence analysis of various light chains has revealed two distinct regions (Figure 2), a highly variable N-terminal half (VL) and a constant C-terminal half (CL). Similar studies on heavy chains have shown that they are also comprised of variable and constant regions (VH and CH, respectively). However, IgG heavy chains contain one variable region (found at the N-terminus) and three constant regions (Figure 2). Unsurprisingly, the variable regions of both chains localize to the Fab domains, providing the structural basis for the antigen and epitope selectivity of antibodies.

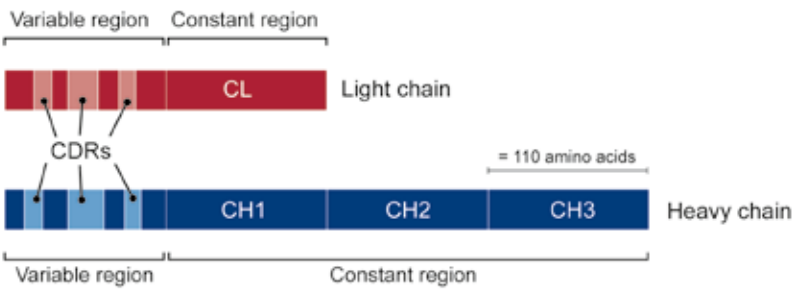

FIGURE 2 **Light and Heavy Chain Structure.** Light chains are approximately 220 amino acids in length and can be divided into two equal variable and constant regions. Heavy chains are approximately 440 amino acids in length and are subdivided into four 110 amino acid segments, with one variable and three constant regions.

The sequence heterogeneity within the variable regions is most pronounced at three short segments (5–30 amino acids in length) within each chain called the hypervariable regions. Since the residues in hypervariable regions form the actual binding site for the antigen, they are also known as the complementarity determining regions (CDRs) (Figure 2).

Advances in Antibody Technology

Although the development of hybridoma technology was a landmark event, the methodology still has a number of limitations. Developing hybridomas requires considerable time, expense, and expertise, as well as specialized cell culture and animal facilities. Additionally, antigens can be toxic or poorly immunogenic in mammals, and therefore, do not elicit an immune response.

Moreover, mouse monoclonal antibodies are unsuitable for use as therapeutic agents because they are rejected by the human immune system. Various approaches to overcome this therapeutic limitation have been tried, such as:

- Combining mouse monoclonal binding sites with human antibody sequences to create chimeric or humanized antibodies
- Using genetically engineered mice to produce antibodies with human sequences
- Developing fully human antibodies directly with *in vitro* recombinant antibody technology

Recombinant Antibodies

Recombinant antibody technology – such as HuCAL® – is a rapidly evolving field with a number of major benefits over conventional antibody generation and production methods.

First, the availability of gene sequences enables antibodies to be genetically engineered, allowing for the following:

- Study of the structural and functional properties of antibodies (binding, folding, stability, catalysis, etc.)
- Generation of smaller antibody fragments that retain the entire antigen binding site, such as the Fab and single-chain variable fragment (scFv) seen in Figure 3
- Enhancement of antibody affinity or specificity via mutagenesis
- Production of novel antibody fusion proteins which couple the antibody binding capacity to additional properties (enzymes, toxins, multimerization modules, and epitope tags)
- Direct creation of partially or fully human antibodies

Second, recombinant monoclonal antibodies can be produced in bacteria. This is easier, faster, and less expensive than using animals (ascites) or mammalian cell culture techniques.

Third, the technology enables the use of *in vitro* selection steps that facilitate the isolation of antibodies with desired characteristics, e.g. antibodies that distinguish closely related antigens.

Finally, the method does not rely on the use of laboratory animals and does not require immunogenicity of the antigen. Antibodies can be produced from non-animal derived materials and therefore are free from animal pathogens.

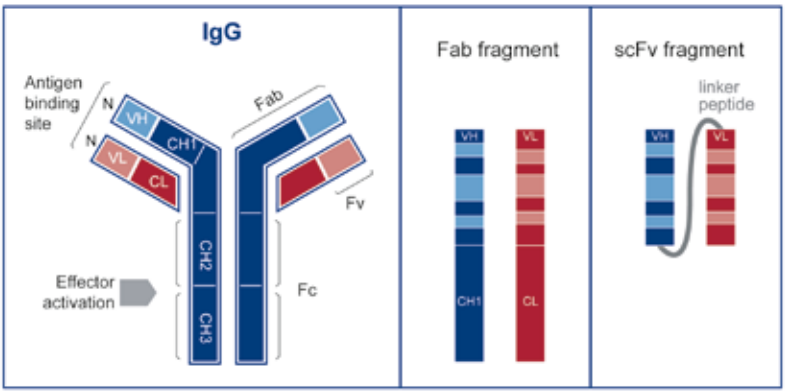

FIGURE 3 **Commonly Used Recombinant Antibody Molecules.** **IgG**: Full length molecules exactly matching the natural antibody composition. **Fab fragment**: The variable domain and the first constant domain of one heavy chain, plus one complete light chain of an antibody. The VH and VL chains can be covalently linked by a disulfide bond, and are also linked by strong non-covalent interactions. **scFv fragment**: A single-chain antibody consisting of one VH and one VL chains expressed as a single polypeptide joined by a peptide linker. The polypeptide linker stabilizes the interaction between VH and VL chains.

Methods for the Generation of Recombinant Antibodies

In the past, the initial step in generating recombinant antibodies was to isolate the relevant genetic material from a pre-selected hybridoma cell line and clone the antibody genes into a bacterial expression vector. Another option was to amplify the antibody repertoire of an immunized animal by polymerase chain reaction (PCR), using degenerate primers, and to clone the sequences into a bacterial selection system such as phage display (see page 15).

Modern techniques to generate recombinant antibodies start instead from very large (>1 billion) libraries of antibody genes. These libraries usually contain human antibody gene sequences since they were originally designed to produce human antibodies for therapeutic purposes. Such libraries can be derived from B-cells taken from non-immunized donors, or they can be generated *de novo* by gene synthesis (such as with HuCAL®). Therefore, they are called unselected or naïve libraries. If they contain a sufficient number of functional members, these libraries can be used to select specific antibodies against virtually any antigen.

Since large gene libraries cannot be screened for the binding properties of individual members, the antibodies that bind a given antigen are identified instead by selection. All selection methods to date physically link the antibody protein in the library (the phenotype) with the genetic information that encodes the given antibody (the genotype). There are a number of techniques, of which the most commonly used are phage display, ribosome display, and yeast display. Phage display is the most popular and best established of the selection methods.

Phage Display Selection

Phage display is used to select *E. coli* host cells that express the desired antibody fragment, i.e. the antibody that binds a given antigen[3,4]. Filamentous *E. coli* phage, such as M13 (which is non-lytic), are most commonly used (Figure 4). For phage display, the antibody library must be cloned into a phagemid vector with the following properties:

- Has both a bacterial and a phage origin of replication (ORI)
- Contains an antibiotic resistance marker
- Allows expression of the encoded antibody
- Links the expressed antibody to a phage surface protein (typically protein III or protein VIII) by genetic fusion

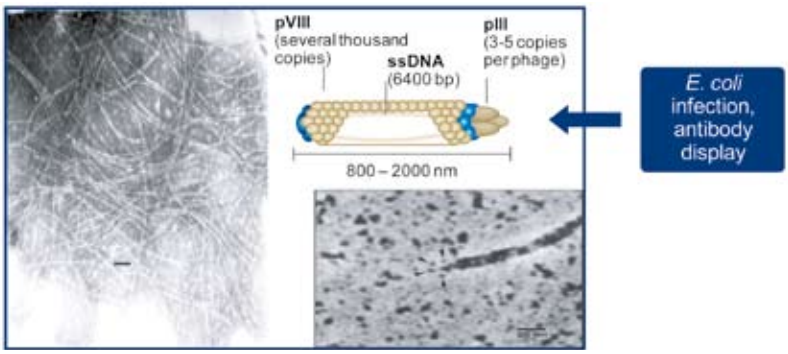

FIGURE 4 **Filamentous Phage M13.** **Left**: Electron microscopy image of filamentous phage. **Top right**: Schematic drawing of M13 phage. **Bottom right:** Electron microscopy image of the tip of M13 phage with arrows marking the pIII surface protein.

[3] Kretzschmar, T. and von Rüden, T. (2002) Antibody discovery: phage display. *Curr Opin Biotechnol* **13**:598–602

[4] Smith, G.P. and Scott, J.K. (1993) Libraries of peptides and proteins displayed on filamentous phage. *Methods Enzymol* **217**:228–257

The process begins by using helper phage to infect *E. coli* host cells containing phagemid vectors causing them to display antibody gene fragments. The fragments are fused to the phage coat protein gene III (known as gIII), which will later form part of the phage particle. The helper phage genome causes the cell to produce daughter phage. In these daughter phage, the cloned antibody genes are transcribed and then translated as a fusion protein consisting of a bacterial 'leader sequence', the antibody gene fragment, and the gIII phage coat protein. The leader sequence directs the proteins to the periplasmic space of the bacterial cell, where the fused antibody fragment is incorporated into viable phage particles via the protein coat. Since the helper phage contains a defective ORI in its genome, which does not replicate, the daughter phage incorporate the phagemid into their capsid. Finally, phage that contain the antibody's genetic information and simultaneously display the corresponding antibody binding sites on their surfaces are secreted through the bacterial outer membrane. About 10^{13} phage particles are harvested and subsequently used for the antibody selection steps.

Phage displaying the desired antibodies are selected by 'phage panning', which shares similarities with solid-phase immunoassays[5] (Figure 10, page 31). In this process, the antigen of interest is immobilized on a solid support, such as microplate wells or magnetic beads. The phage particles are then added to allow binding of phage that display appropriate antibodies. After extensive washing to remove all non-specifically bound material, the bound phage are eluted and amplified by replication in new host cells. The selection procedure is repeated several times, resulting in a population that consists to a high degree of phage that express the desired antibodies (i.e. those that bind the antigen of interest). After this selection step, the antibody genes are isolated from the enriched pool, inserted into an expression vector, and introduced in an expression host (usually *E. coli)*.

Expression and Screening of Antibody Fragments

E. coli cells transformed with the pool of enriched antibody genes are grown on agar plates, forming colonies. Each colony contains the genetic information for one antibody (heavy and light chain) enriched by the previous phage display selection. Therefore, each colony contains the genetic information for one monoclonal antibody.

[5] Barbas, C.F. and Lerner, R.A. (1991) Combinatorial immunoglobulin libraries on the surface of phage (phabs): Rapid selection of antigen-specific Fabs. *METHODS: A Companion to Methods in Enzymology* **2**:119–124

At this stage, colonies are picked and grown, and antibody expression is induced. Cells are subsequently lysed and the antibody-containing lysate is tested using an ELISA for the presence of antigen-specific antibody material. The plasmid DNA from ELISA-positive colonies is isolated and the antibody gene is sequenced. At this point, the panning method typically results in the isolation of a number of different antigen-specific monoclonal antibody fragments from the original large library of antibody genes. The overall procedure takes about 4–6 weeks, after which the antibodies can then be expressed and purified. (See figure 9, page 30)

Purification of Antibody Fragments

Soluble antibody fragments (e.g. Fab or scFv) produced by bacterial cultures are usually purified by one-step affinity chromatography using epitope tags that have been genetically fused to the C-terminus of the antibody heavy chain. Epitope tags commonly used for this purpose are the Strep-tag®, a short peptide with 8–10 amino acids that binds to streptavidin[6]; or the Histidine tag (His-6), which is a series of six histidines that can be complexed by metal chelates such as nickel nitriloacetic acid (Ni-NTA). Since Strep and His-6 epitope tag antibodies are commercially available, the tags can also be used for highly-specific immunodetection.

It should be noted that the antibody fragments which have been isolated will include the complete antigen binding site, and as a result they usually have the same intrinsic antigen-binding affinity as the complete antibody. However, full-length antibodies contain two binding sites per molecule, which increases their avidity and apparent affinity in some assays. To match the affinity and avidity of natural Immunoglobulins (Igs), recombinant antibody fragments can be dimerized or further multimerized by engineering.

Affinity is the strength of binding of an epitope to an antibody binding site.

Avidity is the overall stability of the antibody-antigen complex, which is dependent on affinity, valency, and structural arrangements.

[6] Strep-Tactin®, a mutated Streptavidin with higher affinity to the Strep-tag is also used.

Advantages of Recombinant Antibodies

Recombinant antibodies offer many advantages over conventional monoclonals as described in the following sections. These stem from several properties inherent in the system:

- All stages occur *in vitro*
- Candidates can be engineered using readily available DNA sequences
- Technology is based on a bacterial expression system

In Vitro Selection

The ability to select antibodies *in vitro* is especially valuable for generating antibodies to non-immunogenic and toxic antigens which cannot be used in animals. Furthermore, it is an open system which allows modification of the selection conditions. Using guided selection, the process can be pushed towards the required specificities to enable the generation of epitope-specific antibodies, e.g. those that are phospho-specific.

Once recombinant antibodies with desired specificities are found, they can be further enhanced by generating new combinations of heavy and light chains, or by mutating individual CDRs[7].

Engineering

Peptides and proteins with new functions can be linked by genetic fusion to the antibody. Examples include:

- Epitope tags for purification, immobilization, and detection
- Enzymatic activities, such as alkaline phosphatase (AP), for direct detection
- Multimerization modules which increase avidity
- Heterodimerization modules that allow for the creation of bi-specific antibodies
- Toxins for the elimination of tumor cells or enzymes that convert a pro-drug into a drug at the site of a tumor (ADEPT)

E. coli Production

Antibody genes can be designed for high expression in *E. coli*[8], with demonstrated production yields of more than 1 gram per liter *E. coli* culture.[9]

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[7] Karu, A.E. *et al.* (1995) Recombinant antibody technology. *ILAR Journal* **37**:132–141.

[8] Ge, L. *et al.* (1995) Expressing antibodies in *Escherichia coli*. In: Borrebaeck, C.A.K., *Antibody Engineering, 2nd edition*, 229–236. Oxford University Press

[9] Carter P. *et al.* (1992) High level Escherichia coli expression and production of a bivalent humanized antibody fragment. *Biotechnology* (N.Y.) **10**:163–167

Bacterial production is also less expensive than mammalian cell culture and can be performed without the use of animal-derived components. In addition, the bacterial system is fully-defined, and free from animal-based viruses, prions, and contaminating immunoglobulins. The high purity of antibodies from *E. coli* is accomplished using one-step affinity chromatography.

Antibody Fragments

Antibody fragments, such as Fabs which lack the Fc domain, often show benefits over full-size IgGs, including:

- Elimination of non-specific binding to cellular Fc receptors leading to lower background and increased sensitivity during cellular isolation and IHC
- Avoidance of cytokine release in functional assays with immunocompetent cells, since complement cannot bind
- Increased diffusion through tissue and cell membranes with smaller Fab fragments, leading to faster and more efficient staining
- Creation of fewer artifacts in receptor studies because monovalent Fabs do not cross-link receptors
- Reduced non-specific binding to the solid phase in heterogeneous ELISA experiments due to lower hydrophobicity
- Improved stoichiometry with less interference from serum factors and macromolecules (which mostly bind to the Fc region) in fluorescent or enzyme conjugates
- Simplified co-crystallization of target proteins with antibodies
- Easy determination of monovalent affinities between antigen and antibody

Therapeutics

Antibodies selected from *in vitro* libraries of human antibody genes do not elicit the same immune responses in patients that are seen with non-human antibodies. Therefore, such antibodies can be used for therapeutic development. More and more fully human antibodies obtained from antibody libraries are entering clinical development and are reaching the market[10].

Speed and High-throughput

Antibody selection using phage display of antibody libraries is a process that takes around 3–4 weeks for antigen-specific members to be enriched and isolated. Further production and characterization of the monoclonal antibodies

[10] Weiner L.M. (2006) Fully human therapeutic monoclonal antibodies. *J Immunother* **29**:1–9

is also rapid, due the fast growth rate of *E. coli* cultures. Nevertheless, there are attempts to further accelerate the process with the goal being to perform selections in just a few days[11].

The phage display selection process has been automated to a large degree[12] so that many target antigens can be processed in parallel, further increasing the efficiency of the process and saving the end user significant time.

Considerations When Using Recombinant Antibodies

Some reagents commonly used for immunoassays are not appropriate for protocols involving recombinant antibodies. Anti-mouse and anti-rabbit Ig secondary reagents, for example, are unsuitable for detection because recombinant antibodies are mostly of human origin and lack the Fc domain. Instead, detection of recombinant antibody fragments is usually performed using antibodies directed against epitope tags, such as: Strep-tag®, His-6, V5, c-myc, and FLAG®. These antibodies are usually directly labeled with an enzyme such as horseradish peroxidase (HRP) or a fluorophore. They can also be used in combination with a labeled tertiary antibody. Alternatively, detection of human recombinant Fabs can be achieved using anti-human Fab antisera labeled with AP, HRP, or fluorescent dyes.

In addition, immunoprecipitation using recombinant Fab cannot be performed using Protein A Sepharose® (which relies on the presence of the Fc domain), but can be performed by coupling the recombinant antibody to magnetic beads.

[11] Hogan S. *et al.* (2005) URSA: ultra rapid selection of antibodies from an antibody phage display library. *Biotechniques* **38**:536–538

[12] Krebs B. *et al.* (2001) High-throughput generation and engineering of recombinant human antibodies. *J Immunol Methods* **254**:67–84

HuCAL® Technology

Chapter 2

The HuCAL® Concept

HuCAL® technology is a unique and innovative concept for the *in vitro* generation of highly-specific, fully human antibodies[13]. The technology sets the standard for antibody production, taking the procedure out of the animal house and onto the laboratory bench.

The concept behind HuCAL® technology[14] is to represent the essential features of the natural human antibody repertoire, defined by sub-families of antibody germline gene segments (VH and VL), using the minimum number of sequences that cover the structural diversity of the typical repertoire. This aim to condense the repertoire into a few germline gene representatives is facilitated by three features of antibody genes:

- There are relatively few antibody genes in the human germline as revealed by the human genome project
- Many human antibody germline sequences are very rarely used during an immune response
- Germline genes can be grouped into families with high sequence and structural similarity

Generation of the HuCAL® Library

Detailed analysis of the human antibody repertoire at MorphoSys revealed that its structural diversity is best represented by seven heavy chain and six light chain variable region genes, which give rise to 42 framework combinations in the master library. The genes encoding these combinations were chemically synthesized and unique restriction sites were incorporated at the CDR boundaries (Figure 5). Highly variable genetic cassettes encoding the diversity of CDRs, which were generated using trinucleotides[15], were

[13] Ostendorp, R. *et al.* (2004) Generation, engineering and production of human antibodies using HuCAL®. Subramanian, G. (Ed.). *Antibodies Volume 2: Novel Technologies and Therapeutic Use.* 13–52

[14] Knappik, A. *et al.* (2000) Fully synthetic human combinatorial antibody libraries (HuCAL®) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J Mol Biol* **296**: 57–86

[15] Virnekäs, B. *et al.* (1994) Trinucleotide phosphoramidites: Ideal reagents for the synthesis of mixed oligonucleotides for random mutagenesis. *Nucl Acid Res* **22** 5600–5607

superimposed onto these frameworks to mimic the entire human antibody repertoire. The resulting libraries contain billions of functional human antibody specificities in a Fab format, which are selectable using *in vitro* methods, such as phage display.

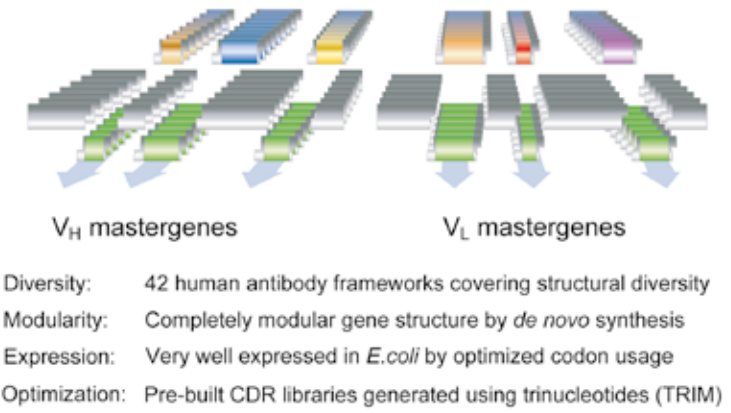

FIGURE 5 **The HuCAL® Concept.** The structural diversity of the human antibody repertoire is represented by seven heavy chain and six light chain variable region genes, which are combined to produce 42 antibody frameworks in the master library. Superimposing highly variable genetic CDR cassettes on these frameworks effectively mimics the entire human antibody repertoire.

Benefits of HuCAL® Technology

HuCAL® is one of the leading recombinant antibody libraries. It offers many advantages when compared to conventional monoclonal antibody generation and production as summarized in Table 1, as well as benefits that extend beyond both conventional methods and other recombinant libraries.

Since the antibody selection is performed *in vitro* in an open system, the process can be adjusted to the requirements of the project. Guided selection approaches, such as applying blocking strategies or usage of more than one antigen, lead to excellent antibody specificities (please refer to page 43). Antibody generation against toxic antigens, immunosuppressants and non-immunogenic antigens is also possible.

TABLE 1 **Advantages of HuCAL® Over Conventional Monoclonal Antibodies.**

Parameter	Conventional Monoclonals	HuCAL®
Selection	Random	Guided
Production	Complicated	Easy
Animal-based	Yes	No
Guaranteed Supply	No – source dies with the cell line	Yes – clones can be resynthesized
Flexible Formats	No	Yes
Engineering and Optimization	No	Yes
Humanness	No	Yes
Throughput	Mostly manual	Automated
Success Rate	~75%	98%
Speed of Delivery	4–9 months	8 weeks

The production of the antibodies occurs in *E. coli*, thus the entire process is free from laboratory animals. There is also no disease transmission risk normally associated with mammalian cell culture and the antibodies can be produced without the use of animal-derived components.

Furthermore, a secure supply is guaranteed by a double backup storage system consisting of bacterial antibody clones and their plasmid DNA. Even worst case scenarios will not impact the supply, since the antibody genes of selected candidates are sequenced so that every antibody produced can always be resynthesized.

The unique modular structure of the HuCAL® antibodies allows for a rapid change of format and engineering of selected antibodies, e.g. adding epitope tags or dimerization domains. For more information on antibody formats, see page 33. The modularity also allows for the optimization of antibodies with respect to affinity by exchanging certain CDRs with CDR libraries followed by stringent selection (see affinity maturation on page 27).

Another unique feature, the complete humanness of the antibodies, becomes an exceptional advantage when characterizing human immune responses in diagnostic and clinical settings, or when developing therapeutically active compounds.

Finally, the highly automated process reduces the risk of failures and provides excellent traceability of single steps. Clients benefit from fast production timelines and unrestricted capacity. With a project timeline of 8 weeks, this technology is considerably faster in delivering purified and characterized antibodies than any animal-based system.

Application of HuCAL® Technology

While HuCAL® technology was originally developed for the generation of fully human therapeutic antibodies, it has also proved to be highly valuable for the generation of research and diagnostic antibodies.

For many applications, the versatility of HuCAL® selection protocols, the specificities and affinities achieved, and the engineering flexibility have proved to be critical. For other applications, the humanness of HuCAL® antibodies has been decisive. HuCAL® offers a seamless transition from a rapidly generated antibody fragment with outstanding binding characteristics to a completely human full length antibody (IgG, IgM, IgA, IgE), immediately ready for assessment as human diagnostic standards in autoimmune testing[16]. They are also suitable for use in many other immune assays that currently rely on human sera as a control. Finally, human Fab and Ig molecules developed from HuCAL® are perfect tools for analyzing patient responses to a drug[17].

AbD Serotec has successfully generated and licensed HuCAL® antibodies for a wide range of purposes, including:

- *In vitro* and *in vivo* research
- Diagnostic immunoassay development (as detection reagents, sera replacements, and fully human controls)
- *In vivo* diagnostic assay development
- Preclinical and clinical drug monitoring in pharmacokinetic (PK) and anti-drug antibody assays (ADA)
- Quality control (QC) release assays for drugs and vaccines
- Purification of drugs
- Food testing
- Biodefense assays
- Medical devices

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[16] Knappik, A. *et al.* (2009) Development of recombinant human IgA for anticardiolipin antibodies assay standardization. *Ann N Y Acad Sci* **1173**:190–198

[17] Tornetta, M. *et al.* (2007) Isolation of human anti-idiotypic antibodies by phage display for clinical immune response assays. *J Immunol. Methods* **328**: 34–44

More than half of the top twenty pharmaceutical companies and many leading biotechnology companies have integrated HuCAL® technology into their R&D processes (see **www.morphosys.com**). As both an antibody-based drug and technology development company, MorphoSys is committed to spearheading the latest advances in antibody development technology. With this in mind, innovations in antibody sourcing technologies are constantly being developed in-house, certain of which may be available for licensing in the future.

The HuCAL PLATINUM® Platform

The HuCAL PLATINUM® antibody library is the latest and most powerful antibody library developed by MorphoSys. It contains several improvements over previous versions of HuCAL®, including a large increase in library members and a completely new approach to reproducing the natural composition of the key antigen recognition region (HCDR3). Both lead to a substantial increase in the diversity of selected antibodies as compared to the previous library. They also provide a wider range of promising antibody candidates to choose from during an initial screening of the library, with respect to affinity and specificity. Furthermore, expression rates in *E. coli* and mammalian cells were improved by gene optimization.

Features of HuCAL PLATINUM® Technology

- 45 billion fully human antibodies in the proven HuCAL® design – one of the largest high-quality antibody libraries worldwide
- Maximum variability for the antigen binding site through diversification of all six CDRs
- Enhanced functionality by length-dependent design of HCDR3
- Optimized selection of frameworks
- TRIM technology applied to generate a high-quality library with over 74% functional clones
- Optimized codons for *E. coli* and eukaryotic expression systems
- Removed mRNA secondary structures that may interfere with expression
- Avoided negative regulatory sequence motifs in prokaryotic and eukaryotic systems
- Significantly reduced number of potential glycosylation sites in CDRs
- Minimized number of non-germline positions

The high diversity of the library guarantees the successful generation of high-quality antibodies.

The new library is wholly compatible with the well-established screening and selection methods at AbD Serotec. As with the previous library, HuCAL PLATINUM® combines the advantages of the Fab format with the superior selection properties of the proprietary CysDisplay® screening technology that is used to select individual antibodies from the library (Figure 6).

CysDisplay® Selection Technology

CysDisplay® technology is a novel and efficient display method for selecting high-affinity binders from antibody libraries using filamentous phage[18]. It combines the advantages of monovalent phage display technology, such as phenotype–genotype linkage, with the additional feature of a cleavable disulfide bond. Therefore, it allows the efficient elution of interacting partners during the panning procedure (Figure 6).

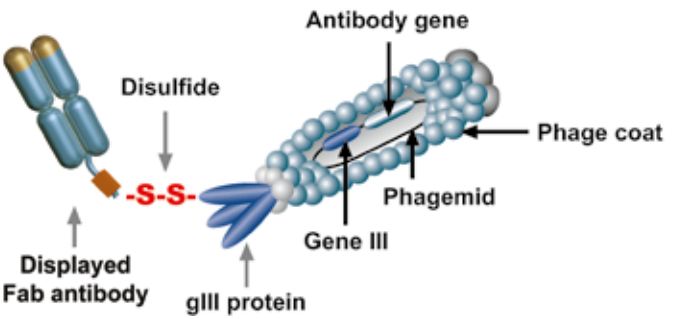

FIGURE 6 **CysDisplay® Screening Technology.** Fab fragments are linked to phage particles by a disulfide bond rather than a peptide bond. This allows elution of phage with reducing agents during antibody selection.

[18] Rothe, C. *et al.* (2008) The human combinatorial antibody library HuCAL GOLD combines diversification of all six CDRs according to the natural immune system with a novel display method for efficient selection of high-affinity antibodies. *J. Mol. Biol.* **376**:1182–1200

CysDisplay® is based on the simultaneous periplasmic expression of engineered phage coat proteins and antibody fragments, each containing an unpaired cysteine residue. Disulfide bonds form between these partners, resulting in heterodimerization. The heterodimers are incorporated into phage particles, leading to the display of monovalent functional antibody fragments on the phage surface. Since the disulfide linkage of the antibody fragments is sensitive to reducing agents, an efficient elution protocol can be used to recover phage displaying specific high-affinity antibody fragments.

This protocol can be used for any type of antigen and is, therefore, well-suited for high-throughput applications. The elution of phage particles from the antigens during the selection process is independent of the affinity of the binder to the antigen. This ensures that all phage displaying specific binders are eluted from the antigen, including the ones displaying very high-affinity antibodies, which cannot be easily retrieved using traditional elution methods.

Affinity Maturation of HuCAL® Antibodies

In contrast to animal-derived antibodies from serum or hybridoma supernatant, HuCAL® antibodies can be rapidly optimized both in affinity and biological activity, since the antibody DNA sequence is known and available. The specificity of the antibody usually remains unchanged during the antibody maturation, while the affinity can be routinely improved. Indeed, several thousand-fold increases in affinity have been achieved using this approach[19,20]. Optimization is facilitated by several features of HuCAL® antibodies:

- Modular design enables replacement of CDRs by highly variable CDR cassettes in a simple cloning step, thereby creating new antibody libraries based on existing HuCAL® specificities
- CDR cassettes are built using TRIM technology, ensuring high functional quality maturation libraries
- Highly diversified affinity maturation cassettes exist for all six CDRs and are compatible with all HuCAL® frameworks

In most antibodies, HCDR3 contains the most binding contacts to the antigen and, therefore, determines to a large extent the epitope bound by the antibody. Consequently, HCDR3 has the highest diversity in the HuCAL® library. Affinity maturation of selected antibodies concentrates on one or more of the remaining CDRs, typically LCDR3 or HCDR2.

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[19] Steidl, S. *et al.* (2008) *In vitro* affinity maturation of human GM-CSF antibodies by targeted CDR-diversification. *Mol Immunol* **46**:135–144

[20] Prassler, J *et al.* (2009) *In vitro* affinity maturation of HuCAL® antibodies: complementarity determining region exchange and RapMAT technology. *Immunotherapy* **1**:571–583

Affinity maturation is an optional step that can be performed once HuCAL® antibodies have been selected that bind a specific antigen. Binding characteristics are optimized by inserting a pre-built CDR library cassette (usually LCDR3 or HCDR2 as in Figure 7), which has been diversified according to the natural repertoire of CDR sequences at unique flanking restriction sites.

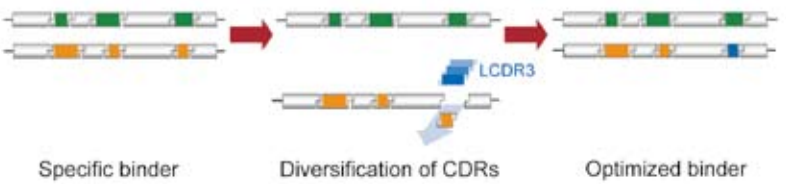
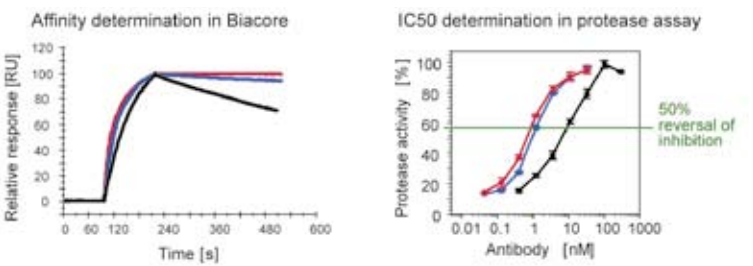


FIGURE 7 **Optimization of a HuCAL® Antibody's Binding Characteristics.** Optimization occurs via CDR library creation for specific binders in a simple cloning step.

This simple and efficient 'mix and match' process generates a new antibody library. Subsequent highly stringent phage display selection then permits rapid affinity maturation (Figure 8) of single antibodies ('lead optimization'), or even of antibody pools ('pool maturation' or RapMAT®).



	HuCAL antibody	K_D[nM]	IC50 [nM]
Selected for biological function	MOR 17 Fab	13.0	11.0
1st round	MOR 17.1 Fab	0.8	1.0
Conversion	MOR 17.1 IgG1	0.04	0.8

FIGURE 8 **HuCAL® Antibodies with Biological Function are Identified during Standard Selection.** New CDR sequences are inserted at unique flanking restriction sites to augment binding characteristics. Antibodies with improved binding characteristics are identified in further rounds of selection. Optimized antibody candidates can then be converted into various immunoglobulin formats. This leads to antibodies that have both higher binding affinities and improved biological function, such as blocking a rat protease inhibitor.

Sequential exchange of CDRs may lead to improvements in affinity by a factor of greater than a thousand-fold as compared with non-optimized parental binders, generating antibodies with affinities below 10^{-12} M[19].

TRIM Technology

TRIM technology is used to facilitate targeted diversification of CDRs. Pre-assembled trinucleotides are used for the chemical synthesis of the CDR sequences, which provides complete control over the amino acid composition and avoids stop codons. Consequently, the resulting CDR libraries display significantly higher quality than those made using conventional approaches.

Developing Therapeutic Antibodies and Accessing MorphoSys Technologies

MorphoSys employs its powerful HuCAL® technology primarily for therapeutic antibody development and grants certain licenses to its technology. The development of therapeutic antibodies and access to our proprietary technologies is handled by MorphoSys AG. Companies have the following partnership options (visit **www.morphosys.com** for further information):

- **Technology Subscription Licensing:** Transfer and installation of the HuCAL® technologies at partner premises, with comprehensive training included. Such transfers may occur for the purposes of *in vitro* and *in vivo* research, development of diagnostic reagents and assays, as well as preclinical and clinical drug monitoring. Subscription licenses in infectious disease indications may also be extended to include therapeutic antibody development and use.
- **Collaborations in Therapeutic Antibody Generation**: Funding of MorphoSys personnel to generate highly optimized antibodies against targets of interest to the partner. This work typically includes full maturation of the antibody with a focus on the therapeutic benefits (high affinity, low immunogenicity, solubility, and half-life).
- **Co-development of Therapeutic Antibodies:** Joint MorphoSys / partner funding and resource allocation throughout the therapeutic antibody generation project in targets of mutual interest to both parties.
- **Product In-licensing:** MorphoSys in-licensing of partner developed preclinical or early clinical stage antibodies for mutually agreed upon terms.
- **Product Out-licensing:** MorphoSys also has a pipeline of antibody drug candidates. These will be available for out-licensing when they reach clinical proof of concept.

[19] Steidl, S. *et al.* (2008). See page 27.

The HuCAL® Antibody Generation Process

Generation of antibodies using HuCAL® technology involves seven steps as shown in the figure below:

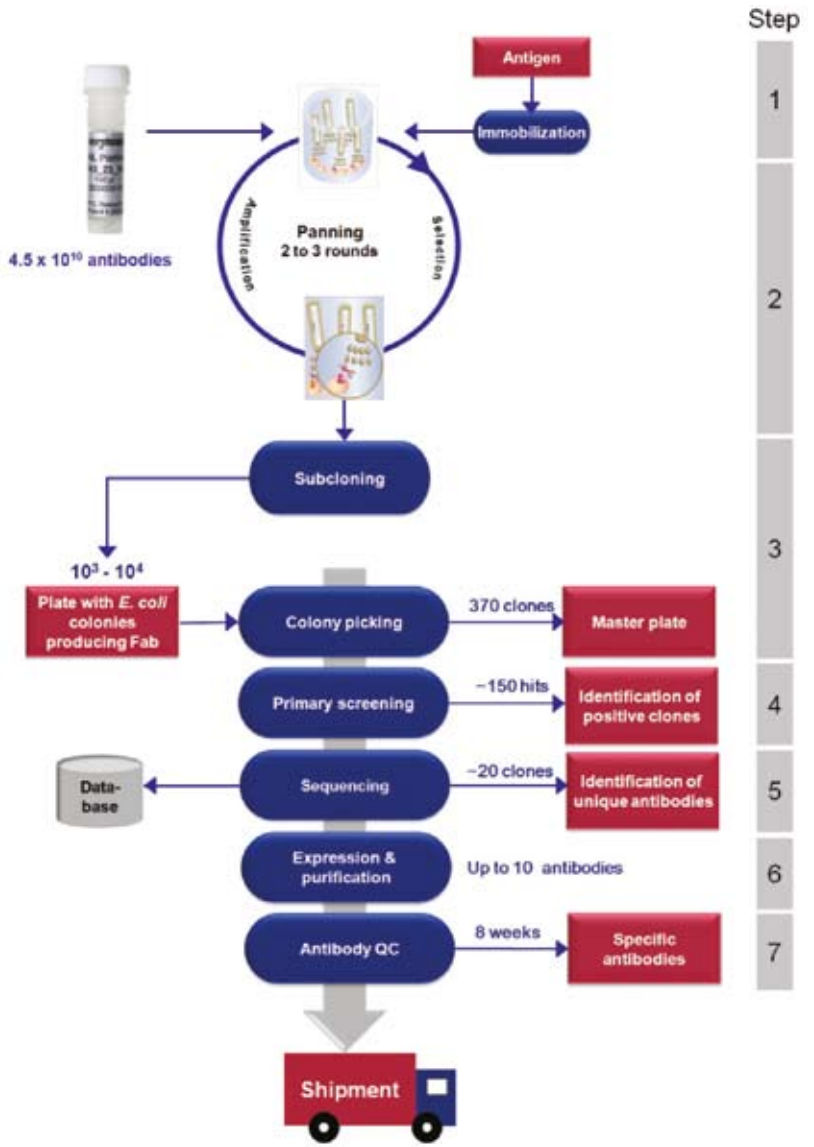


FIGURE 9 HuCAL® Antibody Generation Process Overview.

1. Antigen immobilization

The first step in antibody generation is the immobilization of the antigen on a solid support. The standard method uses covalent coupling to magnetic beads. An alternative strategy is coating polystyrene ELISA microtiter plates. Antigen presentation by passive adsorption may be beneficial for certain applications, and is the only option if the antigen is in a buffer incompatible with the bead-coupling chemistry.

2. Phage display selection – panning

The HuCAL® library presented on phage particles is incubated with the immobilized antigen. Nonspecific antibodies are removed by extensive washing and specific antibody phage are eluted by adding a reducing agent. An *E. coli* culture is infected with eluted phage and helper phage to generate an enriched antibody phage library for the next panning round. Typically, three rounds of panning are completed (Figure 10).

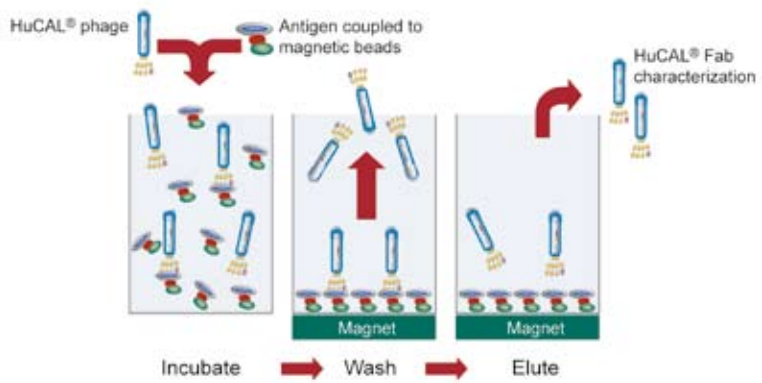


FIGURE 10 **Phage Panning.** Selection round of phage-displayed HuCAL® Fab fragments on antigen-coated magnetic beads.

3. Subcloning into antibody expression vector

After panning, the enriched antibody DNA is isolated as a pool and subcloned into a Fab expression vector. Different vector formats are available for monovalent or bivalent Fab fragments with a choice of epitope tags. *E. coli* are transformed with the ligation mixture and plated on agar plates. Each growing colony represents a monoclonal antibody at this stage.

4. Primary screening

Colonies are picked and grown in a 384-well microtiter plate. Antibody expression is induced and the culture is lysed to release the antibody molecules. Cultures are screened for specific antigen binding either by ELISA or a bead-based homogenous fluorometric microvolume assay technology (FMAT®) assay (see page 120 for more information on FMAT® assays).

5. Sequencing

Hits from the primary screening experiment are sequenced to identify unique antibodies. The sequences not only guarantee the uniqueness of any HuCAL® antibody, they also serve as an ideal antibody storage backup, and a guarantee of reproducibility. Every sequence can be rebuilt by chemical synthesis, if necessary.

6. Expression and purification

The unique antibodies are expressed and purified using one-step affinity chromatography.

7. Antibody QC

Purified antibodies are tested by ELISA, or in a bead-based fluorescence-linked immunosorbent assay (FLISA – usually FMAT®), for required specificity. Purity is assessed by Coomassie® staining of a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and yield is measured by UV absorbance at 280 nm.

Purified antibodies are typically shipped 8 weeks after commencement of a project.

A Typical HuCAL® Project Includes:

- Three rounds of panning
- Subcloning into selected antibody format
- Screening of 368 clones
- Sequencing of up to 20 clones
- Shipment of antibodies (250 µg each) and – if available – 75 µg of each antigen (if produced or modified by AbD Serotec)
- **No antibody –no charge guarantee**[21]

Antibody Generation Parameters

For a successful HuCAL® antibody project, it is important to be aware of all available options that can be used to obtain the desired antibody specificities. Over the course of the following pages, parameters and options are explained in more detail. Our scientists will be happy to offer advice on the best project strategy.

Antibody Formats

The HuCAL PLATINUM® library is based on the human IgG1 Fab format, which consists of the first two domains of the antibody heavy chain (VH and CH1) plus the complete light chain (from either the κ or λ family). The Fab format is truly monovalent (one heterodimeric molecule has one antigen binding site). This format, together with the monovalent phage display selection method, ensures that the *in vitro* selection of antibodies from the library is driven by intrinsic affinity and not by avidity effects.

Antigen-antibody interactions depend on the following characteristics:

- **Affinity** – binding strength of a single antigen binding site to a single antigen epitope
- **Avidity** – binding strength of a potentially multimeric antibody (e.g. whole IgG) to a potentially multimeric antigen

After phage display selection of candidate antibodies, the antibody Fab genes are cloned into an *E. coli* expression vector. The choice of expression vector determines:

[21] If we don't find an ELISA-positive antibody against your antigen, there is no charge for library screening.

- Whether the final antibody is monovalent or bivalent
- Whether an enzyme such as AP is fused to the antibody
- Which peptide tag will be attached to the C-terminus of the antibody heavy chain

Antibody formats can be changed by subcloning the gene into a new expression vector. It is also possible to convert the antibody into a full-length Ig format by choosing an expression vector that adds the Fc region (CH2 and CH3 domains). Full-length antibody constructs are usually expressed in mammalian cells to achieve sufficient quantities of Ig in a functional format.

Choosing the Fab or IgG Format

Many applications and assays benefit from monovalent or bivalent Fab fragments over classical full-size antibodies, as discussed on page 19. For certain types of experiments, however, it will be necessary to convert the recombinant antibody into an IgG format. This is necessary in the following circumstances:

- In experiments that depend on desired effects of the Fc region of the antibody (such as for binding or coating)
- When the class- and subclass-specific antigen determinants located in the Fc region are required
- Where the Fc region is essential to sustain precipitation or agglutination reactions

It is also important to remember that:

- Fab preparations generated from *E. coli* expression systems contain endotoxins and cannot be used in some cellular assays without an additional purification step
- Since most commercial secondary detection systems are directed against the Fc region of the primary antibodies, HuCAL® Fabs use a secondary detection system directed against epitopes on the Fab portion or against the epitope tags
- In IHC on human tissues, the detection of human Fab or $F(ab')_2$ requires an epitope tag antibody, since endogenous Igs will also be recognized by anti-human Fab secondary reagents

Choosing the Best Fab Format

When starting a project, our scientists will help to decide which format is most suitable for the application. Options include the monovalent Fab format or the bivalent format of AbD Serotec's mini-antibodies (functionally equivalent to a

F(ab')$_2$ fragment) (Figure 11 and Table 2). Certain dimerization domains, like the dHLX domain, when fused to the C-terminus of the heavy chain fragment create non-covalently linked Fab-homodimers which are also called mini-antibodies.

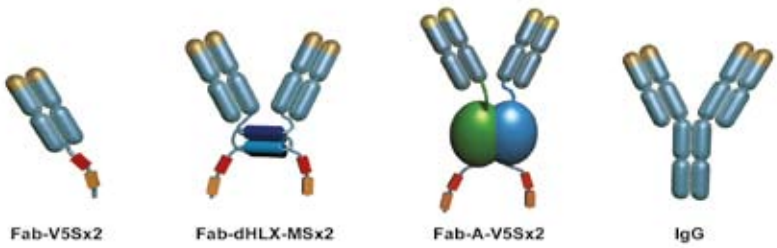


FIGURE 11 **Schematic of the Main Antibody Formats Available.** **Fab-V5Sx2:** Monovalent Fab (shown with two tags). **Fab-dHLX-MSx2:** Bivalent Fab, dHLX domains shown as blue cylinders (with two tags). **Fab-A-V5Sx2:** Bivalent Fab formed by dimerization of bacterial AP (blue/green) with two tags. **IgG:** Complete IgG antibody. *V5= V5 tag; M= c-myc tag; Sx2= two extended Strep-tags.*

Bivalent Fab fragments are preferred to monovalent Fab fragments for most applications that detect surface-bound antigen (Western blots, IHC, and fluorescence-activated cell sorting [FACS™]), because they possess two antigen binding sites and display an avidity effect. Monovalent Fabs work better in some cellular assays because they avoid cross-linking of antigens, i.e. the signal strength is a better match for the binding stoichiometry. Furthermore, monovalent Fabs are usually preferred for crystallization experiments.

Some applications require special considerations. For example, AbD Serotec recommends a monovalent Fab format when comparing affinities or IC50 values of different antibodies; or for use on an affinity chromatography column, since elution of the Fab is prevented under conditions where the elution buffer disrupts the dHLX helix.

Since the bivalent format (Fab-dHLX) gives the best performance in most assays, it is the standard antibody form used, with the heavy chain expressed as a fusion with the dHLX domain[22, 23]. Two dHLX domains dimerize in a

[22] Pack, P. *et al.* (1993) Improved bivalent miniantibodies, with identical avidity as whole antibodies, produced by high cell density fermentation of *Escherichia coli. Biotechnology* (NY) **11**:1271–1277

[23] Plückthun, A. and Pack, P. (1997) New protein engineering approaches to multivalent and bispecific antibody fragments. *Immunotech* **3**:83–105

helix-turn-helix structure to form the bivalent mini-antibody. However, AbD Serotec also offers bivalent Fab fragments in the Fab-A format, where the heavy chain is fused to bacterial alkaline phosphatase (AP). Since bacterial AP forms a homodimer naturally, it creates a bivalent Fab fragment. This format also allows direct detection via the AP activity and without the use of a secondary antibody. It has advantages for applications requiring immobilization of the antibody by random chemical coupling, due to its large size. Further information on the molecular weight of these domains can be found in Table 3 and Table 4.

With some applications, it is useful to have even higher valencies. In those instances, AbD Serotec offers a tetravalent antibody format in which the Fab is fused to the p53-tetramerization domain. Note that hetero-oligomerization with native human p53 protein has been observed after incubation with human p53 containing lysates.

For applications where the Fc part of the antibody is required, we offer subcloning of the antibodies into the human IgG1, IgG2, IgG4, IgA, IgM, and IgE formats, followed by transient expression in mammalian cells. If mouse antibodies are required, AbD Serotec also offers production of chimeric human/mouse IgG2a with human variable regions and mouse constant regions. For rat, human/rat IgG1, Ig2a, and Ig2b chimeras are available.

Choosing the Matching Epitope Tag

It is important to select the tag best-suited for the application. The tag is an additional feature that can be used for immunodetection of the Fab in the assay or for immobilization on a matrix. Refer to Table 2 and Table 3 to identify the most suitable tag. AbD Serotec offers a wide selection of common epitope tag antibodies, either unconjugated or labeled with enzymes and fluorescent dyes (see appendix).

TABLE 2 **Recommended Fab Antibody Formats by Application.**

Application	Mono-valent	Bivalent	Recommended Secondaries	Comments
Western Blot		✔	Anti-human Fab Anti-Strep-tag® Anti-His-6 Anti-V5 Anti-BAP Anti-FLAG® Anti-c-myc	Recommended protocols found in chapter 3
ELISA		✔	Anti-human Fab Anti-Strep-tag® Anti-His-6 Anti-V5 Anti-BAP Anti-FLAG® Anti-c-myc	Recommended protocols found in chapter 4
Immuno-precipitation		✔	Anti-human Fab Anti-Strep-tag® Anti-His-6 Anti-V5 Anti-FLAG®	Recommended protocols found in chapter 5
IHC		✔	Anti-human Fab (for non-human tissue) Anti-Strep-tag® Anti-His-6 Anti-FLAG® Anti-BAP Anti-V5	Recommended protocols found in chapter 6
FACS		✔	Anti-human Fab (for non-human tissue) Anti-Strep-tag® Anti-FLAG® Anti-His-6	Recommended protocols found in chapter 7
Immuno-fluorescence		✔	Anti-human Fab (for non-human tissue) Anti-Strep-tag® Anti-His-6 Anti-FLAG® Anti-BAP Anti-V5	Recommended protocols found in chapter 8
Affinity Determination	✔			Recommended protocols found in chapter 10
Chromatography	✔			Use a Fab-ds or IgG format to avoid column leakage of the light or heavy chain
Co-crystallization	✔			Use Fab-H or Fab TC-MH: use Thrombin to cleave off tags

All available Fab formats contain either a Strep-tag®, an extended Strep-tag® (Strep II-tag with a Ser-Ala linker), or a His-6 tag, which is used for purification of the antibody. A second tag is added in many formats to provide additional options for detection (Table 3). Please note that Ig formats do not contain any epitope tags.

His-6 is a widely used epitope tag that can be used for protein purification on a Ni-NTA matrix. Several antibodies against His-6 are commercially available with different labels. For example, AbD Serotec offers MCA1396 in the following formats: Alexa Fluor® 488, Alexa Fluor® 647, AP, biotin, DyLight® 549, fluorescein isothiocyanate (FITC), HRP, and R-phycoerythrin (RPE).

The Strep-tag® is an artificial epitope tag with 8 amino acids that was originally generated to bind streptavidin. However, Strep-Tactin®, an engineered streptavidin, should be used instead due to its higher affinity. The extended Strep-tag® has two additional amino acids which allow capture of the tag on a solid support by the anti-Strep-tag® Immo antibody (MCA2488), which has a very high affinity. Both tags can be detected with good sensitivity and specificity using the anti-Strep-tag® Classic antibody (MCA2489).

The extended Strep-tag® is generally preferred over His-6 because it yields superior results in affinity purification.

Our standard format is the bivalent mini-antibody with a FLAG® and two extended Strep-tags, referred to as Fab-dHLX-FSx2, which works well in most applications. The molecular weight of these mini-antibodies is approximately 115 kDa. Each Fab is genetically fused to a small homodimerization domain (termed dHLX; molecular weight 5 kDa), a FLAG® tag, and two extended Strep-tags at the C-terminus of the heavy chain (see Table 4 for tag size and sequences).

TABLE 3 **Fab Antibody Formats and Epitope Tag Combinations.**

Short Name	Description	Approx. MW (kDa)
Monovalent		
Fab-MSx2	Fab antibody (c-myc- and StrepX-StrepX-tags)	55
Fab-FSx2	Fab antibody (FLAG®- and StrepX-StrepX-tags)	55
Fab-V5Sx2	Fab antibody (V5- and StrepX-StrepX-tags)	55
Fab-MH	Fab antibody (c-myc- and His-6-tags)	53
Fab-FH	Fab antibody (FLAG®- and His-6-tags)	52
Fab-V5H	Fab antibody (V5- and His-6-tags)	53
Fab-S	Fab antibody (Strep-tag®)	51
Fab-H	Fab antibody (His-6-tag)	51
Fab-H-Cys	Fab antibody (His-6-tag with Cys)	51
Fab-MH-Cys	Fab antibody (c-myc- and His-6-tags with Cys)	53
Fab-MS-Cys	Fab antibody (c-myc- and Strep-tag® with Cys)	53
Fab-ds-H	Disulfide linked Fab antibody (His-6-tag)	51
Fab-ds-FS	Disulfide-linked Fab antibody (FLAG® and Strep-tag®)	52
Fab-Tc-MH	Fab antibody (Thrombin cleavable, c-myc- and His-6-tags)	53
Bivalent		
Fab-dHLX-MSx2	Mini-antibody (c-myc- and StrepX-StrepX-tags)	115
Fab-dHLX-FSx2	Mini-antibody (FLAG®- and StrepX-StrepX-tags)	115
Fab-dHLX-MH	Mini-antibody (c-myc- and His-6-tags)	112
Fab-dHLX-FH	Mini-antibody (FLAG®- and His-6-tags)	112
Fab-dHLX-S	Mini-antibody (Strep-tag®)	111
Fab-dHLX-H	Mini-antibody (His-6-tag)	111
Fab-A-MSx2	Fab bacterial alkaline phosphatase fusion antibody (c-myc- and StrepX-StrepX-tags)	205
Fab-A-FSx2	Fab bacterial alkaline phosphatase fusion antibody (FLAG®- and StrepX-StrepX-tags)	205
Fab-A-V5Sx2	Fab bacterial alkaline phosphatase fusion antibody (V5- and StrepX-StrepX-tags)	205
Fab-A-S	Fab bacterial alkaline phosphatase fusion antibody (Strep-tag®)	201
Fab-A-FH	Fab bacterial alkaline phosphatase fusion antibody (FLAG®- and His-6-tags)	202
Fab-ds-A-FS	Disulfide-linked Fab bacterial alkaline phosphatase fusion antibody (FLAG®- and Strep-tag®)	202
Tetravalent		
Fab-p53-H	Mini-antibody (His-6-tag)	231
Fab-p53-S	Mini-antibody (Strep-tag®)	231
Fab-p53-V5Sx2	Mini-antibody (V5- and StrepX-StrepX-tags)	235

TABLE 4 **Tag Sequences and Homodimerization Domains.**

Short Name	Description	Approx. MW (kDa)
Domains		
Fab	Heavy chain variable and first constant domain, and complete light chain	50
dHLX	Synthetic double helix loop helix motif (dimer)	5
A	Bacterial alkaline phosphatase (dimer)	47
p53	Domain derived from human p53 (tetramer)	5.8
Strep-Tag® Versions		
S	WSHPQFEK	1
FS	DYKDDDDKGAPWSHPQFEK	2.3
FSx2	DYKDDDDKGAPSAWSHPQFEKGGGSGGGSGGSAWSHPQFEK	4.3
MS-Cys	EQKLISEEDLNGAPWSHPQFEKC	2.7
MSx2	EQKLISEEDLNDAPSAWSHPQFEKGGGSGGGSGGSAWSHPQFEK	4.7
V5Sx2	GKPIPNPLLGLDSTDAPSAWSHPQFEKGGGSGGGSGGSAWSHPQFEK	5
His-Tag Versions		
H	HHHHHH	1
H-Cys	HHHHHHC	1
FH	DYKDDDDKGAPHHHHHH	2.1
MH	EQKLISEEDLNGAPHHHHHH	2.4
MH-Cys	EQKLISEEDLNGAPHHHHHHC	2.5
Tc-MH	LVPR↓GSGAPEQKLISEEDLNDAPHHHHHH ↓: Indicates Thrombin cleavage (Tc) site	3.3
V5H	GKPIPNPLLGLDSTDAPHHHHHH	2.9

Besides the standard purification (His-6 or Strep) and detection tags (c-myc, FLAG®, or V5), others are also available to provide additional functionality. For example, we offer a single cysteine or a thrombin cleavage site (Fab-Tc), which enables the removal of the tags after purification.

Introduction of new sequences and tags for special applications is possible via a simple cloning step. If the preferred tag or a combination of tags is not listed in the tables above, please contact your account manager at **sales.muc@abdserotec.com** or visit **www.abdserotec.com/HuCAL** for more information. For further guidance regarding specific applications, refer to the relevant chapter of this manual, or consult the technical support scientists at AbD Serotec.

Antigens

The majority of substances used as antigens for antibody development are proteins. Since HuCAL® technology does not involve immunization of animals, only 0.5 mg of protein antigen is required for the entire antibody generation

project (under certain conditions even as little as 100 µg of protein may be sufficient). If purified protein is not available, it is possible to generate antigen by using our AgX® antigen expression technology, or to express the antigen as an Fc fusion protein in a mammalian expression system. Alternatively, peptides or other antigens, such as small molecules (haptens), can also be used for HuCAL® antibody development.

Protein Antigens

Using the whole protein as antigen is usually the method of choice, providing that a sufficient amount of purified protein is available. Proteins should be at least 80% pure, as judged by Coomassie®-stained reducing SDS-PAGE of a 3 µg sample. The sample buffer must not contain primary amines such as tris or glycine, additives such as bovine serum albumin (BSA), or detergents, since these reagents interfere with the immobilization reaction (covalent coupling to magnetic beads). 4 M guanidine hydrochloride or low amounts of free sulfhydryl groups (e.g. 1 mM Dithiothreitol [DTT]) are compatible with the coupling chemistry. The scientists at AbD Serotec are available to provide advice on buffer compatibility. If it is not possible to change buffer composition, the antigen would be included in a procedure called 'solid-phase panning', where it is passively adsorbed onto an ELISA plate, and subsequently handled with PBS.

If the protein contains a linker or purification tag, then 0.25 mg to 0.5 mg of an unrelated protein with the same linker or tag should also be provided. This is used for counter-selection and screening to make sure that the antibodies delivered will be against the protein of interest, and are not binding to the linker or tag.

The AgX® Antigen Expression Service

When the protein is not available in purified form, the proprietary MorphoSys AgX® antigen expression system is an ideal starting point for generating antigens from cDNA or PCR fragments[24]. If purified DNA is unavailable, the sequence can be used by AbD Serotec for gene synthesis. This will add 2 weeks to the timeline of AgX® antigen production. We use a bioinformatics toolbox to determine the optimal protein domain for expression, and then subclone the DNA fragment into a proprietary *E. coli* N1-fusion protein expression vector.

[24] Frisch, C. *et al.* (2003) From EST to IHC: human antibody pipeline for target research. *J Immunol Methods* **275**:203–212

After subcloning and sequence verification, the antigens are expressed as inclusion bodies in *E. coli*, and purified under denaturing conditions, via the His-6 epitope tag, by one-step metal-affinity chromatography (Figure 12).

Next, the antigen is refolded. The AgX® approach has a high success rate, with more than 90% of all antigens expressed, and it adds just 3 weeks to the antibody generation procedure. This system has two advantages over peptide antigens:

- Antibodies against protein domains are more likely to recognize the parental protein than anti-peptide antibodies
- Many more epitopes are available for antibody recognition; hence, the diversity of antibodies selected is usually higher

For antigens that require a mammalian system for solubility or proper folding, AbD Serotec offers the production of antigens as Fc-fusion proteins.

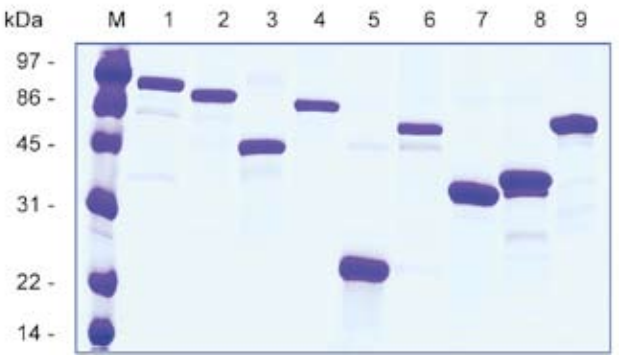


FIGURE 12 **Antigens Expressed as AgX® Fusion Proteins.** 15% SDS-PAGE (reducing conditions) of N1-fusions of nine protein fragments ranging in length from 100 to 700 amino acids. N-terminal fusion of the AgX® to the N1 domain of the pIII protein results in strong expression of protein fragments, while the C-terminal His-6 enables purification of protein samples under denaturing conditions.

Peptide Antigens and Haptens

Peptides have limitations as antigens, due both to the reduced number of epitopes available and to their flexible structure. Antibodies against peptides are often of lower affinity than antibodies against proteins, and there is no guarantee that the anti-peptide antibody will recognize the natively folded parent protein. However, in some cases they are the only option. Furthermore,

selection of certain epitope-specific antibodies (e.g. phospho-specific) often requires a peptide antigen. AbD Serotec has a very good success rate in generating antibodies against peptides, and our technical specialists can help choose the best peptide sequence for antibody generation.

AbD Serotec routinely couples peptides to two carrier proteins (using different linkers) for panning against the library. The standard carrier proteins are BSA and human transferrin (Trf), with coupling via an N- or C-terminal cysteine, but alternative coupling strategies can be used where necessary. We can provide peptide synthesis and coupling services, or just coupling for existing peptides. Contact your account manager at **sales.muc@abdserotec.com** for more information.

Using guided selection (see below), HuCAL® technology offers great advantages for the selection of anti-hapten antibodies with demanding specificities. Similar to peptide antigens, the first step involves coupling of the hapten, or a homolog, to two different carriers. Contact us to discuss the options available for hapten antigens.

Guided Selection Strategies

Selection of antibodies using HuCAL® technology is performed entirely *in vitro* (Figure 9). This enables greater flexibility for antibody generation than is available with conventional methods based on the immunization of animals. All selection strategies used for the identification of antibodies to certain epitopes, or epitope areas, are termed guided selection.

Selection of Epitope-specific Antibodies

The HuCAL PLATINUM® library is highly suited for the selection of antibodies against specific epitopes, such as phosphorylated sites. Chemically synthesized peptides are often used to ensure the purity and homogeneity of the antigen (which is phosphorylated) and the counter-antigen (a closely related antigen [CRA], which is non-phosphorylated). All antibodies in the library that bind the non-phosphorylated peptide are efficiently blocked by incubation with an excess of the non-phosphorylated peptide. The cleared library is then used for the selection of epitope-specific antibodies (Figure 13).

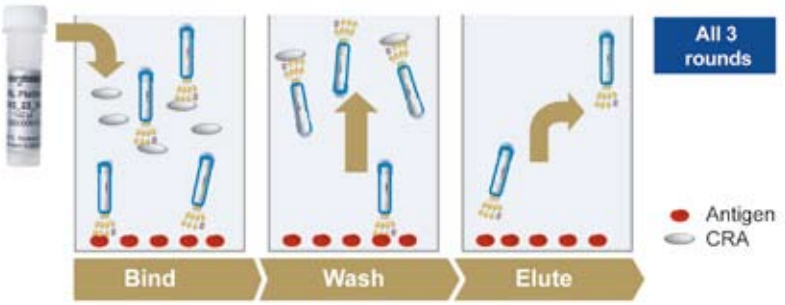


FIGURE 13 **Guided Selection with Blocking.** A closely related antigen is used to remove cross-reactive antibodies (counter-selection).

Antibodies Lacking Cross-reactivity to Closely Related Proteins

A subtraction strategy is used to eliminate cross-reactive antibodies from the selection process. Pre-adsorption steps and intelligent counter-selection blocking drive the selection to unique epitopes on the antigen. This strategy is highly suitable for various procedures, including the generation of anti-idiotype antibodies.

Antibodies that Bind Homologous Proteins

Alternate selection using two homologous antigens allows identification of antibodies that react with both antigens (Figure 14). This strategy is useful for generating cross-species antibodies or for recognizing two isoforms of a protein.

This strategy is also used to select antibodies that bind peptides. The peptide is first coupled to two carrier proteins using two different linkers. By alternating the peptide carrier conjugate during the panning rounds, only antibodies that bind the peptide, and not the linker or the carrier are selected from the library.

Antibodies that Bind Antigens under Special Conditions

AbD Serotec can alter the selection conditions to ensure that the antigen is presented to the library in the form in which it will be assayed – denatured, captured, soluble, or masked. It is even possible to alter the buffer conditions during binding to simulate the assay buffer of the relevant experimental system.

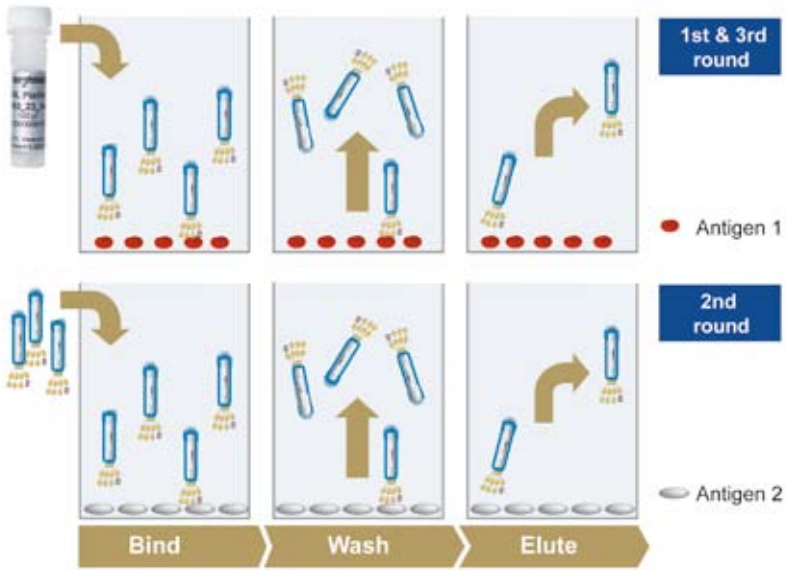


FIGURE 14 **Guided Selection with Alternating Antigens.** The antigen is alternated during the three rounds of selection, thereby enriching antibodies that bind to both antigens. Primary screening on both antigens will confirm the desired cross-reactivity.

Generation of ELISA Sandwich Pair Antibodies

Two strategies are typically used for the generation of sandwich pair antibodies.

1. A standard panning against the antigen is followed by testing all possible combinations of the selected antibodies as capture and detection antibodies in a sandwich ELISA using biotinylated detection antibodies. The best combination of capture and detection antibodies is identified by this method. However, this method depends on the number of selected antibodies and their properties (which, in turn, depends on the antigen used), and does not always result in a good sandwich pair.
2. This method is used if the technique above is unsuccessful. Standard panning against the antigen is followed by ELISA screening of the purified antibodies for the best capture antibody. Labeled (e.g. biotinylated) antigen is required for detection. The best capture antibody is then used to capture the antigen and a subsequent panning is performed on the antibody-antigen complex. The capture antibody is used to pre-clear the library and an isotype-control antibody is used for blocking, ensuring

depletion of capture-antibody specific antibodies. Selected antibodies will bind the captured antigen and therefore are matched detection antibodies (Figure 15).

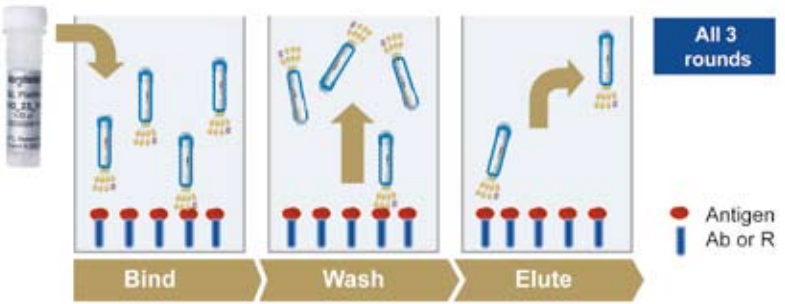


FIGURE 15 **Selection with a Captured Antigen.** The antigen is presented as an antigen-ligand complex. The ligand is used as a CRA to pre-clear the library.

Screening Options

After panning, the enriched antibody gene pool is subcloned into an expression vector to produce the final antibody format. For screening, *E. coli* colonies containing single antibody genes are picked and transferred into a 384-well microtiter plate. By default, one plate (368 colonies, because one row is left blank for controls) is picked. It is possible to pick and screen multiple plates if a maximum diversity of antibodies is required. At this stage, antibodies with the required specificities will be identified and selected for further processing. Therefore, it is important to carefully choose all required antigens for this primary screening experiment. For instance, for peptide and AgX® antigen projects it is advisable to include the purified protein, to demonstrate binding of the resultant antibodies to the native protein, if at least a small amount of purified native protein antigen is available.

The master plate with all 368 clones is stored for two years (as frozen glycerol stocks) if at least one positive hit was found in the primary screening. This allows additional screenings later, e.g. if new antigen material becomes available.

The standard assay for primary screening is an indirect ELISA with coated antigen, antibody-containing *E. coli* lysates, and labeled secondary antibody. It is also possible to screen the antibodies in a capture setting. In that case,

the antibodies are captured from *E. coli* lysates by coated anti-human Fab antibody and then incubated with biotinylated antigen and detected via streptavidin-HRP. For haptens, an inhibition ELISA (or competition ELISA) is strongly recommended. Since the antibody selection is performed with conjugated hapten carrying a linker, an inhibition ELISA with free hapten (without linker) helps to identify only those antibodies which do not cross-react with the linker or the carrier.

If only very small amounts of antigen are available, or if the desired final antibody application is a bead-based assay, the primary screening can be performed as an FLISA assay on the FMAT® 8200 CDS instrument (see page 120). With FMAT® screening, 100 µg of antigen may be sufficient for the entire antibody generation project.

Sequencing

Sequencing of up to 20 primary screening hits is included in any standard project. It identifies the truly unique antibodies and allows storage of the antibody data in an electronic format. To increase the chances of finding the perfect antibody for the application, it is possible to increase the number of sequenced clones by multiples of 10 (providing sufficient primary screening hits are available). Since the master plate with all 368 clones is stored for two years, it is also possible to sequence additional clones later.

Antibody QC

The purity of all antibodies after one-step affinity purification is routinely monitored by SDS-PAGE and subsequent Coomassie-staining of a 1.8 µg sample. Concentration is determined by measuring UV absorbance at 280 nm.

Activity and specificity of the purified antibodies are tested by a QC ELISA before shipment (Figure 16). All antibodies are tested on three non-specific standard antigens and on all positive and negative control antigens, as defined in the project plan. Since only small amounts of antigen are required at this step, more precious antigens can be reserved for this ELISA.

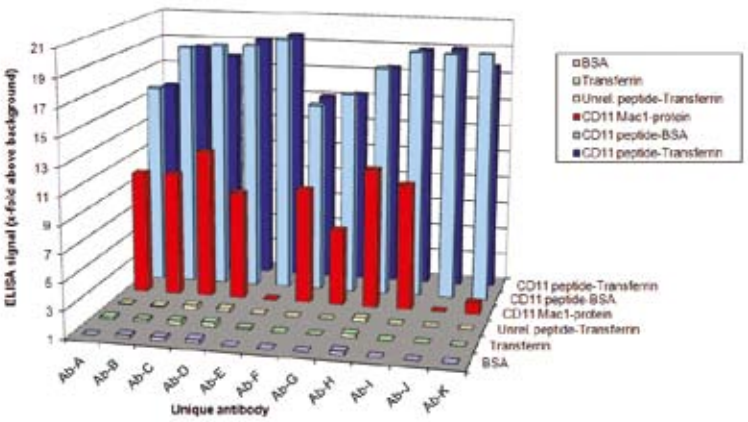


FIGURE 16 **QC ELISA of Anti-peptide Antibodies Show Binding of Parental Protein.** The HuCAL® antibody library was panned against the M18 peptide and 11 binders were expressed as bivalent mini-antibodies. QC ELISA showed that all 11 antibodies bound specifically to the M18 peptide but not to BSA and Trf carrier proteins, or to an unrelated peptide bound to Trf. The I-domain of the parental Mac1 protein is also recognized by eight of the 11 antibodies. Differences in signal intensity between peptide and protein antigens reflect coating densities of peptide vs. protein antigen (several peptides are conjugated to one BSA or Trf molecule), and do not represent differences in affinity.

The QC ELISA is a single, one-point measurement and is not useful for ranking the antibodies according to affinity. Consequently, AbD Serotec always recommends testing all available antibodies in the final application(s). On request we measure the antibody affinities or perform additional assays, such as capture or inhibition ELISAs, as the final QC step. Alternatively, the assay can be performed as a bead-based FLISA assay on the FMAT® instrument.

Additional Custom Services

The laboratories at AbD Serotec are setup to run many additional assays and services for a large number of antibodies in parallel. These assays and services include:

- Antigen generation using proprietary AgX® technology, expression as Fc fusion proteins, or with various other systems
- Antibody testing in a range of typical assays (Chapters 3–8)
- Small- and large-scale antibody conjugation to biotin, common reporter enzymes (HRP and AP), or fluorescent dyes
- Screening for antibody sandwich pairs (in 384-well format)
- Bead-based assays on a Luminex® or FMAT® 8200 CDS instrument
- Affinity ranking (k_{off} ranking) and determination using several different technologies (Chapter 10) including:
  - Surface plasmon resonance (SPR) on a Biacore™ instrument from GE Healthcare
  - Quartz crystal microbalance technology (QCM) on an Attana A200® instrument
  - Solution equilibrium titration (SET) on a Meso Scale Discovery™ (MSD™) instrument

For more information, please contact your account manager at **sales.muc@abdserotec.com**.

Applications: Western Blotting

Chapter 3

Western blotting is widely used for the detection and identification of protein antigens in samples such as solubilized cell pellets, cell culture supernatants, and tissue homogenates. The procedure involves separation of the proteins by size using gel electrophoresis, followed by transfer of the proteins onto a solid support membrane where they can be detected with antibodies.

Samples for Western blotting are denatured by boiling in a buffer containing SDS and a reducing agent prior to loading, which helps to unfold the protein and disrupt disulfide bonds. It is customary to measure the protein content of each sample before denaturation, so that equivalent amounts are loaded into the wells of the gel.

After the gel electrophoresis, the proteins are transferred to a blotting membrane using an electric field. The membrane is then blocked to prevent non-specific binding of proteins, before incubation with a primary antibody recognizing the antigen of interest. A labeled secondary antibody is then applied to detect the target antigen. The most common detection system relies upon the reporter enzyme HRP, which cleaves a substrate to generate a chemiluminescent product that is detectible by an imager or on X-ray film.

Use of HuCAL® Antibodies in Western Blotting

HuCAL® antibodies can be used for Western blotting by following the standard protocols developed for conventional antibodies. The only difference is that the secondary antibody cannot be directed against the Fc domain since this is lacking in HuCAL® Fab antibodies. An anti-human Fab secondary antibody (e.g. STAR126P) is recommended instead, due to the signal amplification achieved with this polyclonal secondary.

Alternatively, antibodies against the epitope tags (which are typically part of the HuCAL® Fab antibody), such as Strep-tag®, His-6, V5, FLAG®, and c-myc are suitable. In addition, if the Fab antibody contains an AP domain, then an antibody against bacterial AP can 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 with our easy-to-use

LYNX Rapid Conjugation kits® (see page 119). Antibodies conjugated with LYNX kits achieve comparable sensitivity to polyclonal antibody detection with a significantly reduced assay time.

The sensitivity of an antibody in a given application depends on its affinity and avidity. 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 since their avidity is greater. The bivalent format of HuCAL® antibodies is, therefore, recommended for most applications, including Western blotting.

AbD Serotec is equipped to run a large number of Western blots in parallel, allowing us to offer the testing of all HuCAL® antibodies resulting from an antibody generation project as an additional service. Contact your account manager at **sales.muc@abdserotec.com** for more information.

AbD Serotec also offers a free, detailed guide dedicated to Western blotting, visit **www.abdserotec.com/westernblot** to learn more.

General Tips

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

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

It is also important to use an appropriately matching 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 5. For other conjugates, please consult **www.abdserotec.com.**

It is also possible to use colorimetric detection substrates instead of chemiluminescent detection; this is described in Example 3, (page 55).

When working with antibodies that have been labeled with LYNX Rapid Conjugation kits®, we strongly recommend HiSpec buffer (BUF049) for antibody dilution. It is necessary to test for optimal antibody concentration to achieve sensitivities and background levels equal to detection with the most favorable secondary antibodies.

TABLE 5 **Selection of HRP-conjugated Secondaries for Western Blotting.**

Secondary Antibody	Recommended Dilution	Product Code
Anti-human F(ab')$_2$:HRP	1:5000	STAR126P
Anti-Strep-tag® Classic:HRP	1:500–1:4000	MCA2489P
Anti-His-6:HRP	1:1000–1:5000	MCA1396P
Anti-V5:HRP	1:1000	MCA1360P
Anti-bacterial AP:HRP	1:8000–1:10000	AHP1108P
Anti-FLAG®:HRP		MCA4764P
Anti-FLAG®:HRP	1:1000–1:5000	AHP1074P
Anti-c-myc:HRP	1:100–1:500	MCA2200P

For other conjugates, visit **www.abdserotec.com/HuCAL**

Protocol

1. Run the samples on SDS-PAGE and transfer onto a polyvinylidene fluoride (PVDF) membrane. For initial testing, use 40 μg of cell lysate proteins or 100 ng of pure antigen.
2. Block the membrane with 5% non-fat dried milk in tris-buffered saline-Tween® 20 (TBST), for 1 hour on a shaker at room temperature (RT), or overnight at 4°C.
3. Rinse the membrane with TBST.
4. Dilute the HuCAL® antibody (primary antibody) in TBST with 1% non-fat dried milk and add to the membrane. The optimal amount of HuCAL® antibody 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.
5. Incubate for 1 hour at RT on a shaker. Make sure that the membrane is completely immersed in the buffer.
6. Wash the membrane three times (5 minutes per wash) with a generous amount of TBST.
7. Add secondary antibody to the membrane. Anti-human Fab:HRP (STAR126P) is recommended at a 1:5000 dilution, either in TBST with 1% non-fat dried milk or in HiSpec buffer (BUF049).

8. Shake for 1 hour at RT.
9. Wash the membrane three times (5 minutes per wash) with a generous amount of TBST.
10. Develop the membrane using Amersham™ ECL Plus or ECL Advance™ (GE Healthcare) according to the manufacturer's instructions. Capture the image with an imager or X-ray film.

Alternative Protocol Using the SNAP i.d.® Protein Detection System

1. Run the samples on SDS-PAGE and transfer onto a PVDF membrane. For initial testing, use 40 μg of cell lysate proteins or 100 ng of pure antigen.
2. Insert the blotting membrane into the SNAP i.d. ® system (Millipore) according to the manufacturer's instructions.
3. Block the membrane using 0.05–0.1% non-fat dried milk in TBST. Use 30 ml, 20 ml, or 10 ml for the one, two, or three blot holder (Millipore), respectively. Run the blocking solution through the membranes without prior incubation.
4. Apply the primary antibody at a 3-fold higher concentration compared to the conventional protocol above, in TBST with 0.05% non-fat dried milk (e.g. 15 μg/ml is recommended for initial experiments). Incubate for 10 minutes with the pump switched off. Then apply vacuum for ~ 20 seconds. Use 10 ml, 6 ml, or 3 ml antibody solution for the one, two, or three blot holder, respectively.
5. Wash the blot three times by applying 30 ml, 15 ml, or 10 ml TBST for the one, two, or three blot holder, respectively, with the pump switched on.
6. Apply the secondary antibody, also at a 3-fold higher concentration compared to our conventional protocols. An anti-human Fab: HRP (STAR126P) at a 1:1500 dilution in HiSpec buffer (BUF049) is recommended. Incubate for 10 minutes with the pump switched off. Then apply vacuum for ~ 20 seconds. Use 10 ml, 6 ml, or 3 ml of antibody solution for the one, two, or three blot holder, respectively.
7. Wash the blot three times by applying 30 ml, 15 ml, or 10 ml TBST for the one, two, or three blot holder, respectively, with the pump switched on.
8. Develop the membrane using Amersham™ ECL Plus or ECL Advance™ according to the manufacturer's instructions. Capture results with an imager or X-ray film.

Examples

Human cyclophilin A from HEK293 cell lysate was detected on a Western blot using a HuCAL® bivalent mini-antibody (Fab-dHLX-MSx2) as the primary antibody, and anti-human F(ab')$_2$: HRP (STAR126P) as the secondary antibody. The blot was developed using Amersham™ ECL Plus (Figure 17).

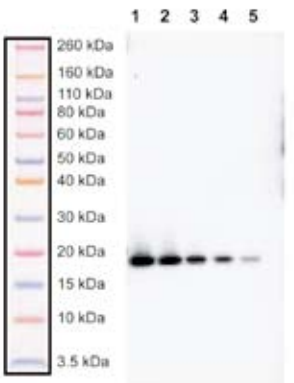


FIGURE 17 **Western Blot Analysis of HEK293 Lysates.** HuCAL® Anti-cyclophilin A Fab-dHLX-MSx2 Antibody (5 µg/ml) and Anti-F(ab')$_2$:HRP were used to probe the blot. Detection was performed using Amersham™ ECL Plus. Molecular Weight Standard at left. **Lanes 1–5**: 1:1 serial dilutions of HEK293 lysate from 4000 cells to 250 cells.

It is possible to omit a secondary antibody by using a primary antibody labeled with LYNX kits, thereby avoiding background issues associated with secondary antibodies. The protocol is significantly faster and provides sensitivities that are comparable to those achieved using optimal secondary antibodies. The anti-cyclophilin A antibody was conjugated to HRP with a LYNX kit (e.g. LNK002P) and used for immunodetection as described in Example 1 (without a secondary antibody) (Figure 18).

FIGURE 18 **Western Blot Analysis of HEK293 Lysate Using a Directly Labeled Antibody.** The blot was probed with HRP-conjugated (LYNX) HuCAL® Anti-cyclophilin A Fab-V5Sx2 antibody (5 μg/ml). Detection was performed using Amersham™ ECL Plus. Molecular weight markers at left. **Lanes 1–5**: 1:1 dilutions of HEK293 lysate from 4000 cells to 250 cells.

The sensitivity of the anti-cyclophilin A HuCAL® mini-antibody (HCA005) was determined by dot blotting. Pure cyclophilin A was spotted onto a PVDF membrane and detected using the HuCAL® anti-cyclophilin A bivalent mini-antibody and an anti-human F(ab')$_2$:HRP (STAR126P) (Figure 19).

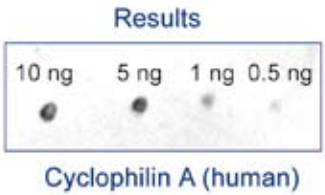


FIGURE 19 **Cyclophilin A Dot Blot.** Dot blot using HuCAL® anti-cyclophilin Fab-dHLX-MH as the primary antibody, anti-F(ab')$_2$:HRP (STAR126P) as the secondary antibody, and Amersham™ ECL Plus for detection.

A second dot blot (Figure 20) was prepared using the antigen, farnesyl diphosphate synthetase (FDPS), and detected with a HuCAL®-bacterial AP fusion antibody in the Fab-A-FH format (AbD02012). This format offers the advantages of a bivalent antibody (dimerization occurs via AP domain)

together with enzymatic activity for detection. The blot was developed with 5-bromo-4-chloro-3′ indolylphosphate p-toluidine salt/nitro-blue tetrazolium chloride (BCIP/NBT) substrate (BUF045A), without the use of a secondary antibody.

FIGURE 20 **FDPS Dot Blot.** Dot blot using HuCAL® anti-FDPS Fab-A-FH (AbD02012) without a secondary antibody and BCIP/NBT for detection.

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. ECL detection reagents are contaminated. Make up 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 or dot 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. Film exposure time (for ECL detection) was too short. Re-expose the blot for a longer time.
White (negative) bands on the film after detection with ECL system	1. Too much protein loaded or antibody concentrations too high, leading to depletion of substrate. Load less antigen 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 protocol. 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 use larger volume of wash buffer. 7. Insufficient amount of Tween® 20 in the buffers. Use TBS with 0.1% Tween® 20. 8. Film overexposed or blot developed for too long. Reduce exposure and/or development times. 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-reacting secondary with substitute that is suitable for the HuCAL® antibody format in use as the primary.

Applications: ELISA

Chapter 4

An ELISA is used to detect antigens or antibodies in samples, either qualitatively or quantitatively. It is usually performed using two antibodies for detection, one specific to the antigen, and the second specific to the first antibody. The second 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.

There are several types of ELISA:

- **Indirect ELISA** is used to confirm binding of the antibody to its antigen, but not to unrelated antigens. Since 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, 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.
- **Competition (or Inhibition) ELISA** detects antigens and monitors their concentration using competitive binding of an antibody to free or immobilized antigen.

Use of HuCAL® Antibodies in ELISAs

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 employed. Since HuCAL® antibodies do not contain the Fc domain, an anti-human Fab secondary antibody is recommended (the use of polyclonal antiserum amplifies the signal). Monoclonal antibodies against the 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 6.

TABLE 6 **Selection of Secondary Antibodies for ELISA.**

Secondary Antibody	Recommended Dilution	Product Code
Anti-human F(ab')$_2$:AP	1:5000	STAR126A
Anti-human F(ab')$_2$:HRP	1:5000	STAR126P
Anti-human F(ab')$_2$:Biotin	1:5000	STAR126B
Anti-Strep-tag® Classic:AP		MCA2489A
Anti-Strep-tag® Classic:HRP	1:5000	MCA2489P
Anti-Strep-tag® Immo (for Fab immobilization)		MCA2488
Anti-His-6:HRP	1:20–1:1000	MCA1396P
Anti-V5:HRP	1:1000	MCA1360P
Anti-bacterial AP:HRP	1:3500	AHP1108P
Anti-FLAG®:HRP	1:10000–1:50000	AHP1074P
Anti-FLAG®:HRP		MCA4764P
Anti-c-myc:HRP	1:100–1:500	MCA2200P

For other conjugates, visit **www.abdserotec.com/HuCAL**

All HuCAL® antibodies are routinely tested by indirect ELISA. The staff at AbD Serotec are highly experienced and well equipped to run all types of ELISAs (e.g. testing for sandwich pairs, inhibition ELISAs, etc.) at high throughput. We offer additional ELISA services in conjunction with any HuCAL® project. Contact your account manager at **sales.muc@abdserotec.com** for more information.

General Points

- 96-well plates can be used instead of 384-well plates, (e.g. black, flat-bottom MaxiSorp™ PS, NUNC, 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.
- In the examples shown, an AP-conjugated antibody was used, together with the AttoPhos® (Roche, 11681982) fluorescence detection system (excitation: 440 ± 25 nm, emission: 550 ± 35 nm). Alternatively, an HRP-conjugated antibody can be used together with QuantaBlu® (Thermo Fisher Scientific, 15169) fluorescence detection reagent (excitation: 320 ± 25 nm, emission: 420 ± 35 nm). If detection is performed using a chromogenic substrate (e.g.TMB Plus [BUF042A]), then transparent plates must be used.

- 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 antibody for capture. The anti-Strep-tag® Immo antibody (MCA2488) offers an extremely 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 secondaries 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 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. This is typically conjugated to an enzyme such as HRP or AP, and binds the antibody-antigen complexes. The plate is washed and the appropriate substrate is applied. Finally, the resulting chromogenic or fluorescent signal is viewed using a spectrophotometer (Figure 21).

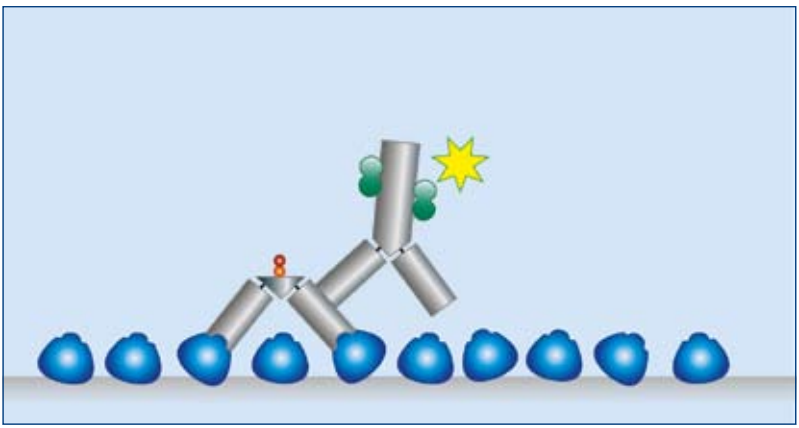


FIGURE 21 **Indirect ELISA.** The antigen (blue) is coated to a surface. The primary antibody (gray mini-antibody) binds to the antigen and an enzyme-linked secondary anti-Fab antibody (gray full-size antibody) is used for detection. The star represents the detection substrate.

Protocol

Indirect ELISA with Fluorescence Readout

1. Coat the antigen by adding 20 µl of a 5 µg/ml antigen solution diluted in phosphate-buffered saline (PBS) to several wells of a 384-well microtiter plate, and incubate overnight at 4°C. Use a black, 384-well ELISA microtiter plate with a square-well and flat-bottom, such as MaxiSorp™ PS (NUNC, 460518).
2. Wash the microtiter plate twice with phosphate-buffered saline-Tween® 20 (PBST).
3. Block the microtiter plate by adding 100 µl 5% non-fat dried milk in PBST to each well. Incubate for 1–2 hours at RT.
4. Wash the microtiter plate twice with PBST.
5. Transfer 20 µl HuCAL® antibody to each well. A standard concentration of 2 µg/ml in PBST or HiSpec buffer (BUF049) is recommended. Incubate for 1 hour at room temperature.
   Note: Optimize the concentrations of each HuCAL® antibody by titration.
6. Wash the microtiter plate five times with PBST.
7. Add 20 µl of the secondary antibody to each well and incubate for 1 hour at room temperature. Anti-human Fab: AP (STAR126A), diluted 1:5000 in HiSpec buffer (BUF049) is recommended.
8. Wash the microtiter plate five times with PBST.
9. Add 20 µl AttoPhos® (Roche, 11681982) to each well. Measure fluorescence after 10 minutes (excitation: 440 ± 25 nm, emission: 550 ± 35 nm).

Example

The antigen (a peptide derived from the pKi-67 protein and coupled to a carrier protein) was detected by ELISA with different concentrations of HuCAL® antibody (bivalent mini-antibody format Fab-dHLX-MH, (HCA006) (Figure 22).

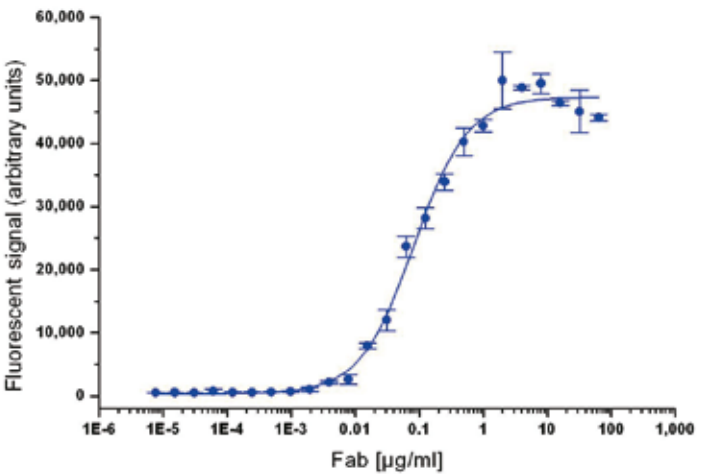


FIGURE 22 **Indirect ELISA.** The experiment was carried out with a HuCAL® Anti-pKi-67 Fab-dHLX-MH Antibody and Anti-F(ab')$_2$:AP, with AttoPhos® detection.

Direct ELISA

In a direct ELISA, the primary antibody is labeled, for example, by genetic fusion with AP or with LYNX Rapid Conjugation kits® (see page 119). This 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.

LYNX-conjugated antibodies show excellent sensitivity, often similar or even better than those achieved in indirect ELISAs. However, the AP genetic fusion system is usually less sensitive than indirect detection via secondary antibodies (Figure 23).

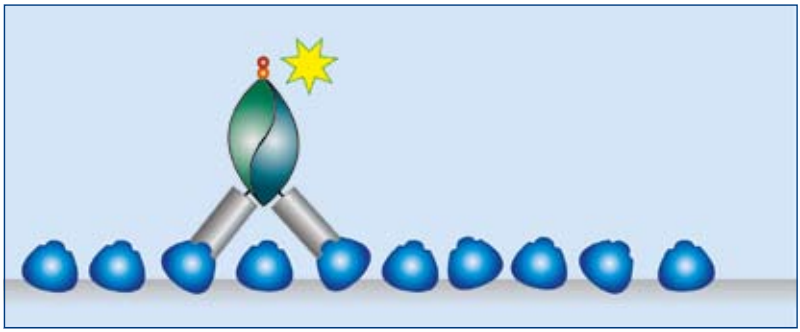


FIGURE 23 **Direct ELISA.** The antigen (blue) is coated to a surface. An AP-fused primary antibody (gray/green) is used in combination with an AP substrate (yellow star) for detection.

Protocol

Direct ELISA with AP-conjugated Primary Antibody

1. Coat the antigen by adding 20 µl of a 5 µg/ml antigen solution diluted in PBS to several wells of a 384-well microtiter plate, and incubate overnight at 4°C. Use a black, 384-well ELISA microtiter plate: square-well, flat-bottom, such as the MaxiSorp™ PS (NUNC, 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. Use a standard concentration of 2 µg/ml in PBST or HiSpec buffer (BUF049). Incubate for 1 hour at room temperature.
   Note: Optimize the concentrations of each HuCAL® antibody by titration.
6. Wash the microtiter plate five times using PBST.
7. Add 20 µl AttoPhos® (Roche, 11681982) to each well and measure the fluorescence after 10 minutes (excitation: 440 ± 25 nm, emission: 550 ± 35 nm).

Example

The antigen (a peptide derived from the pKi-67 protein coupled to a carrier protein) was detected by both direct and indirect ELISA. Direct ELISA was performed using a HuCAL® Fab-A-FH anti-pKi-67 antibody fused with AP (AbD02815; this is the same antibody as HCA006, but cloned into Fab-A-FH format). Indirect ELISA was performed using the same antibody in the Fab-dHLX-MH format (HCA006), and anti-Fab:AP detection (STAR126A) (Figure 24).

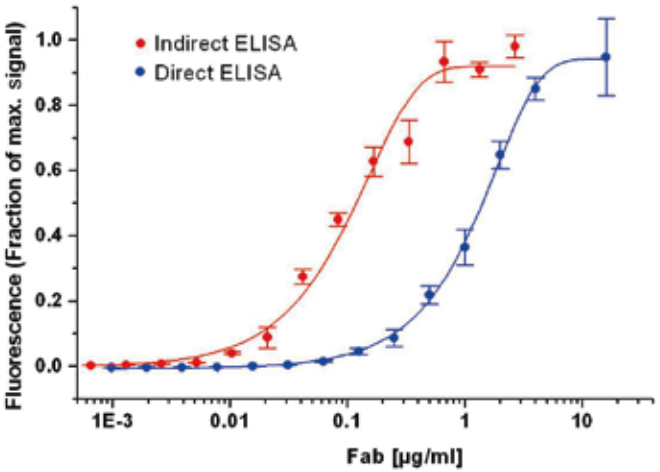


FIGURE 24 **Comparison of Direct and Indirect ELISA.** Direct ELISA was performed using a HuCAL® Fab-A-FH anti-pKi-67 antibody fused to AP. For the indirect ELISA, the same antibody was used in the Fab-dHLX-MH format, and anti-Fab:AP was used for detection. Note that the use of a secondary antibody provides an amplification effect that increases the sensitivity of the assay compared to the AP fusion. Using LYNX kits to conjugate the antibody achieves sensitivities similar to secondary antibody detection.

Sandwich ELISA

Sandwich ELISAs are a highly sensitive and specific method of detecting antigens, which provide fast and accurate determination of the antigen concentration in a given sample. The technique uses two 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 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 (see page 45 for selection strategies for antibody sandwich pairs). The bivalent format of HuCAL® mini-antibodies is highly suitable for both capture and detection. When two HuCAL® antibodies are used, the detection antibody is typically biotinylated (see page 119) for detection via streptavidin. Alternatively, the two HuCAL® antibodies must be cloned in different formats with detection via a tertiary antibody specific for the detection antibody (e.g. Fab-dHLX-MSx2 capture antibody, Fab-A-V5Sx2 detection antibody and HRP-conjugated anti-bacterial AP tertiary antibody (AHP1108P) (Figure 25).

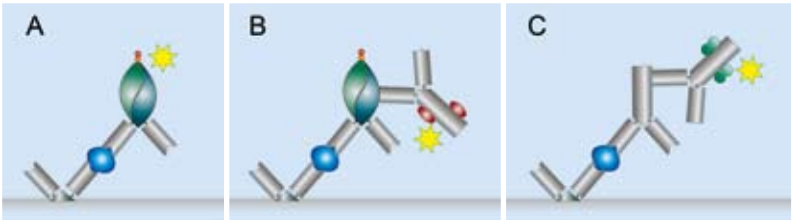


FIGURE 25 **Sandwich ELISA Schematic.** Sandwich ELISAs are carried out using three detection options. **Panel A:** Two HuCAL® antibodies with direct detection. **Panel B:** Two HuCAL® antibodies with indirect detection (using anti-AP:HRP). **Panel C:** A HuCAL® capture antibody and a mouse polyclonal detection antibody plus an HRP-conjugated anti-mouse secondary antibody. The capture antibody (gray bivalent mini-antibody) is immobilized and binds the antigen (blue).

Protocols

Sandwich ELISA Using Two HuCAL® Antibodies

1. Coat the capture antibody by adding 20 µl of a 5 µg/ml solution diluted in PBS to several wells of a 384-well microtiter plate, and incubate overnight at 4°C. Use a black, 384-well ELISA microtiter plate, which has square-wells and a flat-bottom, MaxiSorp™ PS (NUNC, 460518).
2. Wash the microtiter plate twice with PBST.
3. Block the microtiter plate by adding 100 µl 5% BSA in PBST to each well, and then incubate for 1 hour at RT.
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 RT. Use a range of antigen concentrations, diluted in PBST with 1% BSA or in HiSpec buffer (BUF049).
6. Wash the microtiter plate five times with PBST.
7. Add 20 µl HuCAL® detection antibody (1–2 µg/ml concentration in HiSpec buffer (BUF049) to each well, and incubate for 1 hour at RT. Use one of the following options:

   A. Use a biotinylated detection antibody.

   B. Use an antibody in Fab-A format (e.g. Fab-A-V5Sx2) as the detection antibody in combination with Fab-dHLX capture antibody.

   C. Use a detection antibody carrying different tags than 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 detection antibody to each well and incubate for 1 hour at RT. Choose option below to match step 7.

   A. Use either streptavidin HRP (STAR5B) or streptavidin AP (STAR6B) in combination with matching substrate, diluted 1:1000 in HiSpec buffer (BUF049).

   B. Use rabbit anti-bacterial AP HRP (AHP1108P), diluted 1:3500 in HiSpec buffer (BUF049).

   C. Use matching epitope tag antibodies, e.g. anti-Strep-tag® Classic HRP (MCA2489P) diluted 1:5000 in HiSpec buffer (BUF049).
10. Wash the microtiter plate five times with PBST.
11. Add 20 µl QuantaBlu® (Thermo Fisher Scientific, 15169) to each well for HRP-conjugated secondaries or Attophos® (Roche, 11681982) for AP-conjugated secondaries, and measure the fluorescence directly.

Sandwich ELISA with HuCAL® and Polyclonal Mouse Antibodies

1. Coat the plate with the HuCAL® capture antibody by adding 20 µl of a 5 µg/ml solution, diluted in PBS, to several wells of a 384-well MaxiSorp™ microtiter plate. Incubate overnight at 4°C.
2. Wash the microtiter plate twice 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 twice 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 HiSpec buffer (BUF049).
6. Wash the microtiter plate five times with PBST.
7. Add 20 µl biotinylated polyclonal detection antibody (concentration as specified by the manufacturer) in HiSpec buffer (BUF049) to each well, and incubate for 1 hour at RT.
8. Wash the microtiter plate five times with PBST.
9. Transfer 20 µl of AP-conjugated streptavidin (STAR6B) diluted 1:2000–1:10000 in HiSpec buffer (BUF049) and incubate for 1 hour at RT.
10. Wash the microtiter plate five times with PBST.
11. Add 20 µl of AttoPhos® (Roche; 11681982) to each well, incubate for 10 minutes, and measure the fluorescence (excitation: 440 ± 25 nm, emission: 550 ± 35 nm).

Examples

A sandwich ELISA was performed using:

- A HuCAL® sandwich pair
- A HuCAL® capture antibody together with a commercial polyclonal detection antibody

For the experiment with the HuCAL® sandwich pair, a bivalent mini-antibody directed against IFNγ in the Fab-dHLX-MH format (AbD0676, HCA043) was used for capture. A serial dilution of the antigen IFNγ (PHP050) was detected with a bivalent mini-antibody fused to bacterial AP (AbD02503, Fab-A-FH, HCA044). For direct detection (Figure 26), AttoPhos® AP substrate

(Roche; 11681982) was used for signal development. An HRP-conjugated rabbit anti-bacteria AP antibody (AHP1108P) was used together with QuantaBlu® HRP substrate (Thermo Fisher Scientific; 15169) for indirect detection (Figure 27). The use of an anti-AP secondary antibody amplifies the signal, leading to higher sensitivity for this assay.

The mixed sandwich ELISA was performed using a HuCAL® capture antibody against IFNγ (AbD00068, Fab-MH) and a polyclonal anti-IFNγ detection antibody (R&D Systems, BAF285). Since the polyclonal antibody is biotinylated, streptavidin-AP (STAR6B) and AttoPhos® AP substrate can be used for signal development (Figure 28).

Direct Sandwich ELISA

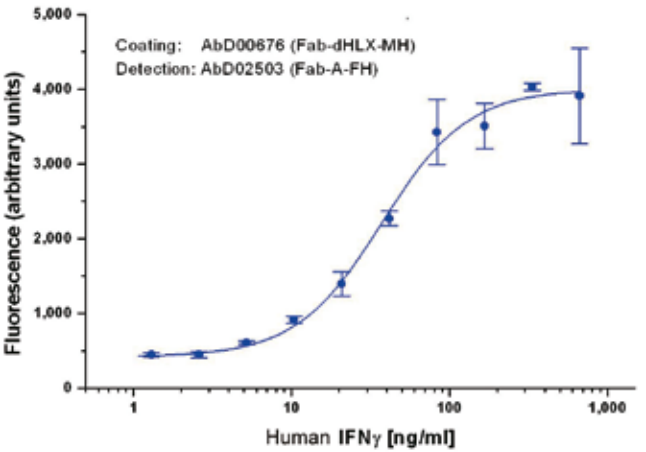


FIGURE 26 **Direct Sandwich ELISA for IFNγ.** The ELISA used HuCAL® capture (Fab-dHLX-MH) and detection (Fab-A-FH) antibodies, with direct detection through the genetic fusion with AP and AttoPhos®.

Indirect Sandwich ELISA

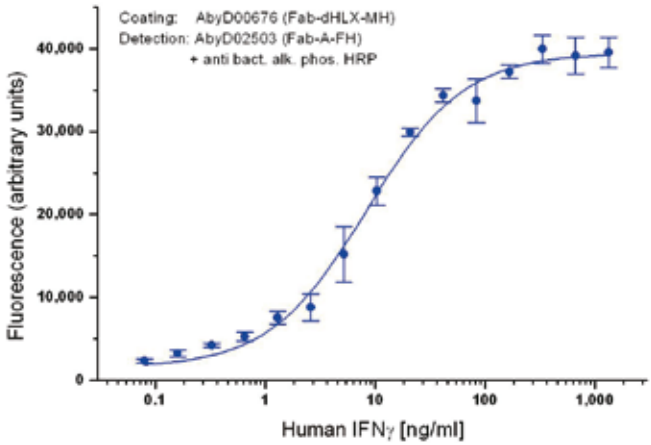


FIGURE 27 **Indirect Sandwich ELISA for IFNγ.** This ELISA used HuCAL® capture (Fab-dHLX-MH) and detection (Fab-A-FH) antibodies, and an HRP-conjugated anti-bacterial AP antibody and QuantaBlu® for indirect detection. Use of a secondary antibody provides an amplification effect that increases the sensitivity of the assay compared with the direct detection experiment shown in Figure 26.

Indirect Sandwich ELISA with a Polyclonal Antibody

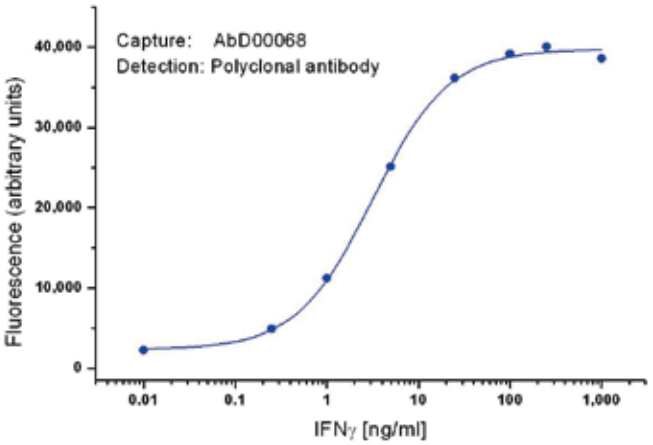


FIGURE 28 **Indirect Sandwich ELISA for IFNγ.** This ELISA used a HuCAL® capture antibody (Fab-MH) and a biotinylated polyclonal detection antibody. Results were visualized with streptavidin-AP and AttoPhos®.

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 binds the immobilized antigen is detected using a secondary antibody linked to a detection label such as HRP. The appropriate substrate is applied and the resulting chromogenic or fluorescent signal is viewed 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. Other variations of a competition ELISA are also possible.

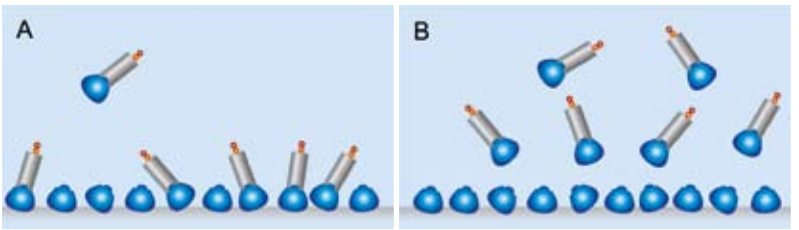


FIGURE 29 **Examples of Competition ELISAs. Panel A:** Competition ELISA with low concentration of free antigen (blue); HuCAL® antibodies (gray) bind to the coated antigen. **Panel B:** Competition ELISA with high concentration of free antigen. More HuCAL® antibodies are kept in solution by binding to free antigen and are washed away before the addition of the secondary antibody.

Protocol

1. Coat the plate with antigen by adding 50 µl of a of 5 µg/ml antigen solution diluted in PBS to several wells of a 384-well MaxiSorp™ 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 RT.

4. Incubate 25 µl of antigen solution with 25 µl of HuCAL® antibody (final concentration 2 µg/ml) for 1 hour at RT. Use a range of antigen concentrations diluted in PBS or HiSpec buffer (BUF049).
5. Wash the microtiter plate twice with PBST.
6. Transfer 50 µl of HuCAL® antibody/antigen mix to each well of the microtiter plate, and incubate for 1 hour at RT.
7. Wash the microtiter plate five times with PBST.
8. Transfer 50 µl of the secondary antibody (anti-human Fab:AP, STAR126A), diluted 1:5000 in HiSpec buffer (BUF049), and incubate for 1 hour at RT.
9. Wash the microtiter plate five times with PBST.
10. Add 20 µl AttoPhos® (Roche, 11681982) to each well. Incubate for 10 minutes at RT and measure fluorescence (excitation: 440 ± 25nm, emission: 550 ± 35nm).

Example

The concentration of the antigen, FITC, in solution was determined by competition ELISA. BSA-conjugated FITC was coated onto a microtiter plate and a mix of primary HuCAL® antibody (AbD00756, HCA002) and varying amounts of free FITC were transferred to the coated plate. Detection was performed using anti-human F(ab')$_2$:AP (STAR126A).

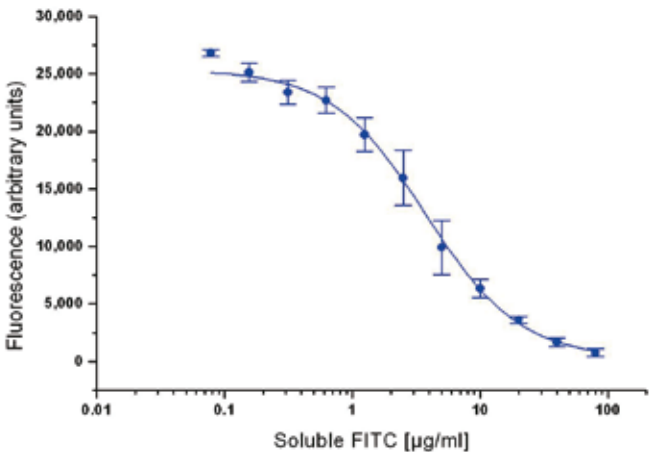


FIGURE 30 **Competition ELISA.** An anti-FITC HuCAL® antibody (AbD00756) is added to coated FITC-BSA. Binding of the antibody to FITC-BSA is inhibited by the addition of varying amounts of soluble FITC.

Troubleshooting

Problem	Possible Cause and Course of Action
No signal in ELISA	1. Assay incorrectly set up or incorrect reagents used. Include a positive control. 2. Improper secondary antibody used. Use 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 fresh aliquot of antibody that has been stored at –20°C or below. 4. Detection reagent contaminated. Use freshly prepared detection reagents. 5. Antigen not coated properly. Try longer coating times, different buffers, or use avidin plates with biotinylated antigen. 6. Incorrect settings on plate reader for this detection system. Check values (wavelength, filters, gain etc.).
Weak signal	1. Insufficient amount of antigen coated to microtiter plate. Use more antigen for coating step. 2. Concentration of primary or secondary antibody too low. Optimize the protocol for the reagents. 3. Detection reagent too old or contaminated. Use fresh detection reagent. 4. Detection reagent diluted. Use a higher concentration of detection reagent. 5. Incorrect settings on plate reader for this detection system. Check values (wavelength, filters, gain etc.).
High background signal	1. Concentration of primary and/or secondary antibody too high. Optimize antibody concentrations. 2. Too much detection reagent used. Repeat with more dilute detection reagent. 3. Number of washing cycles too low. Increase number of washing cycles. 4. Contaminated blocking agent. Use fresh blocking agent.

5. Incorrect concentration of blocking agent. Make sure that the correct concentration is used, as described in the protocol.

6. Incubation time of detection reagent too long. Start measurement soon after addition of detection reagent.

7. Incorrect settings on the reader. Adjust the settings (e.g. gain).

8. Insufficient amount of Tween® 20 in the buffers. Use PBS with 0.05% Tween® 20.

9. Use HiSpec buffer (BUF049) for primary and secondary antibody dilution.

Applications: Immunoprecipitation

Chapter 5

Immunoprecipitation is used to isolate an antigen from cells or tissues with an antigen-specific antibody attached to a sedimentable matrix. The method can also help to identify members of protein complexes in solubilized cell or tissue extracts by targeting any one of the proteins thought to be in the complex. The antibody brings the complex out of solution so that it can be analyzed using standard techniques such as Western blotting and mass spectrometry. HuCAL® antibodies are ideal for immunoprecipitation.

The procedure consists of four stages:

- Immobilization of the antibody onto a matrix
- Solubilization of the antigen
- Immunoprecipitation
- Analysis of the precipitated protein(s)

Basic procedures for each step are outlined in this chapter.

Protein A or G Sepharose® is often used as a matrix for sedimenting conventional antibodies, since these compounds bind to the Fc portion of the antibody. However, since HuCAL® Fabs lack the Fc region, a different strategy is required.

There are three procedures suitable for immobilizing HuCAL® Fabs:

- Covalent coupling on magnetic beads via primary amino groups
- Immobilization on Ni-NTA magnetic beads via the His-6 tag or immobilization on MagStrep Strep-Tactin® beads for Strep-tagged HuCAL® antibodies
- Immobilization of a capture antibody (see Table 7) on magnetic beads and incubation with the HuCAL® antibody

Magnetic beads are preferable to regular beads because they are easier to wash. Ni-NTA beads should not be used with lysis buffer containing metal-chelating compounds (e.g. ethylenediaminetetraacetic acid [EDTA]). Protocols for the first two options are given below.

TABLE 7 **Methods to Pull Down the Immunocomplex.**

Matrix	Method	Advantages	Disadvantages
Primary antibody coupled beads	Fab covalent coupled	Low background, little nonspecific reaction since secondary antibodies are used	Coupling required for every antibody; high primary antibody consumption
Anti-F(ab')$_2$ (STAR126) coupled beads	Capture Fab	Good for testing many different antibodies	Pull-down of intrinsic antibodies in the human samples
Anti-Strep®-tag Immo (MCA2488) coupled beads	Capture Fab via StrepX tag	Excellent antibody affinity, good for testing many different antibodies	None
Anti-Strep®-tag Classic (MCA2489) coupled beads	Capture Fab via Strep tag	Good for testing many different antibodies	None
Anti-His-6 tag (MCA1396) coupled beads	Capture Fab via His-6 tag	Good for testing many different antibodies	None
Anti-V5 tag (MCA1360GA) coupled beads	Capture Fab via V5 tag	Good for testing many different antibodies	None
Anti-FLAG® tag (MCA4764) coupled beads	Capture Fab via FLAG® tag	Good for testing many different antibodies	None
MagStrep type 2 Strep-Tactin® beads (IBA)	Capture Fab via Strep-tag®	Ready-to-use beads, good for testing many different antibodies	None
TALON™ beads (Ni-NTA beads; Invitrogen)	Capture Fab via His-6 tag	Ready-to-use beads, good for testing many different antibodies	Slightly higher background, might require extra washing steps

Methods that use a bead coupling step were tested with Dynabeads® M-450 Epoxy (Invitrogen)

AbD Serotec offers testing of HuCAL® antibodies in immunoprecipitation under customer specified conditions. Contact your account manager at **sales.muc@abdserotec.com** for more information.

Protocols

Covalent immobilization on Dynabeads® M-450 Epoxy

1. Wash 300 µl magnetic Dynabeads® M-450 Epoxy (Invitrogen, 14011) twice using 0.5 ml phosphate buffer, 0.1 M, pH 7.4.
2. Resuspend beads in phosphate buffer and add 100 µg HuCAL® Fab to give a final volume of approximately 350 µl.
3. Incubate on a rotator for 16–20 hours at RT.
4. Place the tube in a magnetic holder for 1 minute and remove the supernatant.
5. Wash the beads three times with 1 ml of PBS.
6. Resuspend beads in 700 µl blocking solution (3% BSA in PBS).
7. Block the beads on a rotator for 30 minutes at RT.
8. Wash the beads once with 700 µl PBS.
9. Resuspend the beads in 300 µl PBS.

Note: This protocol can also be used for the immobilization of capture antibodies. In that case, capture 10 µg of HuCAL® antibody onto 100 µl capture antibody coupled beads. Incubate for 1hour at RT on a shaker or rotator and wash beads with PBST.

Immobilization on Dynabeads® TALON™ via the His-6 tag

(Suitable for Fab formats with a His-6 tag, e.g. Fab-dHLX-MH)

1. Wash 50 µl Dynabeads® TALON™ (Invitrogen, 10101D) twice with 700 µl PBS.
2. Resuspend the beads in 100 µl PBS and add 20 µg HuCAL® Fab. Add PBS to a final volume of 700 µl.
3. Incubate on a rotator for 10 minutes at RT.
4. Place the tube in a magnet holder for 1 minute and remove the supernatant.
5. Wash the beads four times with 700 µl PBS.
6. Resuspend beads in 700 µl blocking solution (3% BSA in PBS).
7. Block the beads on a rotator for a further 30 minutes at RT.
8. Wash the beads once with 700 µl PBS.
9. Resuspend the beads in 100 µl PBS.

Solubilization

Various techniques can be used for this step, depending on the origin and nature of the antigen. Below is a basic protocol suitable for extracting proteins from mammalian cells.

1. Use either a fresh or frozen cell pellet (containing 0.5–2.0 × 10^7 cells) thawed on ice.
2. Resuspend the cells in 1 ml ice-cold non-denaturing lysis buffer (Tris/NaCl/NP 40/EDTA/protease inhibitor [TNEC]) by gentle agitation on a vortex mixer for 30 seconds. Use medium speed to avoid foaming.
3. Store the suspension on ice for 15–30 minutes, then transfer to a microfuge tube.
4. Clear the lysate by centrifugation for 15 minutes at maximum speed, at 4°C.
5. Transfer the supernatant to a fresh microfuge tube. To avoid disturbing the pellet, leave 20–40 µl supernatant in the original tube.
6. Store the supernatant on ice until used for immunoprecipitation.

Immunoprecipitation

This step involves incubation of the immobilized antibodies with the solubilized antigen, followed by extensive washing to remove unbound protein.

1. Add 100 µl bead-coupled antibodies to 1 ml lysate.
2. Incubate on a rotator for 1–2 hours at 4°C.
3. Wash four times with 0.5 ml ice-cold TNEC buffer for a total wash time of approximately 30 minutes. Store samples on ice for 3–5 minutes between washes, if necessary.
4. Wash once with PBS.

Analysis

This step is performed using standard techniques, such as: Coomassie®-stained PAGE, Western blotting (see Chapter 3), or mass spectrometry.

1. Resuspend the pellet in 10 µl reducing SDS gel loading buffer (sample buffer).
2. Heat to 95°C for 5 minutes, and then cool on ice.
3. Run the entire sample on SDS-PAGE and electrotransfer onto a PVDF membrane, and then refer to chapter 3 for Western blot analysis steps.

Example

Granta cells (~1.3 × 10^7 cells per immunoprecipitation) were used for precipitation of vimentin with HuCAL® Fab mini-antibodies. Different formats of the same clone were tested with appropriately matching precipitation methods. Precipitated proteins were detected using Western blot analysis with the same anti-vimentin antibody (Fab-dHLX-MSx2 format, HCA111) conjugated to HRP (using the LNK 002P LYNX kit). Direct detection without a secondary antibody has the advantage that the Fab bands of the precipitating antibody are not visible on the blot.

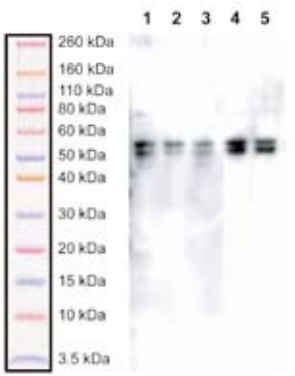


FIGURE 31 **Immunoprecipitation of Vimentin.** Western blot analysis using anti-Vimentin:HRP (Fab-dHLX-MSx2 [HCA111] conjugated to HRP with LNK002P kit) and ECL detection. Different HuCAL® anti-Vimentin antibody formats and beads were used for the immunoprecipitations. **Lane 1:** Fab-dHLX-MSx2 coupled to M450 Epoxy beads. **Lane 2:** Fab-dHLX-MH on Talon™ beads. **Lane 3:** Fab-A-V5Sx2 on anti-V5 coupled beads, **Lane 4:** Fab-dHLX-MSx2 on anti-Strep-tag® Immo (MCA2488) beads. **Lane 5:** Fab-A-V5Sx2 on MagStrep Strep-Tactin® beads.

Troubleshooting

Problem	Possible Cause and Course of Action
No bands on Western blot	1. Insufficient antigen in sample. Increase amount of source material used for immunoprecipitation. 2. Antigen not expressed in the source. Test for antigen expression in source by Western blotting with concentrated lysate. 3. Insufficient antibody used. Use more immobilized antibody. 4. Antigen not properly solubilized. Try different lysis buffers. 5. Antibody not suitable for immunoprecipitation. Test immunoprecipitation with pure antigen. 6. Antibody does not work in Western blotting. Test Western blot with pure antigen (and lysate). 7. 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.
High background signal due to non-specific binding of unrelated protein	1. Use additional washing steps (e.g. 15 times with 0.5 ml for at least 1 hour). 2. Optimize lysis buffer for your cells. 3. Use covalently immobilized antibody or other secondary antibody for precipitation. 4. Try a different blocking agent for bead blocking step. 5. Increase number of washing steps.

Applications: Immunohistochemistry

Chapter 6

IHC is used to detect antigens directly in tissue sections. Experiments can be performed on a wide range of biological specimens using different detection methods. Therefore, it is important to optimize the IHC conditions for each antigen under investigation.

Most samples for IHC are embedded in paraffin because this simplifies storage and provides excellent morphological detail and resolution. Cryostat sections, or cryosections, are typically used if no paraffinized tissue is available. In addition, they may be employed if the antigen cannot be detected after formalin fixation and paraffin-embedding, due to protein cross-linking. This chapter describes the standard procedures used with each type of tissue section, followed by fixation methods and typical staining procedures.

Use of HuCAL® Antibodies in IHC

Monoclonal antibodies are generally preferred over polyclonal antibodies for IHC because they usually have a higher specificity. HuCAL® antibodies offer a number of additional advantages over conventional monoclonal antibodies, including rapid generation time and pre-existing epitope tags to simplify detection.

TABLE 8 **Recommended Secondary Antibodies for IHC.**

Secondary Antibody	Recommended Dilution	Product Code
Anti-human F(ab')$_2$:HRP	1:50–1:500	STAR126P
Anti-Strep-tag® Classic	1:50–1:200	MCA2489
Anti-His-6	1:50–1:200	MCA1396
Anti-His-6	1:60	Roche,1922416
Anti-AP:HRP	1:50–1:100	AHP1108P
Anti-FLAG®:HRP		MCA4764P
Anti-V5		MCA1360
Anti-mouse IgG:Biotin	1:50–1:200	STAR11B

Visit **www.abdserotec.com/HuCAL** for more information. **Note:** Anti-c-myc antibodies are not recommended for use in IHC.

Paraffin Sections

Paraffin tissue sections must be cleared of paraffin (deparaffinized) and rehydrated before use to ensure that the antibodies have full access to the tissue antigen. Samples frequently require pretreatment to unmask the antigen. This is because treatment with formaldehyde fixative often induces intramolecular and intermolecular cross-linking of proteins. As a result, conformational changes may occur that mask the domains of the antigen bound by the antibody, leading to non-specific staining.

Procedure for Deparaffinization and Rehydration

1. Tissue sections are dried 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.
4. Wash the slides twice in 100% ethanol (for 2 minutes per wash).
5. Wash the slides twice in 90% ethanol (for 2 minutes per wash).
6. Wash the slides twice in 80% ethanol (for 2 minutes per wash).
7. Wash the slides twice in 70% ethanol (for 2 minutes per wash).
8. Wash the slides twice in 60% ethanol (for 2 minutes per wash).
9. Wash the slides twice in 50% ethanol (for 2 minutes per wash).
10. Wash the slides twice in TBST (for 2 minutes per wash).

Cryosections

There are two types of cryosections:

- **Fresh or unfixed sections**: quickly frozen tissues are cut, and then air-dried. Sometimes they are fixed before staining.
- **Fixed frozen tissue**: the tissue is fixed first, 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, since this may destroy the antigen
- Use adhesive slides
- Air-dry sections completely before fixation
- All steps can be carried out at RT unless otherwise specified

Fixation

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

Procedure

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.

Mounting

Either aqueous or non-aqueous mounting media is suitable, depending on the chromogenic substrate used to detect the antigen. We recommend a non-aqueous medium for permanent mounting of 3,3' diaminobenzidine (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.
   Important: 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 at RT, or dry in an incubator at 60°C for 15 minutes.

Aqueous Mounting

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

Staining

Cryosections and paraffin-embedded sections were stained with a HuCAL® bivalent mini-antibody (Fab-dHLX-MH, AbD05054, HCA071) against recombinant human desmin expressed in *E. coli.* Desmin belongs to the class III intermediate filaments, which constitute part of the cytoskeleton. It is the characteristic intermediate filament found in all three types of muscle cells (skeletal, cardiac, and smooth muscle).

Structurally, desmin is a 52 kDa protein encoded by nine exons of a gene located on chromosome 2q35. It forms cytoskeletal networks of muscle fibers between the plasma and nuclear membranes, and is found in the subplasmalemmal region and the Z-band. The anti-desmin antibody AbD05054 labels both smooth and striated muscle cells. It is useful for the identification of rhabdomyosarcomas and leiomyomas.

This section describes six alternative staining protocols using a HuCAL® antibody together with different amplification and detection systems.

Staining of Tagged HuCAL® Fabs (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 directly-labeled anti-tag antibody (Figure 32).

Procedure

1. Deparaffinize the slides according to the protocol on page 81.
2. Wash the slides a further three times (for 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 ddH_2O and allow the sections to cool in the buffer for 15 minutes.
6. Wash the slides three times (for 2 minutes per wash) in TBST.
7. Incubate in 3% H_2O_2 in methanol for 10 minutes to quench peroxidase in the tissue.
8. Wash the slides three times (for 2 minutes per wash) in TBST.
9. Transfer slides to a solution of 10% BSA in PBS and incubate for 15 minutes at room temperature to block proteins.
10. Tip excess blocking solutions off the sections, apply 10–25 µg/ml of primary HuCAL® antibody in antibody-diluent (BUF014), and incubate for 1 hour. The concentration of antibody should be determined empirically for each antigen, staining system, and laboratory.
11. Wash the slides three times (for 2 minutes per wash) in TBST.
12. Apply mouse anti-His-6 antibody conjugated to HRP (MCA1396P), diluted 1:50–1:200 in antibody diluent (BUF014), and incubate for 30 minutes.
13. Wash the slides three times (for 2 minutes per wash) in TBST.
14. Add 1 drop (approximately 40 µl) of DAB chromogen per 1 ml of DAB substrate buffer (BUF21A and BUF022), mix by swirling, and apply to tissue. Incubate for 10 minutes.
15. Wash the slides three times (for 2 minutes per wash) in TBST.
16. Counterstain using hematoxylin (Sigma, MHS16) for 1 minute.

17. Rinse slide with tap water to enhance color development.
18. Rinse slides with ddH_2O.
19. Dehydrate with graded alcohol (see mounting procedure on page 82) and apply a permanent coverslip to the slides.

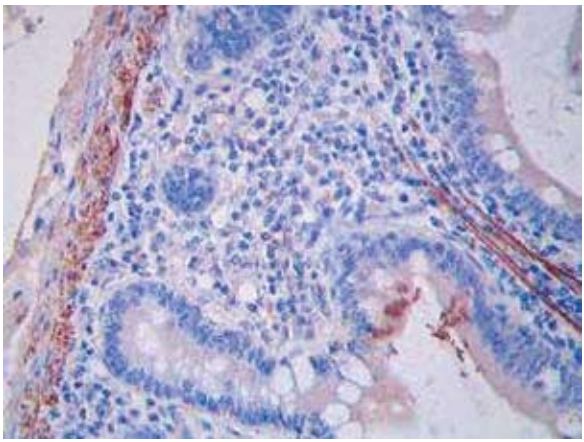


FIGURE 32 **Staining of Human Small Bowel.** 400x magnification, using HRP-labeled HuCAL® Fab AbD05054 at 10 µg/ml and DAB detection.

Staining of Tagged HuCAL® Fabs without Amplification (ABC or LSAB Method)

This straightforward procedure is suitable only 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 enzyme-labeled streptavidin complex (Figure 33).

Procedure

1. Deparaffinize the slides according to the protocol on page 81.
2. Wash the slides three times (for 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 ddH_2O and allow the sections to cool in the buffer for 15 minutes.
6. Wash the slides three times (for 2 minutes per wash) in TBST.
7. Incubate in 3% H_2O_2 in methanol for 10 minutes to quench peroxidase in the tissue.
8. Wash the slides three times (for 2 minutes per wash) in TBST.
9. Transfer slides to a solution of 10% BSA in PBS and incubate for 15 minutes to block proteins.
10. Tip excess blocking solution off the sections, apply 1–5 µg/ml primary HuCAL® antibody in antibody diluent (BUF014), and incubate for 1 hour. The concentration of antibody should be determined empirically for each antigen, staining system, and laboratory.
11. Wash the slides 3 times (for 2 minutes per wash) in TBST.
12. Apply mouse anti-His-6-tag antibody (MCA1396), diluted 1:50–1:200 in antibody diluent (BUF014), and incubate for 30 minutes.
13. Wash the slides three times (for 2 minutes per wash) in TBST.

14. Apply biotinylated anti-mouse antibody (STAR11B), diluted to 1:50–1:200 in antibody diluent and incubate for 15 minutes.
15. Wash the slides three times (for 2 minutes per wash) in TBST.
16. Apply streptavidin-peroxidase complex (STAR5B), and incubate for 15 minutes.
17. Wash the slides three times (for 2 minutes per wash) in TBST.
18. Add 1 drop (approximately 40 µl) of DAB Chromogen per 1 ml of DAB substrate (BUF021A and BUF022), mix by swirling, and apply to tissue. Incubate for 10 minutes.
19. Wash with ddH_2O.
20. Counterstain using hematoxylin (Sigma, MHS-16) for 1 minute.
21. Rinse slide with tap water to enhance color development.
22. Rinse slides with ddH_2O.
23. Dehydrate with graded alcohol (see mounting protocol on page 82) and apply a permanent coverslip to the slides.

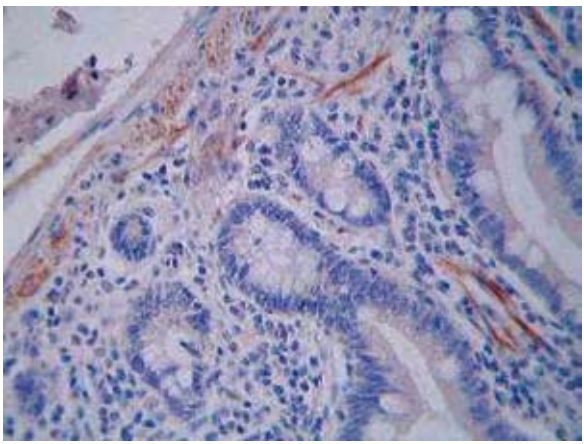


FIGURE 33 **Staining of Human Small Bowel.** 400x magnification, with HuCAL® Fab AbD05054 at 5 µg/ml, detected using the ABC or LSAB method.

Staining of Tagged HuCAL® Fabs with Amplification Using the EnVision™ System

The EnVision™ 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 secondary antibody bound to an HRP-labeled dextran backbone. This system also uses low concentrations of primary antibody, which reduces background staining (Figure 34).

1. Deparaffinize the slides according to the protocol on page 81.
2. Wash the slides twice (for 2 minutes per wash) 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 ddH_2O and allow the sections to cool in the buffer for 15 minutes.
6. Wash the slides three times (for 2 minutes per wash) in TBST.
7. Incubate in 3% H_2O_2 in methanol for 10 minutes to quench peroxidase in the tissue.
8. Wash the slides twice (for 2 minutes per wash) in TBST.
9. Transfer slides to a solution of 10% BSA in PBS and incubate for 15 minutes to block proteins.
10. Tip excess blocking solution off the sections, apply 1–5 µg/ml primary HuCAL® antibody in antibody diluent (BUF014), and incubate for 1 hour. The concentration of antibody should be determined empirically for each antigen, staining system, and laboratory.
11. Wash the slides three times (for 2 minutes per wash) in TBST.
12. Apply mouse anti-His-6-tag antibody (MCA1396), diluted 1:50–1:200 in antibody diluent (BUF014), and incubate for 30 minutes.
13. Wash the slides three times (for 2 minutes per wash) in TBST.
14. Apply ready-to-use EnVision™ anti-mouse HRP (Dako, K4007), and incubate for 15 minutes.
15. Wash the slides three times (for 2 minutes per wash) in TBST.

16. Add 1 drop (approximately 40 µl) of DAB Chromogen per 1 ml of DAB substrate (BUF021A and BUF022), mix by swirling and apply to tissue. Incubate for 5 minutes.
17. Wash with ddH_2O.
18. Counterstain using hematoxylin (Sigma, MHS-16) for 1 minute.
19. Rinse slide with tap water to enhance color development.
20. Rinse slides with ddH_2O.
21. Dehydrate with graded alcohol (see mounting protocol on page 82) and apply a permanent coverslip to the slides.

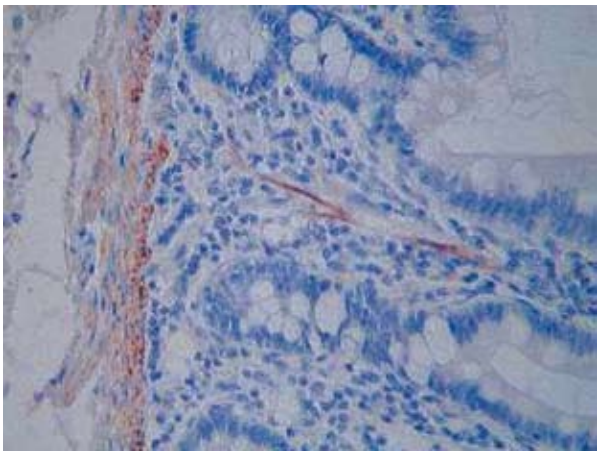


FIGURE 34 **Staining of Human Small Bowel**. 400x magnification, with HuCAL® Fab AbD05054, 1 µg/ml, detected with the EnVision™ system.

Staining of Tagged HuCAL® Fabs with Tyramide Amplification

This procedure avoids the use of tertiary antibodies and is suitable for murine tissues. It uses biotinylated tyramide to amplify the signal, resulting in improved detection sensitivity and requiring 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 bind to tyrosine residues of proteins in the vicinity of the HRP target. The biotin on the bound tyramide is easily visualized using standard ABC techniques[25] (Figure 35).

1. Deparaffinize the slides according to the protocol on page 81.
2. Wash the slides three times (for 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 ddH_2O and allow the sections to cool in the buffer for 15 minutes.
6. Wash the slides three times (for 2 minutes per wash) in TBST.
7. Incubate in 3% H_2O_2 in methanol for 10 minutes to quench peroxidase in the tissue.
8. Wash the slides three times (for 2 minutes per wash) in TBST.
9. Transfer slides to a solution of 10% BSA in PBS and incubate for 15 minutes to block proteins.
10. Tip excess blocking solution off the sections, apply 1–25 µg/ml primary HuCAL® antibody in antibody diluent (BUF014), and incubate for 1 hour.
11. Wash the slides three times (for 2 minutes per wash) in TBST.
12. Apply HRP-labeled mouse anti-His-6-tag antibody, (MCA1396P), diluted 1:50–1:200 in antibody diluent (BUF014). Incubate for 30 minutes.
13. Wash the slides 3 times (for 2 minutes each time) in TBST.

[25] von Wasielewski, R. *et al.* (1997) Tyramine amplification technique in routine immunohistochemistry. *J Histochem Cytochem* **45**:1455–1460

14. Prepare a working solution of activated biotinylated tyramide (see 'Composition of Buffers', page 130) by adding 5 µl tyramide stock solution (freshly thawed) and 2 µl 30% H_2O_2 per 1 ml of TBST.
15. Apply the activated biotinylated tyramide to the slides for 15 minutes.
16. Wash the slides three times (for 2 minutes per wash) in TBST.
17. Apply streptavidin-peroxidase complex (STAR5B), diluted 1:500, and incubate for 15 minutes.
18. Wash the slides three times (for 2 minutes per wash) in TBST.
19. Add 1 drop (approximately 40 µl) of DAB Chromogen per 1 ml of DAB substrate (BUF021A and BUF022), mix by swirling and apply to tissue. Incubate for 10 minutes.
20. Wash with ddH_2O.
21. Counterstain using hematoxylin (Sigma, MHS-16) for 1 minute.
22. Rinse slide with tap water to enhance color development.
23. Rinse slides with ddH_2O.
24. Dehydrate with graded alcohol (see mounting protocol on page 82) and apply a permanent coverslip to the slides.

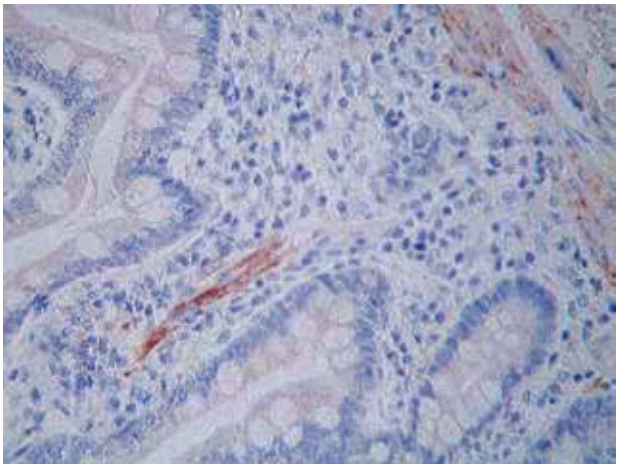


FIGURE 35 **Staining of Human Small Bowel.** 400x magnification, labeled with HuCAL Fab AbD05054 at 0.1 µg/ml using tyramide amplification.

Staining of Tagged HuCAL® Fabs Using a Mouse-on-mouse System

The mouse-on-mouse system is a simple method for staining mouse tissues. A special blocking reagent eliminates background from endogenous mouse Igs. Antigens are detected with a mouse anti-tag antibody and a biotinylated anti-mouse secondary antibody, followed by an enzyme-labeled streptavidin complex.

The protocol is performed using a customized detection kit from Vector Laboratories (PK-2200) (Figure 36).

Important note: before starting the protocol, prepare working solutions according to the manufacturer's instructions.

Procedure

1. Deparaffinize the slides according to the protocol on page 81.
2. Wash the slides three times (for 2 minutes per wash) in TBST.
3. Submerge the slides 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 ddH_2O and allow the sections to cool in the buffer for 15 minutes.
6. Wash the slides three times (for 2 minutes per wash) in TBST.
7. Incubate in 3% H_2O_2 in methanol for 10 minutes to quench peroxidase in the tissue.
8. Wash the slides three times (for 2 minutes per wash) in TBST.
9. Incubate the tissue sections for 1 hour in a working solution of mouse-on-mouse Ig blocking reagent, prepared according to the manufacturer's instructions.
10. Wash the slides three times (for 2 minutes per wash) in TBST.
11. Incubate tissue sections for 5 minutes in a working solution of M.O.M™ diluent, prepared according to the manufacturer's instructions.
12. Tip excess M.O.M™ diluent off slides. Dilute the primary antibody in M.O.M™ diluent to the appropriate concentration and incubate the section with the diluted primary antibody for 1 hour.
13. Wash the slides three times (for 2 minutes per wash) in TBST.

14. Incubate the slides for 30 minutes in a working solution of mouse anti-His-6 antibody (MCA1396), diluted 1:50–1:200 in M.O.M™ diluent.
15. Wash the slides three times (for 2 minutes per wash) in TBST.
16. Apply a working solution of M.O.M™ biotinylated anti-mouse Ig 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: 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 (BUF021A and BUF022), mix by swirling and apply to tissue. Incubate for 10 minutes.
21. Wash the slides three times (for 2 minutes per wash) in TBST.
22. Counterstain using hematoxylin (Sigma, MHS-16) for 1 minute.
23. Rinse slide with tap water to enhance color development.
24. Rinse slides with ddH_2O.
25. Dehydrate with graded alcohol (see mounting protocol on page 82) and apply a permanent coverslip to the slides.

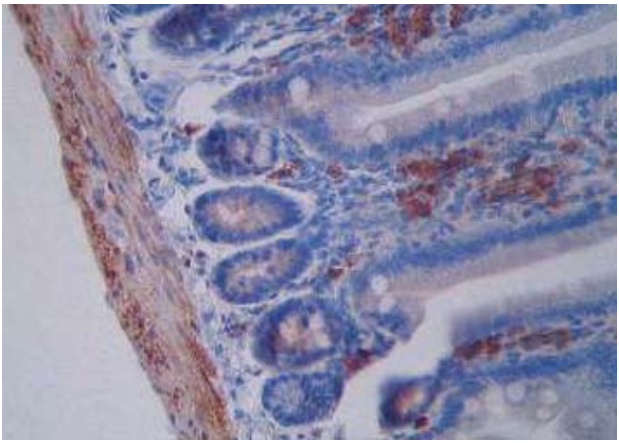


FIGURE 36 **Staining of Mouse Small Bowel.** 400x magnification, labeled with HuCAL® Fab AbD05054 at 5 µg/ml, using mouse-on-mouse system.

Staining of Tagged HuCAL® Fabs with an Anti-Strep-tag® Antibody and HISTAR IHC Detection Kit

AbD Serotec's HISTAR IHC Detection System reagents are designed specifically to enhance the IHC technique with a simple kit format. This kit provides linking and labeling reagents intended for use with species specific primary antibodies for visualizing cellular antigens in tissue specimens. Visualization is accomplished using an ultra-sensitive indirect labeling method that utilizes novel polymer labeling technology. Secondary antibodies are linked directly with HRP into compact polymers bearing a high ratio of enzyme to antibody. This biotin-free system offers enhanced sensitivity and minimal background staining.

All labeling and blocking reagents are pre-diluted for immediate use, and chromogen is provided in concentrated format. The kit is suitable for use with HuCAL® antibodies in combination with a wide range of secondary antibodies. In the example shown in Figure 37, the anti-desmin antibody was used as bivalent mini-antibody with a c-myc and double extended Strep-tag® (Fab-dHLX-MSx2, AbD08860) in combination with an anti-Strep-tag® antibody. The protocol has also been successfully used with the following: an anti-V5 tag antibody (MCA1360) or anti-bacterial AP antibody (AHP1108P) together with anti-desmin in Fab-A-V5Sx2 format.

Procedure

1. Deparaffinize the slides according to the protocol on page 81.
2. Wash the slides twice (for 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 ddH_2O and allow the sections to cool in the buffer for 15 minutes.
6. Wash the slides three times (for 2 minutes per wash) in TBST.
7. Incubate in Peroxide Buffer (BUF017A) for 15 minutes to quench peroxidase in the tissue.
8. Wash the slides three times (for 2 minutes per wash) in TBST.
9. Transfer slides to Serum Block, vial 2 of HISTAR detection kit (STAR3000) and incubate for 15 minutes.

10. Tip excess blocking solution off the sections, apply 5–40 µg/ml primary HuCAL® antibody in TBST or antibody diluent (BUF014) and incubate for 30 minutes. The concentration of antibody must be determined empirically for each antigen, staining system, and laboratory.
11. Wash the slides three times (for 2 minutes per wash) in TBST.
12. Apply Special Block (vial 3; shake before use) and incubate for 20 minutes.
13. Wash the slides three times (for 2 minutes per wash) in TBST.
14. Apply anti-Strep-tag® Classic antibody (MCA2489), diluted 1:50–1:200 in antibody-diluent (BUF014) and incubate for 30 minutes.
15. Wash the slides three times (for 2 minutes per wash) in TBST.
16. Apply Polymer Link (HRP) (vial 4; shake before use), and incubate for 30 minutes.
17. Wash the slides three times (for 2 minutes per wash) in TBST.
18. Add 1 drop (approximately 40 µl) of DAB Chromogen per 1 ml of DAB substrate (BUF021A and BUF022), mix by swirling and apply to tissue. Incubate for 5 minutes.
19. Wash with ddH_2O for 3 minutes.
20. Counterstain using hematoxylin (Sigma, MHS-16) for 1 minute.
21. Rinse slide with tap water to enhance color development.
22. Rinse slides with ddH_2O.
23. Dehydrate with graded alcohol (see mounting protocol on page 82) and apply a permanent coverslip to the slides.

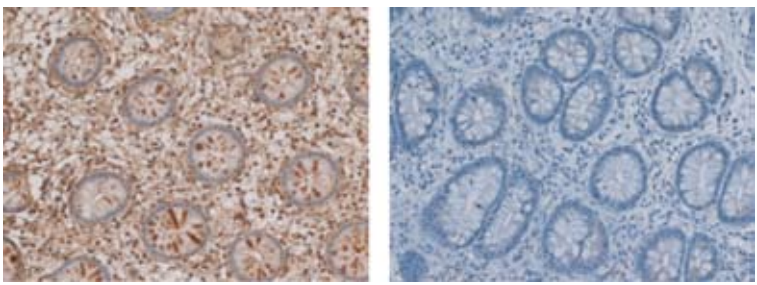


FIGURE 37 **Staining of Human Appendix. Left:** Human appendix at 400x magnification, labeled with HuCAL® Fab AbD08860, 10 µg/ml using an anti-Strep-tag® antibody and the HISTAR Detection Kit. **Right:** Negative control staining without HuCAL® antibody.

Troubleshooting

Problem	Possible Cause and Course of Action
No staining of samples, controls, or background	1. Primary or secondary antibody not used. Repeat experiment according to the protocol. 2. Antibody stored at 4ºC for several weeks or subjected to several freeze–thaw cycles. Repeat experiment using a fresh aliquot of antibody that has been stored at –20ºC or below. 3. Slides prepared with alcohol-soluble chromogen were mounted in alcohol-based medium. Repeat the color reaction and use aqueous mounting medium. 4. Buffers for peroxidase staining contained azide. Repeat experiment with buffers that do not contain azide. 5. Incompatible buffers used for the enzyme and substrate reagents. Repeat the staining reaction with compatible buffers.
Weak or non-specific staining	1. Concentration of primary antibody too low (or too high). Repeat the experiment, adjusting the concentration of primary antibody (often by 2–5 fold), and increasing the incubation time if the background staining was low. 2. Proteolytic digestion omitted. Some antigens require proteolytic digestion, depending on the fixation protocol and the antibody. 3. Too much washing buffer left on slide before incubation with the primary antibody, leading to dilution of the antibody. Repeat the staining procedure and remove the buffer by tapping the slide carefully on a paper towel. 4. Use of expired reagents. Repeat staining with fresh reagents. 5. Incubation times too short. Repeat experiment with longer incubation times.

Applications: FACS™ Analysis – Flow Cytometry

Chapter 7

Fluorescence activated cell sorting (FACS™) analysis or flow cytometry is a technique that measures characteristics, such as surface markers, on single cells. It is also used to detect antigen-expressing cells, to detect antigens bound to cells, and to separate antigen-expressing from non-expressing cells.

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

Contact your account manager at **sales.muc@abdserotec.com** for more information. AbD Serotec also offers a detailed application guide that can be accessed at **www.abdserotec.com/flow**.

Use of HuCAL® Antibodies in Flow Cytometry

HuCAL® antibodies are well suited for use in flow cytometry. Their small size and lack of an Fc region results in low background and avoids potential interactions between the primary antibody and Fc receptors on cells. In addition, HuCAL® antibodies routinely carry epitope tags that allow them to be detected on antibody-expressing cells, such as B-cells. While the bivalent mini-antibody format (Fab-dHLX) is recommended, since it has higher avidity, the monovalent format can also be used. Table 9 lists the recommended secondary antibodies.

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

For cells which inherently display human IgG, detection via the tag on the HuCAL® antibody is required. Check the format of the HuCAL® antibody and use an appropriate secondary antibody from the list in Table 9. A conjugated

anti-tag antibody or an unconjugated secondary antibody followed by a conjugated anti-mouse antibody can be used. Use of an anti-c-myc antibody is not recommended, since it can give higher background due to endogenous c-myc in dead cells.

TABLE 9 **Secondary Antibodies Recommended for Flow Analysis.**

Secondary Antibody	Recommended Dilution	Product Code
Anti-human IgG (H+L):PE $F(ab')_2$ fragment	1:400	STAR97PE
Anti-Strep-tag® Immo		MCA2488
Anti-Strep-tag® Classic		MCA2489
Anti-V5:FITC	Neat-1:5	MCA2894F
Anti-V5:FITC	Neat-1:5	MCA2895F
Anti-FLAG® M2	1:2000	Sigma, F3165
Anti-His-6:FITC	1:10	MCA1396F
Anti-His-6	1:500	MCA1396
Anti-mouse IgG (H+L):PE $F(ab')_2$ fragment	1:5	STAR105PE
Anti-mouse IgG:FITC $F(ab')_2$ fragment	Undiluted – 1:500	STAR9B

Protocols

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

Recommended Controls

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

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

Preparation of Cells

1. **Adherent cells:** Detach cells from their support using Trypsin/EDTA (Versene), and collect in a 50 ml sterile plastic tube.
   Suspension cells: Collect in a 50 ml sterile plastic tube.
2. Wash the cells once in ice-cold flow buffer, add buffer and invert to mix, then centrifuge at 190 × *g* for 5 minutes at 4°C. Preparation of flow buffer is described on page 129.
3. Discard the supernatant and resuspend the cell pellet in the remaining drop. Add *5* ml ice-cold flow buffer and mix by inverting the tube. Centrifuge at 190 × *g* for 5 minutes at 4°C.
4. Discard the supernatant and gently resuspend the cell pellet in the remaining drop. Add a small volume of ice-cold flow buffer.
5. Stain cells with Trypan Blue and count the cells. Add flow buffer and adjust the density of live cells to 0.5×10^6 cells/100 µl.
6. Add 100 µl of cell suspension (0.5×10^6 cells) to each well of a 96-well round bottom tissue culture plate, and centrifuge the plate at 716 × *g* for 2 minutes at 4°C.
7. Carefully remove the supernatant and gently vortex the plate to bring the cells into suspension in the remaining liquid. When using only a few wells, aspirate the supernatant with a pipet. If using an entire 96-well plate, invert the plate quickly but carefully over a tray to remove the supernatant. Then, do not tap the plate on a paper towel, instead turn plate upwards and blot surface moisture from the top with a paper towel.

Procedure for Cell Staining

1. Prepare serial dilutions of HuCAL® antibodies, between 0.1 and 100 µg/ml in flow buffer. (10 µg/ml is a standard concentration.) Preparation of flow buffer is described on page 129.
2. Add 100 µl of diluted antibody and control solutions to the wells containing antigen-expressing cells and negative control cells. Incubate for 1 hour at 4°C with gentle agitation.
3. Centrifuge at 716 × *g* for 2 minutes at 4°C. Remove the supernatant and wash each well 2–3 times with 200 µl flow buffer. When using only a few wells, aspirate the supernatant with a pipet. If using an entire 96-well

plate, invert the plate quickly but carefully over a tray to remove the supernatant. Then, do not tap the plate on a paper towel; instead blot surface moisture from the top of the plate with a paper towel.

4. Add one of the following secondary antibodies:

   A. Add 100 µl RPE-conjugated goat anti-human IgG (H+L) specific $F(ab')_2$ fragment (STAR97PE) diluted 1:400 in flow buffer to the wells (samples and controls) containing antigen-expressing cells and negative control cells.

   B. Add 100 µl conjugated anti-tag secondary antibody (e.g. STAR1396F) diluted in flow buffer to the wells (samples and controls) containing antigen-expressing cells and the negative control cells.

   C. Add 100 µl mouse anti-tag secondary antibody (see Table 9) diluted in flow buffer to the wells (samples and controls) containing antigen-expressing cells and the negative control cells.

5. Incubate for 1 hour at 4°C with gentle agitation.

6. Centrifuge at 716 × *g* for 2 minutes at 4°C. Remove the supernatant and wash 2–3 times with 200 µl flow buffer, as described in step 3.

7. Tertiary antibody incubation (only for unconjugated secondary antibodies, step 4C). Add 100µl of RPE-conjugated goat anti-mouse IgG antibody (STAR105PE) to each well, diluted 1:5 in flow buffer. Incubate for 1 hour at 4°C with gentle agitation followed by centrifugation and wash, as described in step 3.

8. After the final washing step, centrifuge for 2 minutes at 4°C. Resuspend cells in 100–200 µl flow 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 aluminum foil and store at 4°C. Immediately before FACS™ analysis, add flow buffer to the required final volume.

Examples

Transiently transfected HEK293 cells were analyzed by flow cytometry using a monovalent HuCAL® antibody in Fab-FH format (with FLAG® and His-6 epitope tags) against a human transmembrane protein. Detection was performed either with a PE-labeled anti-human IgG heavy and light chain specific secondary antibody, or with an anti-FLAG® or an anti-His-6-tag antibody in combination with a PE-conjugated anti-mouse antibody. A clear shift of the transfected cells is visible for all three detection systems (Figure 38).

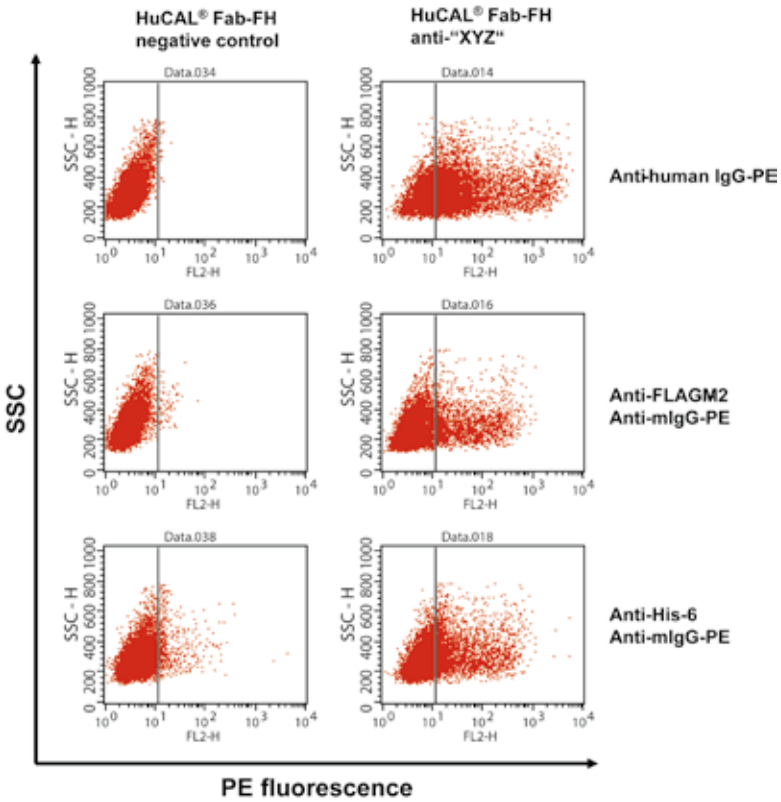


FIGURE 38 **Flow Cytometry of Transiently Transfected HEK293 Cells.**
Left column: Unrelated antibody (control). **Right column:** Antigen-specific antibody. **Row 1:** Detection with a PE-conjugated anti-human IgG antibody. **Row 2:** Detection with a mouse anti-FLAG® M2 antibody and a PE-conjugated anti-mouse IgG. **Row 3:** Detection with a mouse anti-His-6-tag antibody and a PE-conjugated anti-mouse IgG.

CD81-positive cells were detected by FACS™ analysis from washed human blood using an anti-CD81 HuCAL® antibody (AbD06083, HCA086 and AbD06084, HCA087 in Fab-dHLX-MH format or AbD08863, HCA112 and AbD8864, HCA113 in Fab-dHLX-MSx2 format, respectively) together with anti-Strep-tag® Classic (MCA2489) and FITC-conjugated anti-mouse IgG antibody (STAR9B) detection. A clear shift of the peak can be observed when compared to the negative control sample.

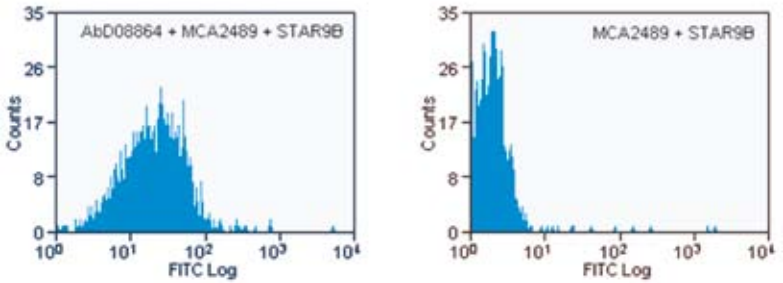


FIGURE 39 **Flow Cytometry of CD81-positive Cells from Washed Human Blood.** **Left:** Anti-CD81 HuCAL® antibody (AbD08864, HCA113) and detection with anti-Strep-tag® Classic (MCA2489) and anti-mouse IgG FITC (STAR9B). **Right**: Negative control without HuCAL® antibody.

Troubleshooting

Problem	Possible Cause and Course of Action
No staining	1. Antibody stored at 4°C for several weeks or subjected to several freeze–thaw cycles. Repeat the experiment using a fresh aliquot of antibody that has been stored at –20°C or below. 2. Incorrect secondary antibody used. Use a conjugated anti-human Fab specific, IgG (H + L), or a suitable anti-tag antibody with a matching tertiary antibody (see Table 9, page 98). 3. Failure of conjugation reaction (primary antibody to PE). Repeat the experiment, following the protocols on antibody conjugation (Chapter 8).
Weak shift	1. Antibodies not stored properly. Repeat the experiment using fresh antibodies. 2. Affinity of secondary antibody for the primary antibody too low. Try a different secondary antibody. If the recommended anti-human IgG (H + L) was used, try the anti-human Fab. 3. Antibody concentrations too low. Use a higher concentration of primary and/or secondary antibody.

Immunofluorescence

Chapter 8

Immunofluorescence is a technique frequently used for the analysis of tissue or cells stained with fluorescence-labeled antibodies. Labeled samples are studied under a fluorescence microscope or by confocal microscopy. Similar to other immunoassays, detection is usually accomplished with a labeled secondary antibody, although direct labeling of the primary antibody may be an advantage if the secondary antibody causes non-specific background signal.

HuCAL® antibodies can be used in immunofluorescence assays in the same manner as conventional antibodies with appropriate secondary reagents. Their exquisite specificity makes them ideal reagents for immunofluorescence analysis. The bivalent mini-antibody format is recommended due to the avidity effect. Recommended secondary antibodies are listed in Table 10.

For a complete list of all available conjugates, contact your account manager at **sales.muc@abdserotec.com** or visit **www.abdserotec.com** for more information.

TABLE 10 **Selection of Secondary Antibodies Recommended for Immunofluorescence.**

Secondary Antibody	Recommended Dilution	Product Code
Anti-human F(ab')$_2$:FITC	1:50–1:200	STAR126F
Anti-human F(ab')$_2$:TRITC	1:50–1:200	STAR126TRC
Anti-Strep-tag Classic	1:50–1:200	MCA2489
Anti-His-6-tag	1:50–1:200	MCA1396
Anti-His-6-tag:Alexa Fluor® 488	Undiluted – 1:100	MCA1396A488
Anti-His-6-tag:Alexa Fluor® 647	Undiluted – 1:100	MCA1396A647
Anti-His-6-tag: DyLight® 549	1:50–1:200	MCA1396D549
Anti-AP	1:50–1:100	AHP1108P
Anti-V5 tag		MCA1360
Anti-V5 tag:Alexa Fluor® 488	1:100	MCA1360A488
Anti-V5 tag: Alexa Fluor® 647	1:100	MCA1360A647
Anti-V5 tag:DyLight® 549	1:100–1:500	MCA1360D549
Anti-mouse IgG:Cy5		OBT1725C
Anti-mouse IgG (H + L):DyLight® 649	1:500–1:1000	STAR117D649

Protocol

Sample Preparation

Cultured cells

1. Grow cells in multi-well plates.
2. Rinse cells briefly with sterile PBS.
3. Aspirate PBS and fix cells with either:

   A. 2–4% paraformaldehyde (PFA) for 10 min at 37°C.

   B. 100% methanol for 10 min at –20°C
   Note: use sufficient solution to cover cells properly.
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 min 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.

Paraffin sections

Refer to the protocol on page 81 for deparaffinization and rehydration. For antigen retrieval, see steps 1–6 of the IHC protocol on page 84

Frozen sections

Refer to the protocol on page 82 for fixation of cells.

Immuostaining

1. Block sample with 3% BSA or fetal calf serum (FCS) 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 FCS 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® mini-antibody (AbD02531, HCA006). Detection was performed with an Alexa Fluor® 488 conjugated anti-His-6 secondary antibody (MCA1396A488). Cells were also stained with Alexa Fluor® 660 Phalloidin (red), and the cell nucleus was stained blue with Hoechst 33342 (Invitrogen, H1399). See Figure 40. The green spots in Figure 40 indicate Ki-67 in growing cells.

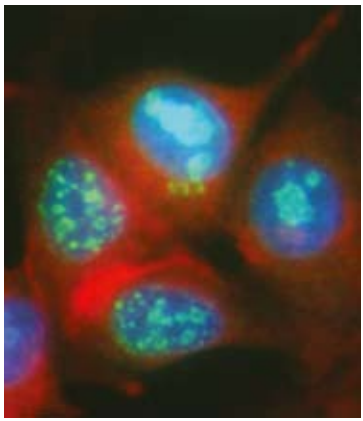


FIGURE 40 **Immunofluorescence Detection of Ki-67 in MCF-7 Breast Cancer Cells.** Cells were stained with HuCAL® Antibody AbD02532 (green spots) and images were taken on an IN Cell Analyzer 1000 (GE Healthcare) at 40x magnification.

Contactin-associated protein, Caspr, was examined in mouse nerve cells using a HuCAL® anti-Caspr mini-antibody (AbD06152). Detection was performed with an Alexa Fluor® 647 conjugated anti-human IgG secondary antibody (red) on an Olympus FluoView® 1000 laserpoint scanning confocal microscope. Ankyrin G delineates the nodes of Ranvier and was stained green with a mouse anti-ankyrin G antibody to make the outline of the nerves visible (Figure 41). Caspr is found at the flanking paranodes.

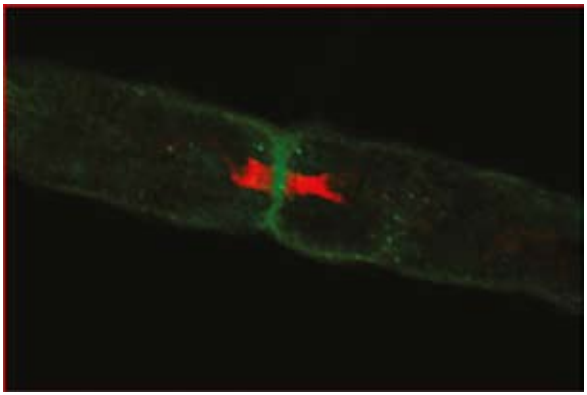


FIGURE 41 **Immunofluorescence Analysis of Caspr and Ankyrin G in Mouse Cerebellum Sections.** Nerves were stained with an anti-ankyrin G antibody to mark the nodes of Ranvier (green). Anti-Caspr HuCAL® antibody (AbD06152) was used in combination with an anti-human IgG antibody to detect Caspr at the flanking paranodes (red). Images were captured with an Olympus FluoView® 1000 laserpoint scanning confocal microscope[26].

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

Applications: Bead-based Assays

Chapter 9

Luminex xMAP® Technology

The Luminex xMAP® technology is popular for antibody assays and diagnostic testing due to its large multiplexing capacity of up to 100 analytes per well. xMAP® is based on flow cytometry technology using fluorescent microspheres (beads). The use of beads with different fluorescent dyes allows differentiation between the types of beads and thus, between different samples that are covalently coupled to the beads. With just one well, it is possible to test the binding of an antibody to different immobilized antigens; or by using immobilized antibodies, the presence of different antigens in one sample can be detected (multiplex assay).

The technology has also proved to be very useful for the detection of sandwich pairs. For example, if 10 different antibodies are to be screened for their suitability as sandwich pairs, it would require at least 100 wells in an ELISA (10 capture antibodies × 10 detection antibodies). With the xMAP® technology, the same assay can be performed in just 10 wells.

HuCAL® antibodies are well suited for this application due to their high specificity. xMAP® technology is well established at AbD Serotec. For more information about timelines and costs, contact your account manager at **sales.muc@abdserotec.com**.

Protocol

This is a standard protocol for a Luminex® xMAP® sandwich assay using a HuCAL® capture antibody together with a biotinylated HuCAL® detection antibody.

Bead Coupling

For coupling to LiquiChip® Carboxy Beads (QIAGEN, 922402), follow the manufacturer's instructions.

To reduce the loss of beads during the coupling steps, AbD Serotec recommends the use of pipet tips from Mettler Toledo (RT-250, RT-1000) and vials from USA Scientific (1415-2500).

xMAP® Assay

1. Prewet filter microtiter plate (MultiScreen$_{HTS}$-BV, 1.2 µm PVDF, Millipore, MSBVN1210) with 200 µl assay buffer [PBST (0.05% Tween® 20) with 1% BSA] per well.
2. Transfer the plate to vacuum manifold base (e.g. Vac-Man® 96 Vacuum Manifold, Promega, A2291) and apply low vacuum. Tap the plate briefly onto a paper towel to remove remaining droplets.
3. Dilute capture antibody coupled beads in assay buffer to 1.5 × 10^5 beads/ml and transfer 1–10 µl of each bead type to the well of a filter plate (150–1500 beads of each type per well).
   Note: Starting with 1500 beads/well is recommended only if a few different beads are used.
4. Add 50 µl antigen diluted in PBS. Use a concentration range of 0–5000 ng/ml. Resuspend beads on a shaker for 1 hour at RT.
5. Transfer the plate to a vacuum manifold, apply low vacuum and wash three times with 200 µl assay buffer. Tap the plate briefly onto a paper towel to remove remaining droplets.
6. Add 70 µl/well biotinylated detection antibody at 2 µg/ml in PBS. Resuspend beads on a shaker for 1 hour at RT.
7. Wash the plate five times with 200 µl assay buffer, as described in step 5.
8. Add 70 µl/well streptavidin-RPE (STAR4B) diluted 1:5 in assay buffer. Resuspend beads on a shaker for 1 hour at RT.
9. Wash plate five times with 200 µl assay buffer, as described in step 5.
10. Add 100 µl assay buffer and vortex the plate for 1 minute.
11. Analyze the samples on a Luminex® 100™ instrument as per the manufacturer's instructions.

Example

A sandwich assay was performed with a HuCAL® capture and detection antibody against human IFNγ. The capture antibody (AbD00676, HCA043) was immobilized on beads and detection was performed using biotinylated detection antibody (AbD02503, HCA044). Human IFNγ (PHP050) was used in a concentration range from ~1 pg/ml to 10 ng/ml. The calculated limit of detection (LOD) was 2.9 pg/ml (Figure 42).

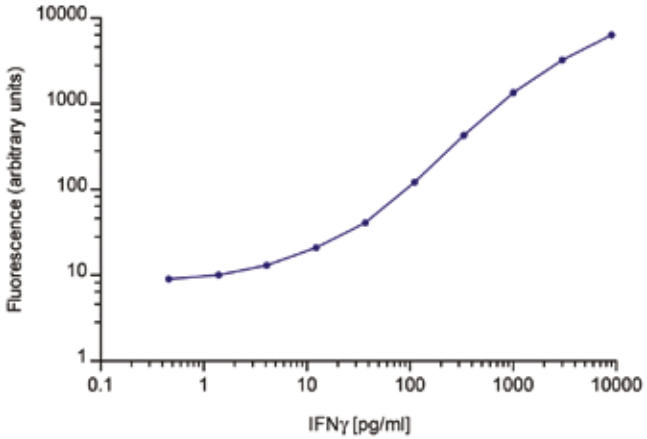


FIGURE 42 **Luminex Sandwich Assay for IFNγ.** The assay used two HuCAL® antibodies, one for capture and one for detection. The detection antibody was biotinylated for further detection by streptavidin-PE.

FMAT® FLISA assays

FMAT® is a fluorescence-linked immunosorbent assay (FLISA) which uses the 8200 Cellular Detection System (8200 CDS; Applied Biosystems). The 8200 CDS is a macroconfocal imager that detects fluorescence on a particle (cell or bead). Speed and simplicity are the main advantages of this technique. In contrast to ELISA, it is a homogenous mix and measure assay without any washing steps. Though this technology was originally developed for cellular assays, it has proved very useful for bead-based assays.

In this example, antigen-coated beads, primary antibody, and fluorescence-labeled secondary antibody are mixed in a 96- or 384-well microtiter plate. A laser scans the bottom 100 μm layer of the well and detects fluorescence of labeled secondary antibody bound to beads. The background from free secondary antibody in solution is automatically subtracted (Figure 43).

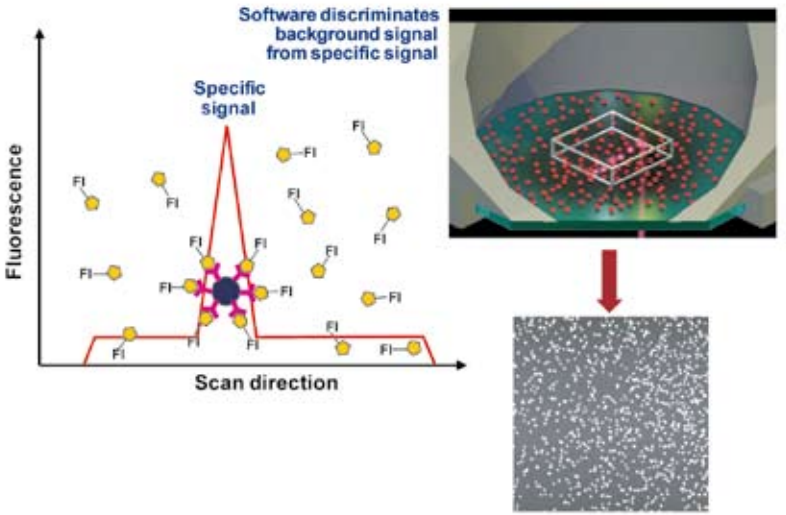


FIGURE 43 **FMAT® Technology.** Schematic drawing of one well of a FMAT® plate with beads (in red). A laser scans a square area and detects the fluorescence-labeled sample on the surface of the beads. Background from sample in solution in the bottom layer is subtracted (red line in the diagram), resulting in images with white spots in front of a black/gray background.[27]

One other advantage of this system is the low antigen consumption required for screening a 384-well plate in comparison to an ELISA. AbD Serotec recommends this assay for all antibody generation projects with limited antigen availability.

Screening in a bead-based assay can also be superior to an ELISA depending on the intended use of the antibodies. The conformation of antigens on beads is believed to be closer to native conformation than is achieved in coating to an ELISA plate. It is known that differences in antigen presentation may lead to differing results with some antibodies. In some cases, an antibody may be positive in one assay, but not in another.

[27] Image included courtesy of Applied Biosystems.

Protocol

Bead Coupling

1. Resuspend M-450 Epoxy Dynabeads® (Invitrogen, 14011) by vortexing for 1–2 minutes.
2. Immediately transfer 300 µl (~1.2 × 10^8 beads) into a 2.0 ml vial or 24-well microtiter plate.
3. Place the vial or plate on a magnet (DynaMag™-2, Invitrogen, 123-21D) for 1 minute and carefully remove the supernatant.
4. Remove vial or plate from the magnet and resuspend beads in 1 ml of 100 mM sodium phosphate buffer, pH 8.0. Mix by inversion with gentle tilting and rotation.
5. Repeat steps 3 and 4, and then remove supernatant.
6. Calculate the required volumes of antigen (60 µg) and buffer to yield a final volume of 400 µl.
7. Resuspend the beads in 100 mM sodium phosphate buffer, pH 8.0, and add the antigen solution as in step 6.
8. Incubate for 16–20 hours on an overhead rotator/shaker at RT. Ensure that the beads do not settle during incubation, and are mixed by inversion with gentle tilting and rotation.
9. Add 20 µl 1M Tris-HCl, pH 7.4, and incubate on a rotator/shaker for 2 hours.
10. Wash beads three times with 500 µl PBS.
11. Resuspend the beads in 500 µl PBS and store at 2–8°C until needed.

Notes: Amount of antigen is calculated for a~30 kDa protein and can be adjusted. Antigen buffer must not contain primary amines (e.g. Tris) or detergents (e.g. Tween® 20, Triton® X-100).

FMAT® Assay

1. Resuspend antigen-coated beads in PBST (0.05% Tween® 20) with 3% BSA and secondary antibody to a final concentration of ~1.3×10^5 – 5.0×10^5 beads/ml. AbD Serotec recommends using a Cy5 conjugated anti-human Fab secondary antibody (Jackson ImunoResearch, 109-175-097 at a 1:5000 dilution).
2. Transfer 40 µl into each well of a 384-well plate.
3. Add 5 µl of the HuCAL® antibody at 45 µg/ml (final concentration of 5 µg/ml).
4. Incubate for 1 hour at RT in the dark. Measure plate with the 8200 CDS, as per the manufacturer's instructions.

Troubleshooting

Problem	Possible Cause and Course of Action
No signal	1. Insufficient beads per well. Prepare a fresh dilution of beads. Resuspend beads before each pipetting step. 2. Antibody stored at 4°C for several weeks or subjected to several freeze–thaw cycles. Repeat the experiment using a fresh aliquot of antibody that has been stored at –20°C or below. 3. Incorrect secondary antibody used. Use a Cy5 conjugated anti-human Fab specific secondary antibody, or a suitable anti-tag antibody. 4. Failure of bead-coupling reaction. Make sure the antigen buffer does not contain primary amines or detergents, or dialyze the antigen against PBS and repeat bead coupling reaction. 5. Insufficient secondary antibody used. Increase the amount of secondary antibody.
Weak signal	1. Antibodies not stored correctly. Repeat the experiment using fresh antibodies. 2. Antibody concentration too low. Use a higher concentration of primary and/or secondary antibody.
High background	1. Reduce the amount of Cy5 conjugated secondary antibody.

Affinity Determinations

Chapter 10

Measuring antibody affinity is a useful tool for characterizing, evaluating, and ranking antibodies. Although there are many different technologies available, there are essentially two different principles applied to determine affinities:

- Kinetic data with association on and off rates (k_{on} and k_{off}), which is acquired by using instruments with a flow cell (e.g. Biacore and Attana)
- Endpoint analysis, which measures concentrations after equilibrium is reached between antigen-bound and free antibody (e.g. ELISA titration)

Flow-cells are often used in label-free technologies where none of the reagents are required to carry a label or dye and no secondary reagents are necessary. For most kinetic measurements, one binding partner (the antigen or antibody) is immobilized on a solid support. Measured affinities for one antibody–antigen pair may vary depending on the method used due to the differences in set-up, antigen presentation, or steric hindrance.

To get true monomeric affinity values, it is important to avoid avidity effects since these may be an issue if the antigen or antibody forms oligomers or shows a tendency to aggregate. Typical examples of oligomeric antigens are Fc-fusion proteins, homodimers, or conjugated peptides. Full IgGs, HuCAL® mini-antibodies, and AP-fusion antibodies are examples of bivalent antibodies. Whenever one of the two partners (or both) is oligomeric, the setup must be chosen with care to avoid avidity influencing the measurement. This is always the case when one molecule can bind the immobilized partner with two binding sites at the same time (e.g. IgG binds with both arms to coated antigen).

Although there are many technologies available for K_D determination, the only ones covered in this book are those that are offered by AbD Serotec for the ranking or affinity determination of HuCAL® antibodies. Contact your account manager at **sales.muc@ abdserotec.com** for more information about these services. The scientists at AbD Serotec can advise which technology (QCM on Attana instruments, SPR on Biacore, or SET) is best suited for the project.

SPR on a Biacore Instrument

For Biacore[28] analysis of HuCAL® Fabs, the antigen (ligand) is usually immobilized on the surface and the Fab (analyte) is in the mobile phase. For most applications, hepes buffered saline (HBS) or PBS is suitable as the running buffer, to which 0.05% Tween® 20 may be added to reduce non-specific binding (if necessary). For affinity determinations, the use of monomeric (i.e. not aggregated) fractions of purified Fab preparations in running buffer are recommended. The affinity determination must be performed with at least five different concentrations of the purified Fab in HBS or PBS, in order to ensure that a measurement with an optimal affinity fit can be obtained.

Immobilization of the Ligand

The CM5 sensor chips (GE Healthcare, BR-1000-14) and the Amine Coupling Kit (GE Healthcare, BR-1000-50) are used for the immobilization of the ligand according to the manufacturer's instructions (BIAapplication Handbook).

The amount of immobilized antigen has to be adapted to its mass and the experiment that will be performed. Immobilization levels of antigens typically range from ~5–3000 RU. A low-density immobilization of the antigen is appropriate for affinity measurements, whereas k_{off} ranking studies may require a higher density of immobilized antigen. Absolute immobilization levels also depend on the activity of the antigen (including loss of activity during immobilization) and stability during regeneration among other factors. The following values may serve as a starting point, but may have to be optimized in the corresponding assay. For example:

- For a 30 kDa protein, immobilize ~300 RU for affinity measurements and ~3000 RU for k_{off} ranking
- For a peptide coupled to BSA or transferrin (with a coupling rate of 9–15 peptides per carrier), immobilize ~300 RU for affinity measurements and ~3000 RU for k_{off} ranking
- For an uncoupled peptide, immobilize ~5 RU for affinity measurements and ~50 RU for k_{off} ranking

[28] Biacore 3000 with BIAevaluation software, version 3.2, from GE Healthcare.

Procedure

1. Set the flow rate to 5 µl/min.
2. Inject 10–20 µl NHS/EDC (Biacore, BR-1000-50) for low-density immobilization and 20–30 µl NHS/EDC for high-density immobilization, depending on the amount of antigen that has to be immobilized.
3. Dilute the antigen to a concentration of ~20 µg/ml (proteins and peptides coupled to proteins) or to a concentration of ~1 µg/ml (uncoupled peptides), using, for example, 10 mM acetic acid. The pH of the acetic acid buffer used should be ~2 pH units below the pI of the antigen. Inject the solution until the desired amount of antigen is immobilized.
4. Deactivation of the surface is achieved by injecting 35 µl of 1 M ethanolamine.

Affinity Determination

Use purified Fab in running buffer and a low-density sensor chip.

1. Dilute the analyte (Fab) to six different concentrations with running buffer, e.g. 1:2 dilutions, starting with 500 nM.
2. Set the flow rate to 20 µl/minute.
3. Using the injection mode 'kinject', inject 20 µl of the analyte (Fab) diluted to the lowest concentration.
4. Measure the binding of the Fab to the low-density chip and allow the analyte to dissociate for at least 100 seconds.
5. Regenerate the chip by injection of 5 µl 10 mM glycine/HCl, pH 1.5, or 5 mM NaOH (for example, depending on the antigen). Several 5 µl pulses may be necessary for complete regeneration.
6. After the regeneration, repeat steps 3–5 with the next concentration of the analyte. Include buffer injections, as well as positive and negative controls, if available.
7. Repeat steps 3–5 for all dilutions.
8. Analyze the data set using the BIAevaluation software, version 3.2, by selecting 'simultaneous fit' and entering the concentrations of the analyte.

Notes:

1. Include a control with a defined affinity as the first and last sample to be measured in order to monitor the quality of the antigen after each regeneration cycle.

2. Include several measurements with buffer as a negative control (at least before and after analysis of the Fabs) for double referencing (subtraction of blank signal to compensate for flowcell-specific artifacts).
3. If the antibody must be captured on the chip, no affinities greater than that of the capture antibody can be measured. AbD Serotec recommends the anti-human Fab secondary antibody (STAR126), although suitable anti-tag antibodies can also be used.

QCM on an Attana Instrument

Affinity determination on an Attana A200® instrument is very similar to measurements on SPR (e.g. Biacore) instruments, even though the physical principles behind the technologies are different. In quartz crystal microbalance (QCM), the frequency of a quartz crystal, which is mass dependent, is measured. When an antibody binds to an antigen immobilized on a quartz chip, the mass on the chip increases and the frequency of the quartz changes. The setup with a flow cell, immobilized sample, and analyte flowing over the chip, as well as the output curves, are very similar to SPR. Thus, the technology allows determination of kinetic parameters (k_{on} and k_{off}) and affinities.

As with SPR, the antigen is usually immobilized on a chip and the antibody in the mobile phase. HBS or PBS with 0.05% Tween® 20 is used as running buffer. For affinity determinations, the use of monomeric, i.e. non-aggregated fractions, of purified Fab preparations in PBS are recommended. The affinity determination has to be performed with at least five different concentrations of the purified Fab in HBS or PBS to ensure that a measurement with an optimal affinity fit can be obtained.

Immobilization of the Ligand

Different chips are available for the Attana instruments. The most commonly used chip is a Carboxyl chip (Attana, 3616-3033) on which the ligand is covalently immobilized. Other available chips include a polystyrene surface similar to an ELISA microtiter plate and a biotin surface for immobilization of biotinylated samples via streptavidin.

Immobilization on Carboxyl sensor chips together with the amine coupling kit (Attana, 3501-3001) can be easily performed by following the manufacturer's instructions.

Affinity Determination

Use purified Fab in HBST or PBST running buffer and a low-density sensor chip.

1. Dilute the analyte (Fab) to six different concentrations with running buffer; e.g. 1:2 dilutions, starting with 500 nM.
2. Set the flow rate to 25 µl/minute.
3. Inject sample (80 seconds) of the analyte (Fab) diluted to the lowest concentration.
4. Measure the binding of the Fab to the antigen on the sensor and allow the analyte to dissociate for 100 seconds.
5. Regenerate by 1–3 injection pulses of 10 seconds of an appropriate regeneration solution. In many cases 10 mM glycine/HCl, pH 1.5, will be appropriate. However, depending on the antigen, other conditions may need to be tested. Alternatives include: 10 mM HCl; 10 mM glycine/HCl, pH 2–3; and 10 mM NaOH.
6. After the regeneration, repeat steps 3–5 with the next concentration of the analyte.
7. Repeat steps 3–5 for all dilutions.
8. Analyze the data set using the Attester Evaluation software and ClampXP fitting software.

SET

Determination of affinities in the low picomolar (pM) range is difficult, since routinely applied methods such as SPR or QCM reach their limit when determining very slow dissociation rate constants (k_{off}). MorphoSys has established an alternate method to overcome this limitation and also offers a way to carry out the affinity determination in a medium throughput fashion.

Solution Equilibrium Titration (SET)[29] in combination with a highly sensitive detection method such as electrochemiluminescence (ECL) on a BioVeris (Roche Group) or MSD™ instrument (Meso Scale Discovery™, SECTOR Imager 6000) allows for the determination of high-affinity antibody to antigen interactions. In contrast to kinetic assays using sensor surfaces, such as SPR or QCM, SET is a method which determines affinities in solution. It is an equilibrium measurement that does not deliver kinetic data.

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[29] Haenel, C. *et al.* (2005) Characterization of high-affinity antibodies by electrochemiluminescence-based equilibrium titration. *Anal Biocemistry* **339**:182–184

In SET, a constant amount of antibody is incubated with different concentrations of antigen until equilibrium is reached. The concentration of free antibody in the equilibrated solution is determined by applying the solution on an antigen coated MSD™ plate (Meso Scale Discovery™, L11XA3), followed by incubation with an ECL-labeled secondary antibody and measurement of signal intensity. At low antigen concentrations, a strong signal is achieved (high concentration of free antibody which binds to the antigen on the plate) whereas for high antigen concentration, the antibody is completely antigen-captured, resulting in a low signal.

If a sufficient number of antigen concentrations in a matching range are available, the titration curve allows for a reasonable determination of the affinity, using the appropriate fit model. For a complete titration, antigen concentrations of at least 10-fold higher than the anticipated K_D have to be applied. The constant concentration of antibody applied in the assay should be in the range of, or below, the K_D.

Antibody Conjugation

Chapter 11

There are two methods for the direct detection of HuCAL® antibodies without the need for secondary antibodies. One is the use of AP-fusion antibodies (e.g. the Fab-A-FH format) together with an AP detection substrate, and the other is by directly-labeling the HuCAL® primary antibody by chemical coupling.

The advantage of using a secondary antibody is that it works with many different antibodies and can enhance sensitivity. However, working with only a primary antibody is often preferred because it reduces the risk of non-specific background staining from cross-reactive secondary antibodies. It also reduces incubation times, washing steps, and costs by avoiding secondary reagents.

Conjugation of HuCAL® antibodies with biotin and various fluorescence dyes works the same as for complete antibodies. To date, in house testing has shown that conjugation with LYNX kits delivers antibodies with excellent sensitivities, which are similar to those achieved with the best secondary antibodies.

For more information on receiving conjugated HuCAL® antibodies, contact your account manager at **sales.muc@abdserotec.com**.

Conjugation Using LYNX Rapid Conjugation Kits®

LYNX kits are based on a revolutionary new technology that offers extremely fast and simple conjugation of antibodies and other proteins to biotin, fluorescent, or enzymatic labels. LYNX kits offer the following advantages:

- Easy protocol with just 30 seconds hands-on time
- No purification required, so there is no loss of antibody
- High-quality conjugates are ready to use
- Assay sensitivities are equal to those achieved with secondary antibodies

LYNX kits are available in a range of sizes for easy, scaleable conjugations from microgram to milligram quantities. In addition, they are available for all commonly used reporter enzymes, fluorescent dyes, and biotin as listed in Table 11.

TABLE 11 **LYNX Rapid Conjugation Kits®.**

	Label	Reaction Size	Product Code
	Biotin	3 × 200 µg 1 mg	LNK041B LNK042B
Enzymes	AP	100 µg 3 × 100 µg 1 mg	LNK011AP LNK012AP LNK013AP
	HRP	400 µg 3 × 400 µg 4 mg 5 × 4 mg 20 mg	LNK001P LNK002P LNK003P LNK004P LNK005P
	Glucose oxidase (GOx)	3 × 100 µg 1 mg	LNK051GOX LNK052GOX
Single Dyes	Allophycocyanin (APC)	100 µg 3 × 100 µg 1 mg	LNK031APC LNK032APC LNK033APC
	Cy5	200 µg 2 mg	LNK101CY5 LNK102CY5
	Fluorescein	200 µg 2 mg	LNK061F LNK062F
	PerCP	100 µg 3 × 100 µg 1 mg	lNK071PERCP lNK072PERCP lNK073PERCP
	RPE	100 µg 3 × 100 µg 1 mg	LNK021RPE LNK022RPE LNK023RPE
Tandem Dyes	APC-Cy5.5	150 µg 3 × 150 µg 1.5 mg	LNK121APCCY5.5 LNK122APCCY5.5 LNK123APCCY5.5
	APC-Cy7	150 µg 3 × 150 µg 1.5 mg	LNK131APCCY7 LNK132APCCY7 LNK133APCCY7
	PerCP-Cy5.5	100 µg 3 × 100 µg 1 mg	LNK141PERCPCY5.5 LNK142PERCPCY5.5 LNK143PERCPCY5.5
	RPE-Cy5	60 µg 3 × 60 µg 600 µg	LNK081C LNK082C LNK083C
	RPE-Cy5.5	60 µg 3 × 60 µg 600 µg	LNK091PECY5.5 LNK092PECY5.5 LNK093PECY5.5
	RPE-Cy7	60 µg 3 × 60 µg 600 µg	LNK111PECY7 LNK112PECY7 LNK113PECY7

In the LYNX procedure, the antibody is mixed with the labeling reagents followed by 3-hour, or overnight, incubation. Next, the reaction is stopped and the antibody is now ready to use (Figure 44). All materials and reagents are provided with the kit and no purification of the antibody is required after the labeling reaction.

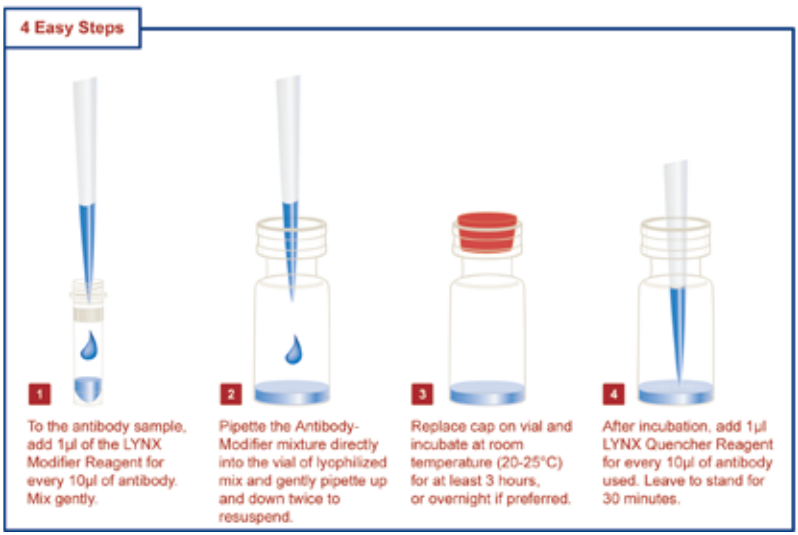

FIGURE 44 **LYNX Kit Procedure.**

Antibodies conjugated with LYNX kits offer excellent sensitivities and, therefore, can often be used at lower concentrations than needed with typical secondary reagents. Conditions that yield the best signal with low background need to be optimized for each antibody. For many assays, we recommend diluting the conjugated antibody in HiSpec buffer (BUF049) in order to obtain superior results.

Example

Western Blotting

An anti-cyclophilin A antibody in Fab-A-V5Sx2 format (AbD08966) was used in Western blot analysis of HEK293 cell lysate, either after direct HRP conjugation with a LYNX kit (LNK001P) or in combination with a HRP-conjugated anti-V5 tag secondary antibody (MCA1360P). The labeled antibody was diluted in HiSpec buffer (BUF049) and the blot was developed with Amersham™ ECL Plus.

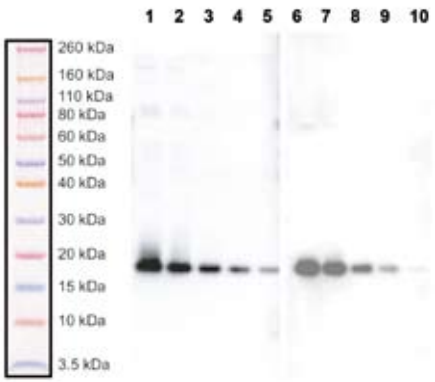

FIGURE 45 **Western Blot Comparison of LYNX-HRP Conjugate Antibody vs. Typical Secondary Antibody.** **Lanes 1–5:** Serial dilutions of HEK293 cell lysate detected with LYNX HRP-conjugated anti-cyclophilin A. **Lanes 7–10:** The same samples detected with unconjugated anti-cyclophilin A and an anti-V5:HRP secondary antibody.

Conjugation to FITC, Cy5, and Biotin Using Conventional Kits

Important points before starting

- The appropriate quantity of reagent dye depends on antibody concentrations and on desired levels of labeling
- For most situations, a 20-fold molar excess of the reagent dye is recommended; this will label each antibody with an average of 2–4 dye molecules
- For antibody concentrations below 0.5 mg/ml, an approximately 30-fold molar excess of reagent dye is recommended
- If lower levels of labeling are required, use a 5–10 fold molar excess of reagent dye
- Sample calculations for the required molar excess of reagent dye are provided for each procedure

Conjugation with Fluorescein Isothiocyanate (FITC)

FITC-labeling of antibodies can be performed using commercially available kits. The protocol described below uses the kit supplied by Thermo Fisher Scientific, and follows the manufacturer's instructions. It describes the conjugation of 1 mg of a bivalent and monovalent antibody, and can be scaled down to provide labeling of smaller amounts.

TABLE 12 **Calculation of Reagent Dye Volumes.**

Compound	Molecular weight	nmol in 1 mg reagent	nmol in 20-fold molar excess of reagent	Required volume of reagent
FITC	389 g/mol	2500 nmol/ml (solution of 1 mg/ml)	–	–
Fab	~50 kDa	20 nmol	20 × 20 nmol = 400 nmol	400 nmol of a FITC reagent solution at 2500 nmol/ml = 160 µl
Fab-dHLX	~120 kDa	8 nmol	20 × 8 nmol = 160 nmol	160 nmol of a FITC reagent solution at 2500 nmol/ml = 64 µl

Procedure

1. Prepare a fresh solution with 1 mg of FITC in 10 ml PBS.
2. The solution is stable for several hours. Higher temperatures, traces of amines (e.g. Tris), and non-neutral pH greatly reduce the stability. Minimize exposure to light, as FITC is light sensitive.
3. For **bivalent** Fab labeling (Fab-dHLX format), add 64 µl reagent solution to 1 mg antibody.
   For **monovalent** Fab labeling (Fab format), add 160 µl reagent solution to 1 mg antibody.
   Antibody concentration should be between 0.5 and 2 mg/ml. Lower concentrations of antibody require a higher reagent excess (see 'Important points before starting').
4. Incubate for 30 minutes at RT, or on ice for 2 hours.
5. Follow the purification protocol described on page 126.

Conjugation with Cy5

TABLE 13 **Calculation of Reagent Dye Volumes.**

Compound	Molecular weight	nmol in 1 mg reagent	nmol in 20-fold molar excess of reagent	Required volume of reagent
Cy5-NHS		6300 nmol/ml (solution of 5 mg/ml)	–	–
Fab	~50 kDa	20 nmol	20 × 20 nmol = 400 nmol	400 nmol of a Cy5 reagent solution at 6300 nmol/ml = 63 µl
Fab-dHLX	~120 kDa	8 nmol	20 × 8 nmol = 160 nmol	160 nmol of a Cy5 reagent solution at 6300 nmol/ml = 25 µl

Procedure

1. Prepare a fresh solution of 5 mg of Cy5 mono NHS ester (Cy5-NHS, GE Healthcare) in 1 ml dimethylsulfoxide (DMSO).
   The solution is stable for several hours. A fresh solution should be prepared for each experiment since DMSO is hygroscopic and traces of water will greatly reduce the stability of the solution. Minimize exposure to light, since Cy5-NHS is light sensitive.
2. For **bivalent** Fab labeling (Fab-dHLX format), add 25 µl reagent solution to 1 mg antibody.
   For **monovalent** Fab labeling (Fab format), add 63 µl reagent solution to 1 mg antibody.
   Antibody concentration should be between 0.5 and 2 mg/ml. Lower concentrations of antibody require a higher reagent excess (see 'Important points before starting').
3. Incubate for 30 minutes at RT, or on ice for 2 hours.
4. Follow the purification protocol described on page 126.

Conjugation with Biotin

Conjugation with biotin can be performed using the EZ-link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, 21335).

TABLE 14 **Calculation of Reagent Dye Volumes.**

Compound	Molecular weight	nmol in 1 mg reagent	nmol in 20-fold molar excess of reagent	Required volume of reagent
Biotin-NHS		1800 nmol/ml (solution of 1 mg/ml)	–	–
Fab	~50 kDa	20 nmol	20 × 20 nmol = 400 nmol	400 nmol of a biotin reagent solution at 1800 nmol/ml = 222 µl
Fab-dHLX	~120 kDa	8 nmol	20 × 8 nmol = 160 nmol	160 nmol of a biotin reagent solution at 1800 nmol/ml = 89 µl

Procedure

1. Prepare a fresh solution with 1 mg of EZ-link Sulfo-NHS-LC-Biotin (Biotin-NHS) in 1 ml PBS.
   The solution is stable for several hours. High temperatures, traces of amines (e.g. Tris), or non-neutral pH greatly reduce the stability of the solution.
2. For **bivalent** Fab labeling (Fab-dHLX format), add 89 µl reagent solution to 1 mg of antibody.
   For **monovalent** Fab labeling (Fab format), add 222 µl reagent solution to 1 mg antibody.
   Antibody concentration should be between 0.5 and 2 mg/ml. Lower concentrations of antibody require a higher reagent excess (see 'Important points before starting').
3. Incubate for 30 minutes at RT, or on ice for 2 hours.
4. Follow the purification protocol on page 126.

Purification

For each type of labeling, there are three options for separating the antibody from the unreacted reagent.

Purification on a PD10-column (GE Healthcare) or Comparable SEC Column

1. Add 50 µl 1 M Tris, pH 7.4, to stop the reaction (this inactivates NHS esters), and use the resulting solution in experiments.
2. Equilibrate the column twice with 8 ml 3x PBS.
3. Adjust the reaction volume to 2 ml using 3x PBS.
4. Apply 2 ml reaction mix to the column and discard the flow-through.
5. Wash the column using 0.5 ml 3x PBS and discard the flow-through.
6. Elute with 2.5 ml 3x PBS.

Note: The antibody yield is approximately 80% of the starting amount.

Dialysis Using 3x PBS

1. Add 50 µl 1 M Tris, pH 7.4, to stop the reaction (this inactivates NHS esters).
2. Perform two dialysis steps using at least a 50-fold excess of dialysis volume. 'Slide-A-Lyzer' cassettes (0.5–3 ml; Thermo Fisher Scientific) with a molecular weight cut-off (MWCO) of 10,000 are recommended.

Dialysis for Small-scale Reactions

1. Add 50 µl 1 M Tris, pH 7.4, to stop the reaction (this inactivates NHS esters).

Perform one dialysis step using 3x PBS using at least a 200-fold excess of dialysis volume. The 'Mini Slide-A-Lyzer' with a MWCO of 3,500 (Thermo Fisher Scientific) is recommended.

Note: For monovalent Fab fragments, 1x PBS can also be used as dialysis buffer.

Examples

FITC conjugation

The successful FITC conjugation of a monovalent antibody, AbD00178 in Fab-MH format, and a bivalent antibody, AbD00908 in Fab-dHLX-MH format, was demonstrated by direct ELISA. The antibodies in this example bind human NFκB (Figure 46).

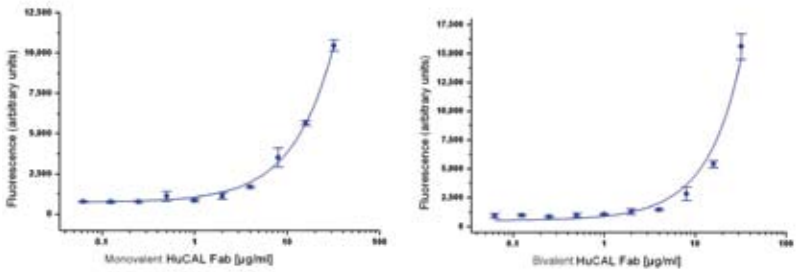

FIGURE 46 **Direct ELISA. Left:** FITC-labeled anti-NfkB HuCAL® bivalent mini-antibody AbD00908 (Fab-dHLX-MH). **Right**: FITC-labeled anti-NfkB HuCAL® monovalent Fab fragment AbD00178 (Fab-MH).

Biotinylation

Successful biotinylation of a mono and bivalent HuCAL® antibody against NFκB was shown by ELISA. The biotinylated antibody was immobilized on a neutravidin plate and detected with an anti-Fab antibody conjugated to AP and AttoPhos® substrate (Figure 47).

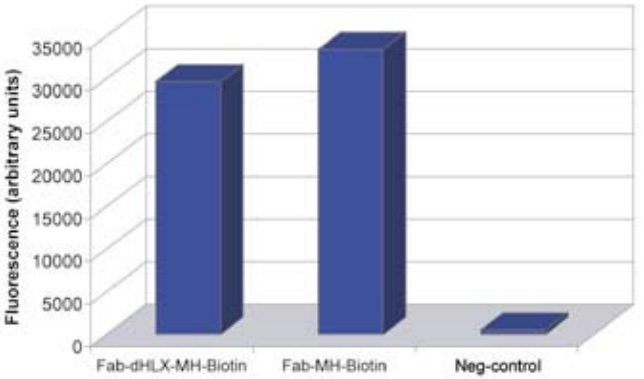

FIGURE 47 **ELISA Detects Biotinylated HuCAL® Fab.** Detection was performed with an anti-Fab polyclonal antibody. The negative control is the same HuCAL® Fab without biotinylation.

Troubleshooting

Problem	Possible Cause and Course of Action
Failed labeling reaction	1. Incompatible buffer used. Use a buffer that does not contain primary amines or SH-groups. 2. Reagent hydrolyzed and non-reactive. Prepare labeling reagent immediately before use, and do not store the reagent in aqueous solution. 3. Reagents expired or not stored correctly. Use fresh reagents and store according to the manufacturer's instructions.
Fluorescence signal too low	1. Too few or too many dye molecules coupled to the antibody. Use a higher or lower ratio of antibody to dye (see 'Important points before starting' page 122).

Appendices

Chapter 12

Buffer Composition

Buffer	Composition	Storage
HBS	20 mM Hepes, pH 7.4 150 mM NaCl	4°C
PBS	136 mM NaCl	RT
	2.68 mM KCl	
	8.1 mM Na_2HPO_4	
	1.46 mM KH_2PO_4	
PBST	PBS with 0.05% Tween®-20	RT
10x TBS	500 mM Tris-HCl, pH 7.4	RT
	1.5 M NaCl	
TBST	50 mM Tris-HCl, pH 7.4	RT
	0.15 M NaCl	
	0.1% Tween® 20	
TNEC buffer	50 mM Tris-HCl, pH 8.0	4°C
	150 mM NaCl	
	1% Nonidet P40	
	2 mM EDTA	
	1 tablet/10 ml buffer of complete mini protease inhibitor (Roche, 1332473)	

Buffer Preparation

Flow Buffer

1. Mix ice-cold PBS, pH 7.4, 3% FCS, and 0.02% sodium azide.
2. Centrifuge at high speed until the suspended matter is pelleted (~ 30 minutes at 17700 × g), at 4°C.
3. Transfer supernatant to a new tube.
4. Store at 4°C for up to one week.

Activated Biotinylated Tyramide Buffer for IHC

1. Add 100 mg EZ-link Sulfo-NHS-LC-biotin (Thermo Fisher Scientific, 21335) to 40 ml of 25 mM borate buffer.
2. Add 30 mg tyramine-HCl (Sigma, T-2879).
3. Shake gently at room temperature for 12 hours.
4. Sterilize by filtration through a 20 µm filter.
5. Divide into single-use aliquots of 50 µl and store at –20°C.

Source of Reagents

Reagent	Supplier	Product Code
4% Paraformaldehyde in PBS	Sigma	P6148
96-well round bottom microtiter plates, sterile	NUNC	163320
96-well, flat-bottom, black MaxiSorp™ PS microtiter plates	NUNC	437111
384-well, flat-bottom, black MaxiSorp™ PS microtiter plates	NUNC	460518
Antibody diluents	AbD Serotec	BUF014
Attana Carboxyl Sensor Chip	Attana	3616-3033
Attana Amine Coupling Kit	Attana	3501-3001
AttoPhos®	Roche	11681982
BCIP/NBT	AbD Serotec	BUF045A
Biacore Sensor Chip CM5	GE Healthcare	BR-1000-14
Biacore Amine Coupling Kit	GE Healthcare	BR-1000-50
BM Blue Soluble Peroxidase Substrate	Roche	1484281
BSA	Sigma	A7960
Cy5 Mono NHS Ester	GE Healthcare	PA15100
DAB substrate	AbD Serotec	BUF021A
DAB substrate buffer	AbD Serotec	BUF022
Dynabeads® TALON™	Invitrogen	10101D
DynaMag™-2	Invitrogen	123-21D
ECL Plus™	GE Healthcare	RPN2132
ECL Advance™ Western Blotting Detection Kit	GE Healthcare	RPN2135
EZ-link sulfo-NHS-LC-Biotin	Thermo Fisher Scientific	21335

Reagent	Supplier	Product Code
FCS	PAN	3302-P241105
FITC labeling reagents	Thermo Fisher Scientific	46110
Hematoxylin	Sigma	MHS16
HiSpec Buffer	AbD Serotec	BUF049
HISTAR Detection System	AbD Serotec	STAR3000
IFNγ	AbD Serotec	PHP050
LiquiChip® Carboxy Beads Set A	QIAGEN	922402
LiquiChip® Carboxy Beads Set B	QIAGEN	922404
LiquiChip® Carboxy Beads Set C	QIAGEN	922406
LYNX Rapid Conjugation Kit® for HRP	AbD Serotec	LNK001P
Dynabeads® M-450 Epoxy	Invitrogen	14011
MagStrep type 2 Strep-Tactin® coated magnetic beads	IBA	2-1611-002
Microcentrifuge tubes for Luminex assays	USA Scientific	1415-2500
Mini Slide-A-Lyzer, MWCO 3,500	Thermo Fisher Scientific	69550
Mouse Envision™ Kit	Dako	K4007
Mouse-on-Mouse (M.O.M™) Kit	Vector Laboratories	PK-2200
MSD Sector Imager 6000 Reader 96-well Plates	Meso Scale Discovery	L11XA3
MultiScreen$_{HTS}$-BV, 1.2 µm PVDF Filter Plate	Millipore	MSBVN1210
PBS	Gibco	14190-094
PD10 SEC columns	GE Healthcare	17-0851-01
Peroxide Blocking Reagent	AbD Serotec	BUF017A
QuantaBlu® Fluorogenic Peroxidase Substrate	Thermo Fisher Scientific	15169
Slide-A-Lyzer, 0.5–3 ml, MWCO 10,000	Thermo Fisher Scientific	66380
Streptavidin-AP	AbD Serotec	STAR6B
Streptavidin-RPE	AbD Serotec	STAR4B
Streptavidin-HRP	AbD Serotec	STAR5B
Tips for Luminex® assays, 250 µl	Mettler Toledo	RT-250
Tips for Luminex® assays, 1000 µl	Mettler Toledo	RT-1000
Trypan Blue	Sigma	T-B154
Trypsin/EDTA	Gibco BRL	25300-054
Tyramide	Sigma	T-2879
Vac-Man®96 Vacuum Manifold	Promega	A2291

Source of Antibodies

Visit **www.abdserotec.com** for additional conjugates or to download detailed datasheets.

HuCAL® Antibodies

Antibody	Clone (Product Code)	Format
Anti-Caspr	AbD06152	Fab-dHLX-MH
Anti-CD81	AbD06083 (HCA086)	Fab-dHLX-MH
	AbD06084 (HCA087)	Fab-dHLX-MH
	AbD08863 (HCA112)	Fab-dHLX-MH
	AbD08864 (HCA113)	Fab-dHLX-MH
Anti-Cyclophilin A	AbD00794 (HCA005)	Fab-dHLX-MH
	AbD08865	Fab-dHLX-MSx2
	AbD08966	Fab-A-V5Sx2
Anti-Desmin	AbD05054 (HCA071)	Fab-dHLX-MH
	AbD08860 (HCA114)	Fab-dHLX-MSx2
	AbD08961	Fab-A-V5Sx2
Anti-FDPS	AbD02012	Fab-A-FH
	AbD02122 (HCA012)	Fab-dHLX-MH
Anti-FITC	AbD00756 (HCA002)	Fab-FS
Anti-IFNγ	AbD00068	Fab-MH
	AbD00676 (HCA043)	Fab-dHLX-MH
	AbD02503 (HCA044)	Fab-A-FH
Anti-Ki-67	AbD02531 (HCA006)	Fab-dHLX-MH
	AbD02815	Fab-A-FH
Anti-NFκB	AbD00908	Fab-dHLX-MH
	AbD00178	Fab-MH
Anti-Rootletin	AbD02085 (HCA009)	Fab-A-FH
Anti-Vimentin	AbD02701 (HCA032)	Fab-dHLX-MH
	AbD08866 (HCA111)	Fab-dHLX-MSx2
	AbD08962	Fab-A-V5Sx2

HuCAL® Negative Control Antibodies (anti-GFP, MOR06391)

Format	Product Code
Fab-MH	HCA051
Fab-V5H	HCA098
Fab-FH	HCA045
Fab-H	HCA124
Fab-V5Sx2	HCA125
Fab-MSx2	HCA100
Fab-FSx2	HCA101
Fab-FS	HCA046
Fab-dHLX-MH	HCA052
Fab-dHLX-FH	HCA097
Fab-dHLX-MSx2	HCA102
Fab-dHLX-FSx2	HCA104
Fab-dHLX-MS	HCA048
Fab-dHLX-FS	HCA047
Fab-A-FH	HCA096
Fab-A-V5Sx2	HCA123
Fab-A-MSx2	HCA106
Fab-A-FSx2	HCA107
Fab-p53-H	HCA099
Fab-p53-V5Sx2	HCA139
hIgG1	HCA049
hIgG2	HCA108
hIgG4	HCA050
h/mIgG2a	HCA110

Secondary Antibodies

Anti-human IgG Antibodies	Supplier	Product Code	Application
Goat anti-human $F(ab')_2$	AbD Serotec	STAR126	E, IP
Goat anti-human $F(ab')_2$:AP	AbD Serotec	STAR126A	E, WB
Goat anti-human $F(ab')_2$:HRP	AbD Serotec	STAR126P	C, E, P, WB
Goat anti-human $F(ab')_2$:Biotin	AbD Serotec	STAR126B	C,E, IP, P, WB
Goat anti-human $F(ab')_2$:FITC	AbD Serotec	STAR126F	C, F, IF, P
Goat anti-human $F(ab')_2$:TRITC	AbD Serotec	STAR126TRC	C, F, IF, P
Goat anti-human $F(ab')_2$:Cy5	Jackson Immuno Research	109-175-097	C, F, IF, P
Goat anti-human IgG (H + L), $F(ab')_2$ fragment:R-PE	AbD Serotec	STAR97PE	F
Goat anti-human kappa light chain	AbD Serotec	STAR127	E
Goat anti-human kappa light chain:HRP	AbD Serotec	STAR127P	C, E, P, WB
Goat anti-human kappa light chain:FITC	AbD Serotec	STAR127F	C, F, IF
Goat anti-human kappa light chain	AbD Serotec	STAR128	E
Goat anti-human kappa light chain:Biotin	AbD Serotec	STAR128B	E, IF, WB
Goat anti-human kappa light chain:HRP	AbD Serotec	STAR128P	C, E, P, WB
Goat anti-human kappa light chain:R-PE	AbD Serotec	STAR128PE	F
Goat anti-human lambda light chain	AbD Serotec	STAR129	E
Goat anti-human lambda light chain:HRP	AbD Serotec	STAR129P	C, E, P, WB
Goat anti-human lambda light chain:FITC	AbD Serotec	STAR129F	C, F, IF
Anti-mouse IgG Antibodies	**Supplier**	**Product Code**	**Application**
Rabbit anti-mouse IgG, $F(ab')_2$ fragment:Biotin	AbD Serotec	STAR11B	C, E, F, P
Goat anti-mouse IgG (H+L), $F(ab')_2$ fragment	AbD Serotec	STAR105	C, E
Goat anti-mouse IgG (H+L), $F(ab')_2$ fragment:HRP	AbD Serotec	STAR105P	C, E, P
Goat anti-mouse IgG (H+L), $F(ab')_2$ fragment:FITC	AbD Serotec	STAR105F	F
Goat anti-mouse IgG (H+L), $F(ab')_2$ fragment:R-PE	AbD Serotec	STAR105PE	F
Rabbit anti-mouse IgG (H+L), $F(ab')_2$ fragment:FITC	AbD Serotec	STAR9B	F
Goat anti-mouse IgG (H+L):Dylight®649	AbD Serotec	STAR117D649	F, IF
Rabbit anti-mouse IgG:Cy5	AbD Serotec	OBT1725C	IF

Anti-tag Antibodies	Supplier	Product Code	Application
Mouse anti-Strep-tag® Classic	AbD Serotec	MCA2489	C, E, F, IF, IP, P, WB
Mouse anti-Strep-tag® Classic:AP	AbD Serotec	MCA2489A	E, WB
Mouse anti-Strep-tag® Classic:HRP	AbD Serotec	MCA2489P	C, E, WB
Mouse anti-Strep-tag® Immo (capture antibody)	AbD Serotec	MCA2488	E, IP
Mouse anti-His-6	AbD Serotec	MCA1396	C, E, F, IF, IP, P, WB
Mouse anti-His-6:Biotin	AbD Serotec	MCA1396B	C, E, F, IF, IP, P, WB
Mouse anti-His-6:HRP	AbD Serotec	MCA1396P	C, E, F, P, WB
Mouse anti-His-6:FITC	AbD Serotec	MCA1396F	F, IF
Mouse anti-His-6:Alexa Fluor® 488	AbD Serotec	MCA1396A488	F, IF
Mouse anti-His-6:Alexa Fluor® 647	AbD Serotec	MCA1396A647	F, IF
Mouse anti-His-6:DyLight® 549	AbD Serotec	MCA1396D549	F, IF
Mouse anti-His-6	Roche	1922416	C, E, F, IF, IP, P, WB
Mouse anti-V5	AbD Serotec	MCA1360	C, E, IF, IP, WB
Mouse anti-V5:AP	AbD Serotec	MCA1360A	E, WB
Mouse anti-V5:HRP	AbD Serotec	MCA1360P	E, WB
Mouse anti-V5:Biotin	AbD Serotec	MCA1360B	C, E, IF, IP, WB
Mouse anti-V5:FITC	AbD Serotec	MCA1360F	IF
Mouse anti-V5: Alexa Fluor® 488	AbD Serotec	MCA1360A488	IF
Mouse anti-V5: Alexa Fluor® 647	AbD Serotec	MCA1360A647	IF
Mouse anti-V5:DyLight® 549	AbD Serotec	MCA1360D549	IF
Mouse Anti-V5	AbD Serotec	MCA2894	E, IF, IP, WB
Mouse Anti-V5:FITC	AbD Serotec	MCA2894F	F, IF
Mouse Anti-V5:DyLight® 549	AbD Serotec	MCA2894D549	F, IF
Mouse Anti-V5:DyLight® 649	AbD Serotec	MCA2894D649	F, IF
Mouse Anti-V5	AbD Serotec	MCA2895	E, IF, IP, WB
Mouse Anti-V5:FITC	AbD Serotec	MCA2895F	F, IF
Mouse Anti-V5:DyLight® 549	AbD Serotec	MCA2895D549	F, IF
Mouse Anti-V5:DyLight® 649	AbD Serotec	MCA2895D649	F, IF
Rat anti-FLAG®	AbD Serotec	MCA4764	E, IF, IHC, WB
Rat anti-FLAG®:HRP	AbD Serotec	MCA4764P	E, IF, IHC, WB
Rat anti-FLAG®:Biotin	AbD Serotec	MCA4764B	E, IF, IHC, WB

Anti-tag Antibodies	Supplier	Product Code	Application
Rabbit anti-FLAG®	AbD Serotec	AHP1074	E, WB
Rabbit anti-FLAG®:HRP	AbD Serotec	AHP1074P	E, WB
Mouse anti-FLAG® M2	Sigma	F3165	E, F, IF, WB
Rabbit anti-bacterial alkaline phosphatase:HRP	AbD Serotec	AHP1108P	C, E, IF, WB
Mouse anti-c-myc (9E10)	AbD Serotec	MCA2200	C, E, F, P, WB
Mouse anti-c-myc (9E10):HRP	AbD Serotec	MCA2200P	C, E, P, WB
Mouse anti-c-myc (9E10):AP	AbD Serotec	MCA2200A	C, E, P, WB
Mouse anti-c-myc (9E10):Biotin	AbD Serotec	MCA2200B	C, E, F, P
Mouse anti-c-myc (9E10):FITC	AbD Serotec	MCA2200F	F
Mouse anti-c-myc (9E10):R-PE	AbD Serotec	MCA2200PE	F
Mouse anti-c-myc (9E10): Alexa Fluor® 488	AbD Serotec	MCA2200A488	F
Mouse anti-c-myc (9E10): Alexa Fluor® 647	AbD Serotec	MCA2200A647	F
Mouse anti-c-myc (9E10):DyLight® 549	AbD Serotec	MCA2200D549	F, IF

Applications: C: IHC – frozen; E: ELISA; F: Flow cytometry; IF: Immunofluorescence; IP: Immunoprecipitation; P: IHC – paraffin; WB: Western blotting.

Technical Assistance

A group of experienced technical advisors is available at AbD Serotec 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, AbD Serotec encourages 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.

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Confidentiality

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For a complete HuCAL antibodies publication list, please visit our website **www.abdserotec.com/HuCAL**.

Gustchina, E. *et al.* (2009) Affinity maturation by targeted diversification of the CDR-H2 loop of a monoclonal Fab derived from a synthetic naïve human antibody library and directed against the internal trimeric coiled-coil of gp41 yields set of Fabs with improved HIV-1 neutralization potency and breadth. *Virology* **39**:112–119

Knappik, A. *et al.* (2009) Development of recombinant human IgA for anticardiolipin antibodies assay standardization. *Ann N Y Acad Sci* **1173**:190–198

Lochmatter, C. *et al.* (2009) *Schistosoma mansoni* TOR is a tetraspanning orphan receptor on the parasite surface. *Parasitology* **136**:487–498

Chernov, A.V. *et al.* (2008) The two-component NS2B-NS3 proteinase represses DNA unwinding activity of the West Nile virus NS3 helicase. *J Mol Biol* **283**:17270–17278

Hillig, R.C. *et al.* (2008) Fab MOR03268 triggers absorption shift of a diagnostic dye via packaging in a solvent-shielded Fab dimer interface. *J Mol Biol* **377**:206–219

Gustchina, E. *et al.* (2007) A monoclonal Fab derived from a human non-immune phage library reveals a new epitope on gp41 and neutralizes diverse HIV-1 strains. *J Virol* **81**: 12946–12953

Jarutat, T. *et al.* (2007) Selection of vimentin-specific antibodies from the HuCAL® phage display library by subtractive panning on formalin-fixed, paraffin-embedded tissue. *Biol Chem* **388**:651–58

Zimmerman, T. *et al.* (2006) Simultaneous metal chelate affinity purification and endotoxin clearance of recombinant antibody fragments. *J Immunol Methods* **314**:67–73

Jarutat, T. *et al. (*2006) Isolation and comparative characterization of Ki-67 equivalent antibodies from the HuCAL phage display library. *Biol Chem* **387**:995–1003

Ooe, H. *et al. (*2006*).* Establishment of specific antibodies that recognize C106-oxidized DJ-1. *Neurosci Lett* **404**:166–169

Ohara, R. *et al.* (2006) Antibodies for proteomic research: comparison of traditional immunization with recombinant antibody technology. *Proteomics* **6**:2638–2646

Rauchenberger, R. *et al.* (2003) Human combinatorial Fab library yielding specific and functional antibodies against the human fibroblast growth factor receptor 3. *J Biol Chem* **278**:38194–38205

Nagy, Z.A. *et al.* (2002) Fully human, HLA-DR-specific monoclonal antibodies efficiently induce programmed death of malignant lymphoid cells. *Nat Med* **8**:801–807

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