HuCAL® Antibodies Technical Manual
HuCAL Technology

HuCAL technology is a unique and innovative concept for the *in vitro* generation of highly specific, fully human antibodies (Ostendorp R et al. 2004). The technology sets the standard for antibody production, taking the antibody generation process out of the animal house and onto the laboratory bench.

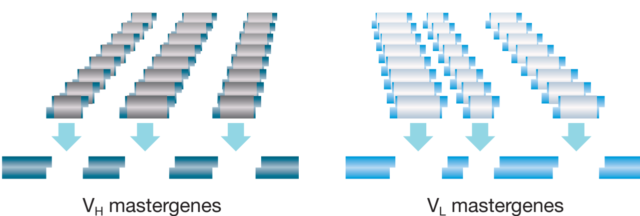
HuCAL stands for Human Combinatorial Antibody Libraries

The concept behind HuCAL technology (Knappik A et al. 2000) is to represent the essential features of the natural human antibody repertoire, defined by sub-families of antibody germline gene segments (VH and VL), and by canonical structures of the CDR loop regions, using a minimum number of sequences that cover the structural diversity of the repertoire. This goal to condense the features of the repertoire into a few germline gene representatives is facilitated by three characteristics of the human antibody germline repertoire:

- There are relatively few antibody genes in the human germline as revealed by genome sequencing
- Many putative human antibody germline sequences are rarely or never 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 on the level of VH and VL amino acid sequences revealed that its structural diversity is best represented by seven heavy chain and seven light chain variable region genes, which give rise to 49 framework combinations in the master library. A later version of the library, HuCAL PLATINUM®, contains seven VH and six VL master frameworks. The genes encoding these combinations were chemically synthesized and unique restriction sites were incorporated at the complementarity determining region (CDR) boundaries (Figure 1). Highly diverse genetic cassettes encoding the human CDR variability, which were generated using trinucleotides (Virnekäs B et al. 1994), were superimposed onto these frameworks to mimic the entire human antibody repertoire. The resulting libraries contain billions of functional human antibody specificities in scFv or Fab format, which are selectable using *in vitro* methods, such as phage display; HuCAL PLATINUM uses the Fab format.



Diversity: 42 human antibody frameworks covering structural diversity
Modularity: Completely modular gene structure by *de novo* synthesis
Expression: Very well expressed in *E. coli* by optimized codon usage
Optimization: Pre-built CDR libraries generated using trinucleotides (TRIM)

Fig. 1. 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.

Table 1. Advantages of HuCAL over conventional monoclonal antibodies

Parameter	Conventional Monoclonals	HuCAL Monoclonals
Animals used	Yes	No
Speed of delivery	4-9 months	8 weeks
Flexible formats	No	Fab antibodies (mono-and bivalent) Full length Igs, many isoptypes
Antibody specificity	Not predictable, extensive screening required	Tailored to customer's needs using Guided Selection
Fully human	No	Yes
Technology	Immunization Complex cell culture	Phage display Bacterial expression
Antibody engineering and optimization	No	Tags for purification and detection, enzyme fusions Affinity maturation
Success rate	~75%	>90%

Since the antibody selection is performed *in vitro* comprising pipetting and washing steps, the process can be adjusted to the requirements of the project. Guided selection approaches, such as applying blocking strategies, use of more than one antigen, or working with antigen complexes, lead to excellent antibody specificities. Antibody generation against toxic antigens, immunosuppressants and non-immunogenic antigens is also possible.

The production of the Fab antibodies occurs in *E. coli*, thus the entire process is free from laboratory animals, 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 all selected candidates are sequenced so that every antibody gene 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 (Table 4: Fab Antibody Formats and Epitope Tag Combinations, p7). The modularity also allows for the routine optimization of antibodies with respect to affinity by exchanging certain CDRs with prebuilt codon-based CDR libraries followed by a stringent selection (Affinity Maturation of HuCAL Antibodies, p3).

Another unique feature, the complete humanness of the antibodies, in combination with the availability of all human isotypes, is a very useful feature when developing positive controls and calibrators in human immune response assays in diagnostic and clinical settings.

Finally, the highly automated process of generating HuCAL antibodies reduces the risk of failures and provides excellent traceability of single steps. Clients benefit from fast production timelines and flexible capacity. With a project timeline of as little as 8 weeks to generate Fab antibodies, this technology is considerably faster in delivering purified and characterized antibodies than animal based systems.

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, which is demonstrated by the large and increasing number of publications using HuCAL derived 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 (Knappik A et al. 2009) or for analyzing patient responses to a drug (Tornetta M et al. 2007). They are also suitable for use in many other immune assays that currently rely on human sera as a control.

Using HuCAL technology, recombinant antibodies have been successfully generated 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

Custom antibodies that we generate for clients using HuCAL technology are for research and diagnostic use only. They are not for use in humans and they are not for therapeutic, prophylactic or palliative use.

The HuCAL PLATINUM® Platform

HuCAL PLATINUM is the latest HuCAL antibody library we use for client custom antibody generation and for new product development. It is a library of 45 billion antibodies in Fab format (Prassler et al. 2011). The antibody genes have been optimized to avoid undesirable glycosylation sites and to increase expression levels in *E. coli* and mammalian cells. A diversification strategy was used to replicate the natural amino acid distribution, with particular attention to the key antigen recognition region, HCDR3. These features lead to a high diversity of selected antibodies and provide a wider range of promising antibody candidates to choose from during an initial screening of the library, with respect to affinity and specificity.

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 designed CDR amino acid composition
- Optimized codons for *E. coli* and eukaryotic expression systems

- Removal of mRNA secondary structures that may interfere with expression
- Avoidance of negative regulatory sequence motifs in prokaryotic and eukaryotic systems
- Significant reduction in the 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 library is wholly compatible with our well-established screening and selection methods. 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 2).

CysDisplay Selection Technology

CysDisplay technology is a proprietary and efficient display method for selecting high affinity binders from antibody libraries using filamentous phage (Rothe C et al. 2008). 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 2).

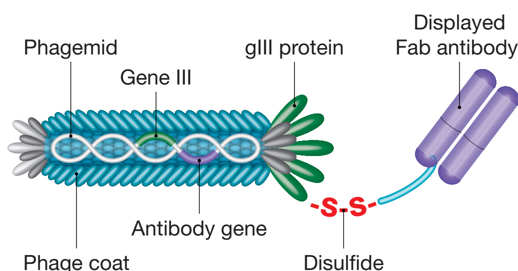


Fig. 2. CysDisplay Screening Technology. Fab fragments are linked to phage particles by a disulfide bond rather than a peptide bond. This allows elution of phage with reducing agents during antibody selection.

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 antibody fragments.

This protocol can be used for any type of antigen and is 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 elution methods such as acidic elution.

Affinity Maturation of HuCAL Antibodies

In contrast to animal-derived antibodies from polyclonal serum or from hybridoma supernatant, HuCAL antibodies can be rapidly optimized in affinity, since the antibody genes are obtained and sequenced after selection as plasmid DNA. 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, resulting in antibodies with affinities below 10^{-12} M (Steidl S et al. 2008; Prassler J et al. 2009). 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 HuCAL antibodies isolated from the initial library
- CDR cassettes have been pre-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.

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 first inserting a pre-built trinucleotide CDR library cassette (usually LCDR3 as in Figure 3, or HCDR2), which has been diversified according to the natural repertoire of CDR sequences at unique flanking restriction sites.

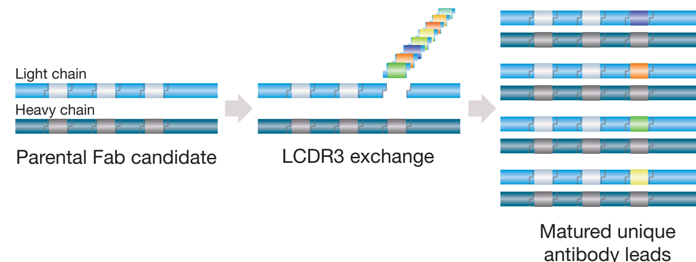


Fig. 3. Optimization of Binding Characteristics of a HuCAL Antibody. Optimization occurs via CDR library creation for specific binders in a simple cloning step.

This simple and efficient 'mix and match' process generates a new antibody library, where most members will still bind to the antigen the parental antibody was selected against. Subsequent highly stringent phage display selection then permits isolation of affinity matured variants of single antibodies ('lead optimization'), or even of antibody pools ('pool maturation' or RapMAT®).

TRIM Technology

TRIM technology (Virnekäs et al. 1994) is used to facilitate targeted diversification of CDRs. Pre-assembled trinucleotide phosphoramidites are used for the chemical synthesis of DNA oligonucleotides encoding CDR sequences, which provides complete control over the amino acid composition at each position and avoids any stop codons or undesired codons. Consequently, the resulting CDR libraries display significantly higher quality than those made using conventional approaches such as NNK libraries.

The HuCAL Antibody Generation Process

Generation of antibodies using HuCAL technology involves seven steps (Figure 4).

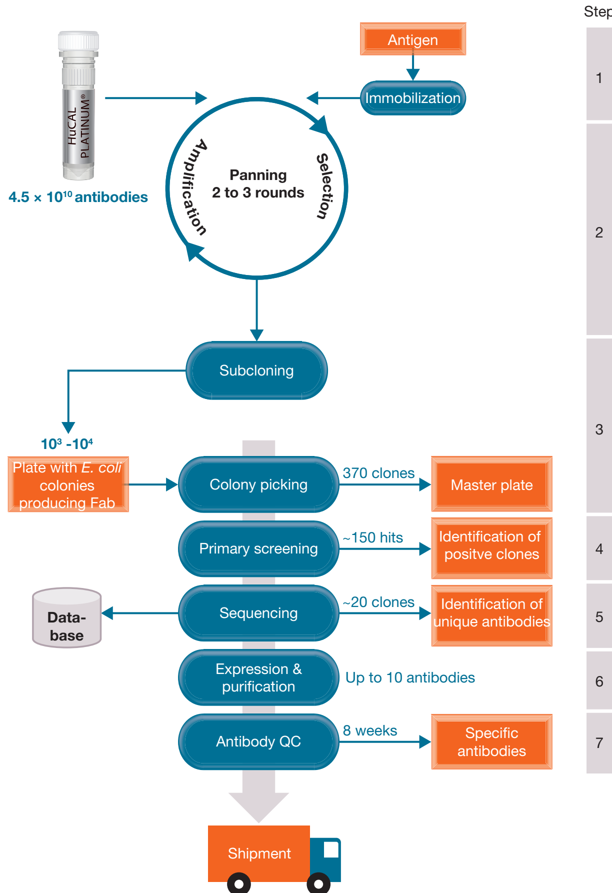


Fig. 4. HuCAL Antibody Generation Process Overview

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 adsorption to polystyrene ELISA microtiter plates. Antigen presentation by passive adsorption may be beneficial for certain applications and is the best 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. Non-specific antibodies are removed by extensive washing and specific antibody phages are eluted by adding a reducing agent (see CysDisplay). An *E. coli* culture is infected with eluted phage and with helper phage to produce phages representing an enriched antibody phage library for the next panning round. Typically, three rounds of panning are completed (Figure 5).

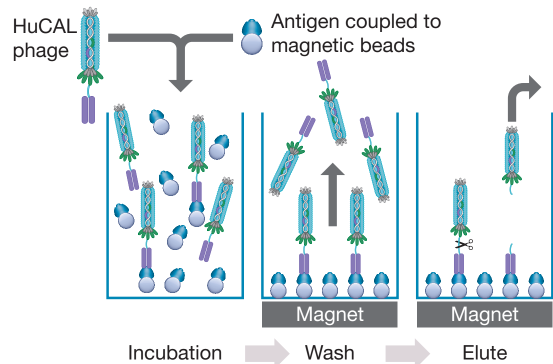


Fig. 5. Phage Panning. Selection round of phage-displayed HuCAL Fab fragments on antigen-coated magnetic beads.

3. Subcloning into antibody expression vector

After panning, the phagemid DNA encoding the enriched antibody population is isolated as a pool and subcloned into a Fab expression vector. Different vector formats are available for expression of monovalent or bivalent Fab fragments with a choice 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.

4.1. Primary screening of crude extracts by ELISA

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 by indirect ELISA. Alternatively, screening can be carried out on antigen coupled magnetic beads, for instance using the Bio-Plex® instrument. High-throughput flow cytometry screening on cells can be performed to identify antibodies that detect the native antigen on cells.

4.2. Optional secondary screening

A secondary screening offers the option to further filter the primary screening hits for certain characteristics. Two options for a secondary screening are currently available:

- Off rate screening to filter according to binding strength
- Western blot screening to identify antibodies that detect the antigen in western blots

5. **Sequencing**
   Hits from the primary or secondary screening experiment are sequenced to identify unique antibodies. The sequences not only guarantee the uniqueness of any HuCAL antibody obtained from the panning, 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 antibodies with unique sequences are expressed in *E. coli* and purified using one-step affinity chromatography.
7. **Antibody QC**
   Purified antibodies are tested by ELISA for required specificity. Purity is assessed by Coomassie® staining of a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and concentration is measured by UV absorbance at 280 nm.

Additional QC assays and cross-reactivity testing can be performed depending on the project requirements.

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

The custom HuCAL recombinant monoclonal antibody service is tailored to the client's project needs. Three packages provide the basis of the antibody generation service; additional services can be added. Working with the customer, our technical specialists provide expertise to design a project to generate and characterize the antibodies required.

Table 2. Antibody generation packages

Fab Package

- Antibody selection using a single customer provided antigen
- QC by specificity ELISA with purified antibodies
- Delivery of 250 µg each of up to 10 unique ELISA positive, purified Fab antibodies

Dispatch of antibodies 8 weeks after project commencement

Guided Selection Package

- Antibody selection using a single customer provided antigen
- Guided selection with up to two further customer provided closely related antigens (CRAs) and human serum
- Cross-reactivity profiling by ELISA with up to two CRAs
- QC by specificity ELISA with purified antibodies
- Delivery of 250 µg each of all unique ELISA positive, purified Fab antibodies, (a typical project generates 11-15 unique antibodies)

Dispatch of antibodies 8 weeks after project commencement

Antibody Maturation Package

- Antibody selection with additional integrated affinity maturation steps (RapMAT), with 1 customer supplied antigen
- Guided selection with up to two further customer provided CRAs and human serum
- Cross-reactivity profiling by ELISA with up to two CRAs
- Affinity ranking and affinity measurement of up to 16 monovalent Fab antibodies
- QC by specificity ELISA with purified antibodies
- Delivery of 250 µg each of up to 16 unique ELISA positive, purified Fab antibodies
- DNA and amino acid sequence of up to 5 Fab antibodies

Dispatch of antibodies 16 weeks after project commencement

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. In the following sections, 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 type). 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, the enriched antibody Fab genes are cloned into an *E. coli* expression vector. The choice of expression vector determines:

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

Antibody formats can also be changed later by subcloning the genes 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 for IgG). Full length antibody constructs are expressed in mammalian cell culture 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 the classical full-size antibody. However, for certain types of experiments 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 endotoxin and cannot be used in some cellular assays without an additional purification step
- Most commercial secondary detection antibodies are directed against the Fc region of the primary antibody. For HuCAL Fabs, secondary detection antibodies directed against epitopes on the human Fab portion or against the epitope tags are used
- If HuCAL antibodies are used to detect antigen in human samples with techniques such as immunohistochemistry (IHC) or western blot, the potential presence of endogenous human antibodies requires the detection of HuCAL Fab with an epitope tag secondary antibody

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 one of the bivalent formats (functionally equivalent to a $F(ab')_2$ fragment) (Figure 6 and Table 3).

A monovalent Fab format is recommended when comparing affinities or IC50 values of different antibodies since the signal is not influenced by avidity effects. For this reason it is the first format in projects with off-rate rankings. Generally this format works well in most assays. 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. The monovalent format is also recommended for immobilization on affinity chromatography columns. The two Fab arms in the bivalent formats are held together by non-covalent interactions which potentially could lead to leaching of one Fab arm from the column during the elution or cleaning step.

Bivalent Fab fragments are preferred to monovalent Fab fragments for most applications that detect surface-bound antigen (some ELISAs, western blotting, IHC, and Flow Cytometry), since the two paratopes binding to surface-bound antigen with sufficient density will lead to an avidity effect and therefore a potentially higher sensitivity.

Certain dimerization domains, when fused to the C-terminus of the heavy chain fragment, create non-covalently linked Fab-homodimers. Bacterial alkaline phosphatase naturally forms a homodimer which can be used to form bivalent antibodies. Resulting antibodies (Fab-A) are larger than IgG molecules, which is of advantage for applications requiring immobilization or conjugation of the antibody by random chemical coupling. Since the alkaline phosphatase in this molecule is an active enzyme it also can be used for direct detection with the appropriate substrate. For targets in which the enzymatic activity might be problematic (e.g. phosphorylated antigens) we also offer this format with a modified protein with inactivated enzymatic activity (Fab-Max).

Further information on the molecular weight of the different formats can be found in Table 4 and Table 5.

In our mini-antibodies, the heavy chain is expressed as a fusion with the dHLX domain. Two dHLX domains dimerize in a helix-turn-helix structure to form the bivalent mini-antibody (Pack P et al. 1993; Plückthun A and Pack P. 1997).

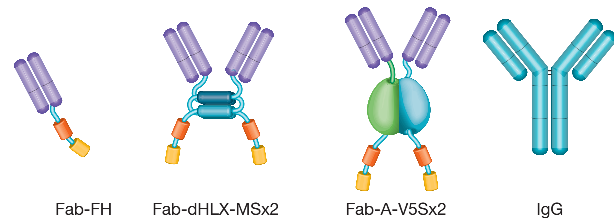


Fig. 6. Schematic of the Main Antibody Formats Available. Fab-FH: Monovalent Fab (shown with two tags). **Fab-dHLX-MSx2:** Bivalent Fab, dHLX domains shown as blue cylinders (with two tags). **Fab-A-V5Sx2:** Bivalent Fab formed by dimerization of bacterial AP (blue/green) with two tags. **IgG:** Complete IgG antibody.

F= DYKDDDDK tag; H= His-6 tag; M= c-myc tag; Sx2= two extended Strep-tags; V5= V5 tag.

For some applications, it is useful to have even higher valencies. For those instances, we offer 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 antibody genes into the human IgG1, IgG2, IgG3, IgG4 (plus the IgG4-Pro variant that does not show Fab arm exchange), IgA, IgM, and IgE formats, followed by transient expression in mammalian cells. If Fc regions from other species are required, we offer production of chimeric human/mouse IgG2a with human variable regions and mouse constant regions. For rat, human/rat IgG1 and human/rat IgG2a and IgG2b 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 3 and Table 4 to identify the most suitable tag. We offer a wide selection of common epitope tag antibodies, either unconjugated or labeled with enzymes and fluorescent dyes.

Table 3. Recommended Fab antibody formats by application

Application	Monovalent	Bivalent	Recommended Secondary Antibodies
Western Blot		√	Anti-human Fab Anti-Strep-tag Anti-His-6 Anti-V5 Anti-BAP Anti-DYKDDDDK Anti-c-myc
ELISA		√	Anti-human Fab Anti-Strep-tag Anti-His-6 Anti-V5 Anti-BAP Anti-DYKDDDDK Anti-c-myc
Immunoprecipitation		√	Anti-human Fab Anti-Strep-tag Anti-His-6 Anti-V5 Anti-DYKDDDDK
Immunohistochemistry		√	Anti-human Fab (for non-human tissue) Anti-Strep-tag Anti-His-6 Anti-DYKDDDDK
Flow Cytometry		√	Anti-human Fab (for non-human tissue) Anti-Strep-tag Anti-DYKDDDDK Anti-His-6
Immunofluorescence		√	Anti-human Fab (for non-human tissue) Anti-Strep-tag Anti-His-6 Anti-V5 Anti-BAP
Affinity Determination	√		
Affinity Chromatography	√		Use a Fab-ds or IgG format to avoid column leakage of the light or heavy chain
Co-crystalization	√		Use Fab-H or Fab TC-MH: use Thrombin to cleave off tags

All available HuCAL Fab expression vectors will add a Strep-tag®, an extended Strep-tag (Strep II-tag with a Ser-Ala linker), or a His-6 tag to the antibody protein, which is used for purification. A second tag is added in many formats to provide additional options for detection (Table 4). Note that Ig formats do not contain epitope tags.

Table 4. Fab antibody formats and epitope tag combinations

Short Name	Description	Approx MW (kDa)
Monovalent		
Fab-FH	Fab antibody (DYKDDDDK- and His-6-tags)	52
Fab-V5H	Fab antibody (V5- and His-6-tags)	53
Fab-MH	Fab antibody (c-myc- and His-6-tags)	52
Fab-V5Sx2	Fab antibody (V5- and StrepX-StrepX-tags)	56
Fab-FSx2	Fab antibody (DYKDDDDK- and StrepX-StrepX-tags)	54
Fab-MSx2	Fab antibody (c-myc- and StrepX-StrepX-tags)	55
Fab-H	Fab antibody (His-6-tag)	51
Fab-S	Fab antibody (Strep-tag)	51
Fab-CysH	Fab antibody (Cys with His-6-tag)	51
Fab-Cys3H	Fab antibody (three Cys with His-6-tag)	51
Fab-ds-H	Disulfide-linked Fab antibody (His-6-tag)	51
Fab-ds-FS	Disulfide-linked Fab antibody (DYKDDDDK- and Strep-tag)	52
Fab-Tc-MH	Fab antibody (Thrombin cleavable, c-myc- and His-6-tags)	53
Bivalent		
Fab-dHLX-FH	Mini-antibody (DYKDDDDK- and His-6-tags)	115
Fab-dHLX-MH	Mini-antibody (c-myc- and His-6-tags)	115
Fab-dHLX-H	Mini-antibody (His-6-tag)	112
Fab-dHLX-FSx2	Mini-antibody (DYKDDDDK- and StrepX-StrepX-tags)	119
Fab-dHLX-MSx2	Mini-antibody (c-myc- and StrepX-StrepX-tags)	120
Fab-dHLX-S	Mini-antibody (Strep-tag)	113
Fab-A-FH	Fab bacterial alkaline phosphatase (BAP) fusion antibody (DYKDDDDK- and His-6-tag	198
Fab-A-V5H	Fab BAP fusion antibody (V5- and His-6-tags)	200
Fab-A-MH	Fab BAP fusion antibody (c-myc- and His-6-tags)	199
Fab-A-Cys3H	Fab BAP fusion antibody (three Cys with His-6-tag)	197
Fab-A-FSx2	Fab BAP fusion antibody (DYKDDDDK- and StrepX-StrepX-tags)	203
Fab-A-V5Sx2	Fab BAP fusion antibody (V5- and StrepX-StrepX-tags)	205
Fab-A-MSx2	Fab BAP fusion antibody (c-myc- and StrepX-StrepX-tags)	203
Fab-A-S	Fab BAP fusion antibody (Strep-tag)	196
Fab-ds-A-FS	Disulfide-linked Fab BAP fusion antibody (DYKDDDDK- and Strep-tag)	199
Fab-Max-FH	Fab modified BAP fusion antibody with inactivated enzymatic activity (DYKDDDDK- and His-6-tags)	198
Fab-Max-V5Sx2	Fab modified BAP fusion antibody with inactivated enzymatic activity (V5- and StrepX-StrepX-tags)	205
Tetravalent		
Fab-p53-V5Sx2	Mini-antibody (V5- and StrepX-StrepX-tags)	246
Fab-p53-H	Mini-antibody (His-6-tag)	227
Fab-p53-S	Mini-antibody (Strep-tag)	228

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, we offer anti-histidine-tag antibody MCA5995P as HRP conjugate and MCA1396 conjugated to Alexa Fluor® 488, Alexa Fluor 647, AP, biotin, DyLight® 549, DyLight 800, FITC and HRP.

The Strep-tag is an artificial epitope tag with 8 amino acids that was originally generated to bind streptavidin (Voss and Skerra, 1997). Strep-Tactin®, which is an engineered streptavidin with higher affinity to the Strep-tag, is usually preferred. 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).

Table 5. 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)	5.2
A	Bacterial alkaline phosphatase (dimer)	47
Max	Modified bacterial alkaline phosphatase with inactivated enzymatic activity (dimer)	47
p53	Domain derived from human p53 (tetramer)	5.8
His-tag Combinations		
H	HHHHHH	0.9
FH	DYKDDDDKGAPHHHHHH	2.1
V5H	GKPIPNPLLGLDSTDAPHHHHHH	2.9
MH	EQKLISEEDLNGAPHHHHHH	2.4
CysH	CHHHHHH	1.1
Cys3H	CCCHHHHHH	1.3
Tc-MH	LVPR ↓ GSGAPEQKLISEEDLNDAPHHHHHH ↓: Indicates Thrombin cleavage (Tc) site	3.3
Strep-tag Combinations		
S	WSHPQFEK	1.2
FS	DYKDDDDKGAPWSHPQFEK	2.3
FSx2	DYKDDDDKGAPSAWSHPQFEK GGGSGGGSGGSAWSHPQFEK	4.3
V5Sx2	GKPIPNPLLGLDSTDAPSAWSHPQFEK GGGSGGGSGGSAWSHPQFEK	5.6
MSx2	EQKLISEEDLNDAPSAWSHPQFEK GGGSGGGSGGSAWSHPQFEK	4.7

Besides the standard purification tags (His-6 or Strep-tags) and detection tags (c-myc, DYKDDDDK, V5), other sequence additions are also available to provide additional functionality. For example, we offer addition of one or three cysteine residues for site-specific coupling or conjugation, 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. If the preferred tag or a combination of tags is not listed in the tables above, please contact us.

Antigens

The main antigen class for antibody development is protein antigen. With HuCAL technology, only 0.5 mg of protein antigen is required for the entire project from antibody generation to screening and futher characterization. Under certain conditions even as little as 100 µg of protein may be sufficient. If purified protein is not available, it is possible to have antigen material generated using our *E. coli* expression platform (AgX® antigen expression technology), or using our services to express the antigen in a mammalian expression system, typically as an Fc fusion protein. Alternatively, peptides or other antigens, such as small molecules (haptens), and a variety of other antigen classes can also be used for HuCAL antibody development.

Protein Antigens

Proteins used as antigens 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).

Up to 4 M guanidine hydrochloride or low amounts of free sulfhydryl groups (e.g. 1 mM Dithiothreitol [DTT]) are compatible with the coupling chemistry. Our scientists are available to provide advice on buffer compatibility. If it is not possible to change buffer composition, immobilization of the antigen for panning will be performed by passive adsorption onto microtiter plate, and subsequently handled with PBS ('solid-phase panning').

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. This step is not required for the His-tag, as we routinely screen for this specificity.

The AgX Antigen Expression Service

When the protein is not available in purified form, the AgX antigen expression system is an ideal starting point for generating antigens starting from DNA sequences or from purified DNA (Frisch C et al. 2003). We use a bioinformatics toolbox to determine the optimal protein domain for expression and then synthesize or subclone the DNA fragment into a proprietary *E. coli* N1-fusion protein expression vector. Gene synthesis will add 2 weeks to the timeline of the AgX antigen production.

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 7).

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 as compared to a peptide antigen; hence, the diversity of antibodies selected is usually higher

Since AgX antigens are refolded protein fragments their conformation might differ from the native protein conformation. Therefore AgX is not a recommended strategy when antibodies against the native protein conformation are required (e.g. sandwich assays, flow cytometry, immunofluorescence etc).

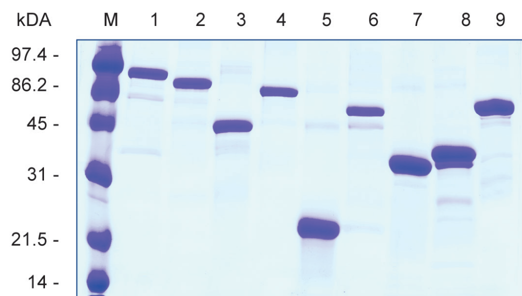


Fig. 7. Antigens Expressed as AgX Fusion Proteins. 15% SDS-PAGE (reducing conditions) of N1-fusions of nine protein fragments ranging in length from 100 to 700 amino acids. N-terminal fusion of the AgX to the N1 domain of the pIII protein results in strong expression of protein fragments, while the C-terminal 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. We have a very good success rate in generating antibodies against peptides and our technical specialists can help choose the best peptide sequence for antibody generation.

Our routine method couples peptides to two carrier proteins (using different linkers) for panning against the library. The two antigen-carrier conjugates are alternated during the panning rounds, which ensures that selection takes place towards the peptide and not the carrier or the linker. The standard carrier proteins are BSA and human transferrin (Trf), with coupling via an N- or C-terminal cysteine added to the peptide sequence, but alternative coupling strategies can be used where necessary. We can provide peptide synthesis and coupling services, or just coupling existing suitable peptides.

Using guided selection, 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 peptide and hapten antigens.

Guided Selection Strategies

Selection of antibodies using HuCAL technology is performed *in vitro* (Figure 4). This enables greater flexibility for antibody generation than is available with conventional methods based on the immunization of animals. Guided selection strategies are strategies that involve blocking steps or the use of two or more antigens, and are typically used for the isolation of epitope-specific antibodies or for antibodies that recognize shared epitopes on different antigens.

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 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 8).

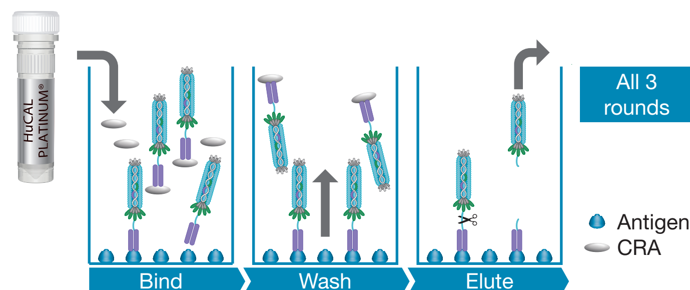


Fig. 8. Guided Selection with Blocking. A closely related antigen is used to remove cross-reactive antibodies (counter selection).

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 antigens allows identification of antibodies that react with both antigens (Figure 9), if the antigens share a common epitope. This strategy is useful for generating cross-species antibodies or for recognizing two isoforms of a protein.

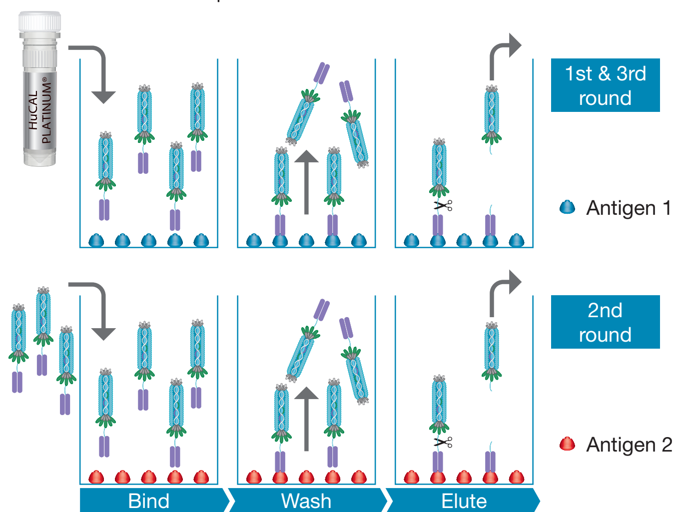


Fig. 9. Guided Selection with Alternating Antigens. The antigen is alternated during the three rounds of selection, thereby enriching antibodies that bind to both antigens. Primary screening on both antigens will confirm the desired cross-reactivity.

Antibodies that Bind Antigens under Special Conditions

Selection conditions can be altered 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.

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 HRP conjugated 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 10).

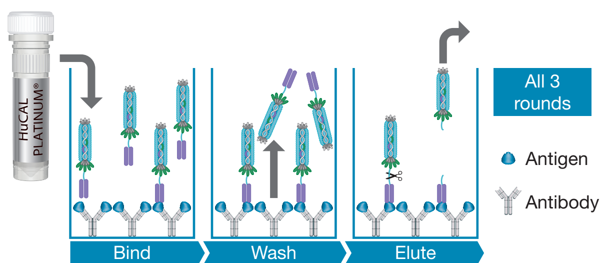


Fig. 10. Selection with a Captured Antigen. The antigen is presented as an antigen-ligand complex. The ligand is used as a CRA to pre-clear the library.

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 full length protein, if possible, 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 by default 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 those antibodies that truly bind the free unconjugated hapten.

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 a bead-based FLISA assay. As little as 100 µg of antigen might be sufficient for the entire antibody generation project.

For Bio-Plex applications, antigen coupled Bio-Plex beads can be used for screening on the Bio-Plex 3D instrument.

For flow cytometry applications a screening on cells enables the selection of flow cytometry-positive candidates, since not all ELISA positive antibodies function well on cells. Positive candidates are sequenced and unique antibodies retested in flow cytometry after purification. For flow cytometry screenings, customers typically provide positive and negative cells.

The primary screening is performed with crude *E. coli* lysates containing unknown and varying amounts of antibody and only one data point per clone is obtained. Consequently, the ELISA result does not necessarily correlate with the antibody affinity as highly expressed antibodies with poor affinity might overshadow weakly expressed candidates with high affinity. To ensure that we continue with the best hits from the primary screening we recommend an off-rate ranking as a secondary screening step (Ylera F et al. 2013).

Up to 95 hits from ELISA are tested by biolayer interferometry (BLI) on immobilized antigen to rank the dissociation rate constants (k_{off}). Only the candidates with the best k_{off} rates are then selected for sequencing. In case of less than 40 primary screening hits, an off-rate ranking is economically not advisable and we would continue with sequencing of all hits.

To ensure that the antibodies detect the protein of interest in lysates on western blots we offer a western blot secondary screening. 96 ELISA-positive antibodies can be screened on lysate provided by the customer. Positive candidates are sequenced and unique antibodies retested after purification.

Sequencing

Sequencing of up to 20 primary screening hits is included in any standard project. It identifies the truly unique antibodies and allows secure storage of the antibody sequence 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 and calculating the concentration using an extinction coefficient calculated from the antibody sequence.

Activity and specificity of the purified antibodies is tested by a QC ELISA before shipment (Figure 11). All antibodies are tested on three non-related standard antigens and on all positive and negative control antigens, as defined in the project plan. Only small amounts of antigen are required at this step, therefore if the amount of antigen material available for the project is very small, the panning and screening steps can be carried out using a surrogate antigen, such as a peptide, and the precious antigen material can be reserved for the QC ELISA step.

The QC ELISA is a single, one-point measurement and is more useful to evaluate specificity rather than affinity. Consequently, we always recommend testing all available antibodies in the final application(s). On request we measure the antibody affinities or perform additional assays, such as capture, inhibition or sandwich ELISAs, flow cytometry assays, western blots, Bio-Plex assays or epitope binning.

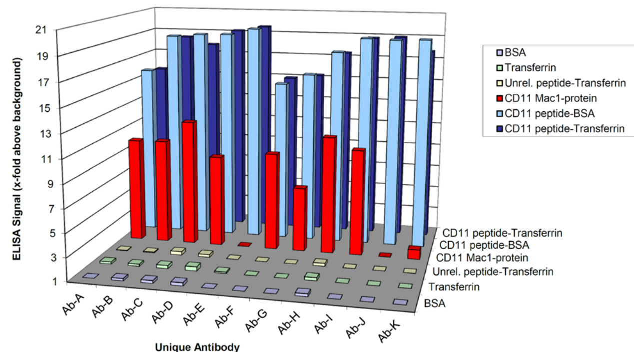


Fig. 11. QC ELISA of Anti-peptide Antibodies Show Binding to Parental Protein. The HuCAL library was panned against a peptide derived from CD11 and 11 binders were expressed as bivalent mini-antibodies. QC ELISA showed that all 11 antibodies bound specifically to the peptide but not to BSA and Trf carrier proteins, or to an unrelated peptide bound to Trf. The parental protein is also recognized by 8 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.

Additional Custom Services

Our laboratories are set up to run many additional assays and services for a large number of antibodies in parallel. These include:

- Antibody testing in a range of typical assays:
  - Different ELISA assays
  - Screening for antibody sandwich pairs (in 384-well format)
  - Assay set-up for biotherapeutical drug development
  - Bio-Plex bead-based assays (incl. sandwich assays)
  - Western blot
  - Flow cytometry
- Small- and large-scale antibody conjugation to biotin, common reporter enzymes (HRP and AP), or fluorescent dyes
- Affinity ranking (k_{off} ranking) and determination by biolayer interferometry (BLI) on a ForteBio Octet® Red384 instrument
- Epitope binning on a ForteBio Octet Red384 instrument

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