TRAILBLAZER ANTIBODIES

Experimental flexibility, reproducible quality

TrailBlazer Antibody Custom Services

We understand that the design and optimization of immunoassays is complex and time consuming. There's never a 'one size fits all' solution. Having one antibody in one format limits your options, and the resulting assay may not answer all your questions. Producing several different conjugates or alternative formats can be tedious and costly, can delay your project, and may still result in a dead end.

TrailBlazer antibodies open up more pathways to successful assay development.

We have incorporated SpyTag technology into our custom recombinant antibodies, enabling site-directed conjugation or assembly into different stable antibody formats. This provides you with an extensive antibody toolbox that enables rapid development of multiple assays, providing the results you need to make progress.

Your custom-made antibodies can be delivered in a variety of configurations at the outset. Test in monovalent or bivalent Fab, and full length immunoglobulin formats, with a choice of isotypes and labels.

You can explore which antibodies work best in your desired applications and on your different technology platforms.

What Is SpyTag Technology?

SpyTag technology is based on the SpyTag peptide and SpyCatcher protein (Zakeri et al. 2012), which are derived from the fibronectin-binding protein (FbaB) of *Streptococcus pyogenes* (Spy). The FbaB protein contains an intrachain isopeptide bond between the sidechains of a lysine and an aspartic acid within the Ig-like collagen adhesion domain. The SpyTag peptide incorporates the aspartic acid residue, and the SpyCatcher has the lysine residue; when the SpyTag and SpyCatcher are mixed, the isopeptide bond is formed between these amino acids. This reaction occurs with high yield in diverse conditions of pH, temperature, and buffer, and following optimization of the two components (SpyTag2 and SpyCatcher2, Keeble et al. 2017) the reaction time was shortened from hours to minutes (Figure 1) and has subsequently been improved to increase the reaction speed even further (SpyTag3 and SpyCatcher3) (Keeble et al. 2019). All versions of SpyTag and SpyCatcher are compatible with each other.

SpyCatcher2
116 aa
15.2 kDa

SpyTag2
13 aa

Ligated proteins

- Spontaneous (autocatalytic) reaction
- Covalent isopeptide bond formation, irreversible
- Rate constant 2.0 × 10^4 M-1 s-1 ($t_{½}$ = 74 sec; 10 µM)
- pH 5 to 8, temperature +4 to +37°C
- Robust to buffer conditions, Ca^{2+}/Mg^{2+} not needed
- Robust to detergents
- Reaction occurs also inside cells (in vivo)

Fig. 1. The reaction between SpyTag2 and SpyCatcher2 requires only mixing, is rapid, high yielding, and shows good specificity.

BIO-RAD

About HuCAL® Technology

HuCAL technology is proven and well published, and has been used by the Bio-Rad Custom Antibody Team to generate antibodies for research and diagnostic applications since 2004. The structural diversity of the human antibody repertoire is represented in the HuCAL PLATINUM® library by seven heavy chain and six light chain variable region genes, which give rise to 42 master framework combinations. Highly diverse genetic cassettes, encoding the complementarity determining regions (CDRs) of the antibody binding sites, are combined with these frameworks to create antibody genes that code for some 45 billion unique antibodies in Fab format (Knappik et al. 2000, Prassler et al. 2011).

Screening of the HuCAL library is performed in vitro enabling the successful selection of antibodies that it is impossible to generate by in vivo immunization of animals; such challenging targets include highly conserved or self-antigens, low immunogenic antigens, and conformational variants. As the sequence of any selected antibody is known, it is possible to reproduce the genes synthetically if needed. This sequence back up secures the future supply of the antibody, and recombinant production methods ensure a high level of consistency between batches.

How Is SpyTag Technology Used with Bio-Rad's Custom Antibodies?

Bio-Rad has incorporated SpyTag technology into the Human Combinatorial Antibody Libraries (HuCAL) antibody phage display platform, introducing completely new versatility. The SpyTag2 is genetically fused to the C-terminus of the recombinant Fab heavy chain (Figure 2b). SpyCatchers have been created with site-specific conjugated labels such as HRP or biotin. Further variants have been designed, including the BiCatcher, where two SpyCatchers are genetically linked to allow formation of bivalent Fab2, and the FcCatcher, where SpyCatcher is fused to an immunoglobulin Fc domain to make a full length Ig-like molecule after the reaction with two SpyTag Fabs (Figure 3). A Fab antibody with a SpyTag forms a covalent isopeptide bond to the chosen Catcher, enabling conjugation or fast conversion to bivalent Fab or a full length immunoglobulin-like molecule within just one hour (Figures 4 and 5).

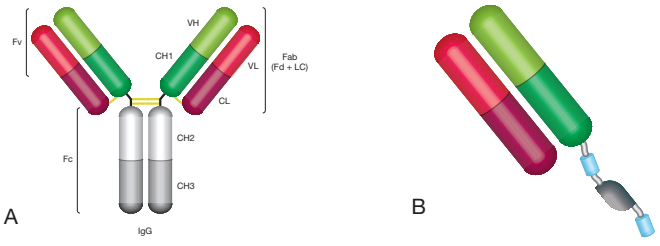


Fig. 2. Schematic images of A, full length immunoglobulin; B, monovalent Fab format with SpyTag (gray) and purification and detection tags (blue).

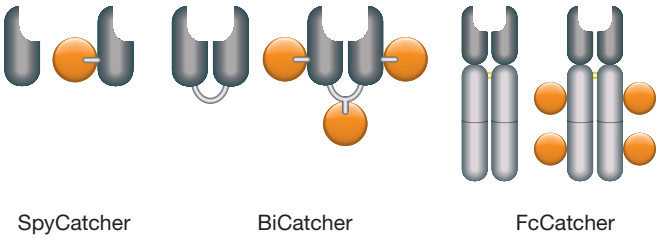


Fig. 3. Schematic images of different Catchers (gray) unlabeled (left) and conjugated (orange circle, right). SpyCatcher and BiCatcher have been modified to include a cysteine residue that enables site-specific conjugation with a known degree of labeling (DOL); FcCatcher is conjugated via primary amines and the DOL measured.

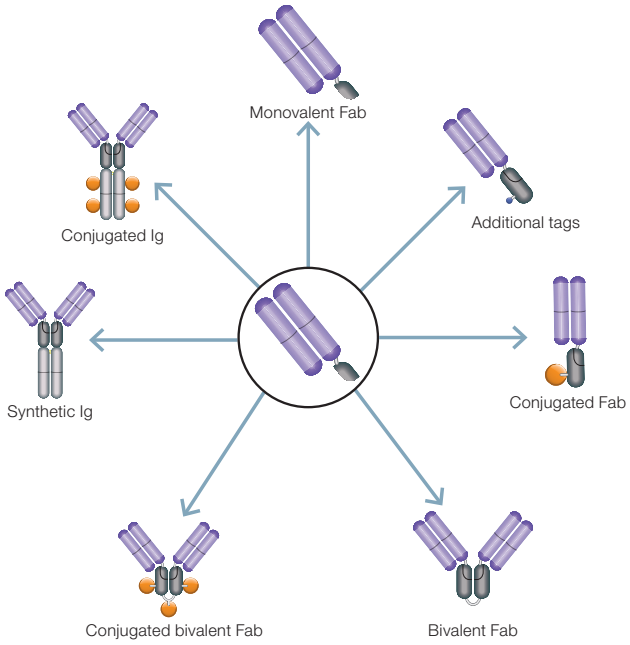


Fig. 4. A monovalent Fab with SpyTag (center) can be converted to multiple different formats via a formation of a covalent isopeptide bond with the chosen Catcher.

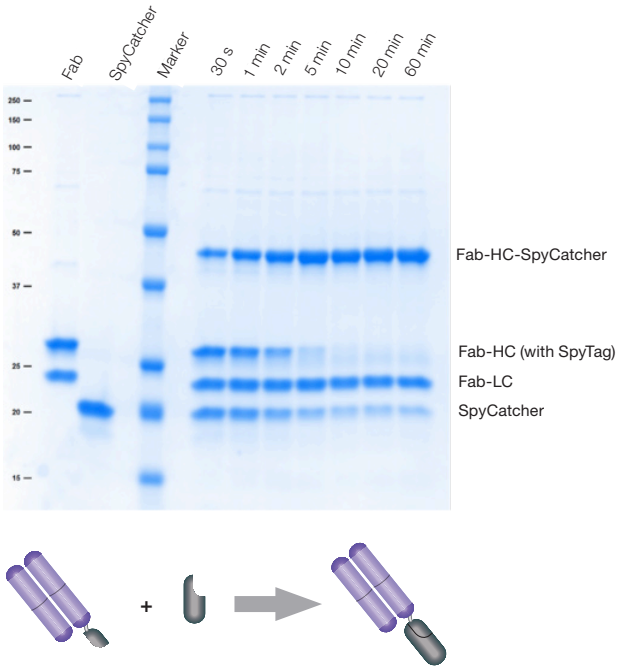


Fig. 5. Fab-SpyTag2 and SpyCatcher2 coupling reaction. In lane 1, both the Fab heavy chain (HC) and light chain (LC) are visible as they are not covalently linked; 30 sec after mixing the Fab-SpyTag2 and SpyCatcher2 the formation of the coupled Fab-HC-SpyCatcher2 can be visualized, and is seen to increase over the time course of one hr; the amount of individual Fab-HC-SpyTag2 and SpyCatcher2 is seen to decrease over the same time period. Coupling at room temperature, ratio Fab:Catcher = 1:1.25, 4 µM Fab-SpyTag2, 5 µM SpyCatcher2, Bio-Rad AnyKD gel, non-reducing conditions.

The Benefits of Site-Directed Conjugation

Several applications require or benefit from directly conjugated antibodies, such as a biotinylated or HRP conjugated detection antibody for a sandwich ELISA. Antibody conjugation protocols can be complex and time consuming, and conjugation is highly antibody specific. The conventional method is based on the random reaction of a label with the primary amines of the antibody. This process can result in some of the antibodies carrying a label at the antibody-antigen binding site, preventing them from binding to the antigen, decreasing the overall functionality of the antibody, and resulting in a sub-optimal assay. The degree of labeling follows a distribution curve that not only means each antibody carries a varying number of labels at different positions, but also that a new batch is likely to vary in the degree of labeling from the last. Site-directed conjugation through SpyTag-SpyCatcher coupling avoids these drawbacks as the antibody is labeled by coupling the already-conjugated SpyCatcher to the SpyTag located at the C-terminus of the heavy chain.

We have modified the SpyCatcher2 and BiCatcher2 by adding a cysteine residue, which facilitates site-specific conjugation, and results in a fixed degree of labeling (DOL). The result is an antibody with a fixed number of labels at a defined position. The labeling of an antibody simply requires mixing the antibody and conjugated Catcher together and leaving them at room temperature for one hour. Table 1 shows a list of currently available Spy- and BiCatchers.

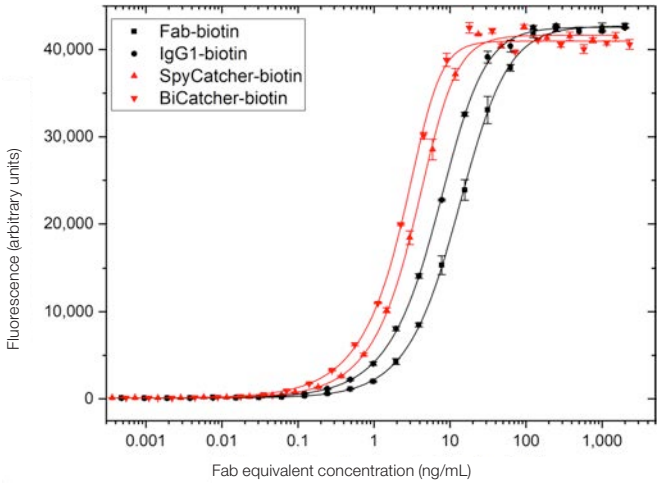


Fig. 6. Site-specific conjugation to biotin leads to better sensitivity. Fab antibody coupled to BiCatcher2-Biotin or SpyCatcher2-Biotin (red) perform better than the same antibody clone in Fab or IgG format (black) when chemically conjugated to biotin. A microtiter plate was coated overnight with adalimumab at a concentration of 1 µg/ml. Four different antibody variants were biotinylated: human anti-adalimumab (clone AbD18655) was biotinylated as Fab and as IgG via the Sulfo-NHS-LC-Biotin reagent; the same antibody with a SpyTag was coupled to biotinylated SpyCatcher2 or biotinylated BiCatcher2. After washing and blocking with PBST + 5% milk, the biotinylated antibodies were titrated to the given concentrations in PBST + 0.5% milk. Detection was performed using Neutravidin-HRP in HISPEC Assay Diluent, and QuantaBlu Fluorogenic Peroxidase Substrate. Data points are shown as the mean of three measurements.

Table 1. Catchers for coupling to recombinant Fab antibodies with a SpyTag. SpyCatcher and BiCatcher are expressed in *E. coli*, FcCatcher is expressed in a mammalian cell line; additional variants are in development, please inquire.

Monovalent	**Ig-like**
SpyCatcher2-HRP	Human
SpyCatcher2-Biotin	IgG1-FcSpyCatcher3
SpyCatcher2-Cys	IgG1-FcSpyCatcher3-HRP
SpyCatcher2-Flag3	IgG1-FcSpyCatcher3-Biotin
	IgG2-FcSpyCatcher3
Bivalent	IgG3-FcSpyCatcher3
BiCatcher2	IgG4-FcSpyCatcher3
BiCatcher2-HRP	IgG4-Pro-FcSpyCatcher3
BiCatcher2-Biotin	
BiCatcher2-Cys	Mouse IgG2a-FcSpyCatcher3
BiCatcher2-Flag3-His	Rabbit IgG-FcSpyCatcher3
BiCatcher2-PE	

Rapid Antibody Assembly into Different Formats

Recombinant antibodies can be converted to different formats by sub-cloning the gene fragments encoding the antibody variable domains into expression vectors containing the desired elements such as IgG constant domains, followed by expression and purification. This method is well established but can take several weeks to achieve. Using the simple SpyTag-SpyCatcher coupling reaction, monovalent Fab fragments can be converted into different antibody formats using BiCatchers and FcCatchers in less than an hour (Figures 3 and 5).

One antibody can be used in alternative formats based on suitability for the application, for instance a monovalent format for intrinsic affinity determination, and a bivalent format in a western blot to take advantage of avidity effects. The FcCatcher can be coupled to the antibody for those assays that require an Fc domain (Table 1). Mouse IgG2a-FcCatcher and rabbit IgG-FcCatcher are suitable for use with species-specific anti-Fc secondary reagents, and can also be used for multiplexing purposes by coupling different antibodies to different FcCatchers (Figure 7).

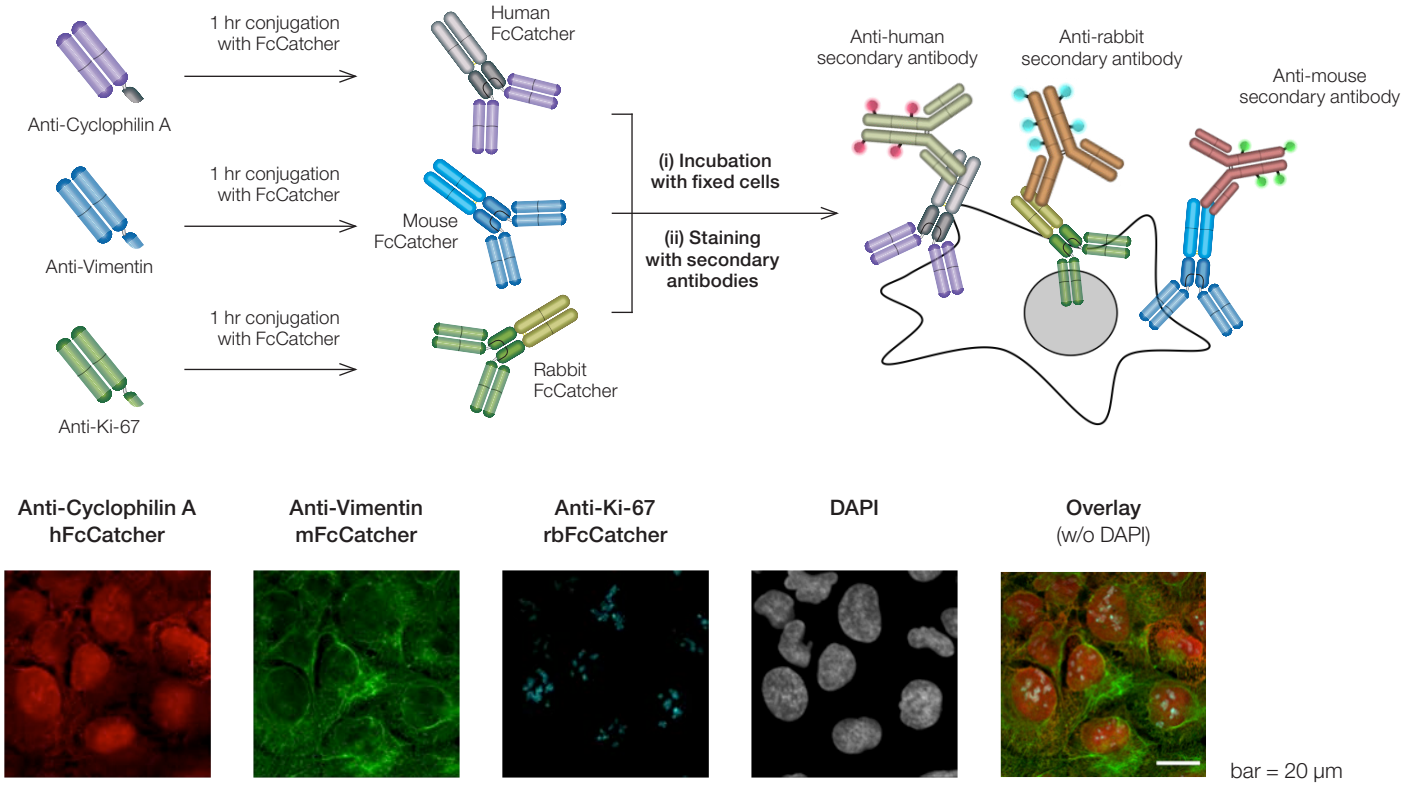


Fig. 7. Multiplexing immunofluorescence experiment. Three different Fab antibodies were coupled with three FcCatchers, simultaneously incubated with the fixed cells (U2OS) and after washing three different fluorescently labeled secondary antibodies were incubated simultaneously with the sample.

SpyTag Antibodies for Bioanalytical Method Development

Optimization of pharmacokinetic bridging ELISA

A robust pharmacokinetic (PK) assay requires a labeled detection antibody that can be produced with batch-to-batch consistency throughout the lifetime of a clinical study. To demonstrate suitability in this application, the performance of SpyTag coupled antibodies was compared with that of a full length immunoglobulin, IgG1 (Figure 8). The results were identical for the three different types of detection antibody.

Moreover, the concentration of the detection antibody coupled to BiCatcher and FcCatcher was reduced by a factor of 4 compared with the full IgG1 detection antibody, adding a reagent cost efficiency to the method.

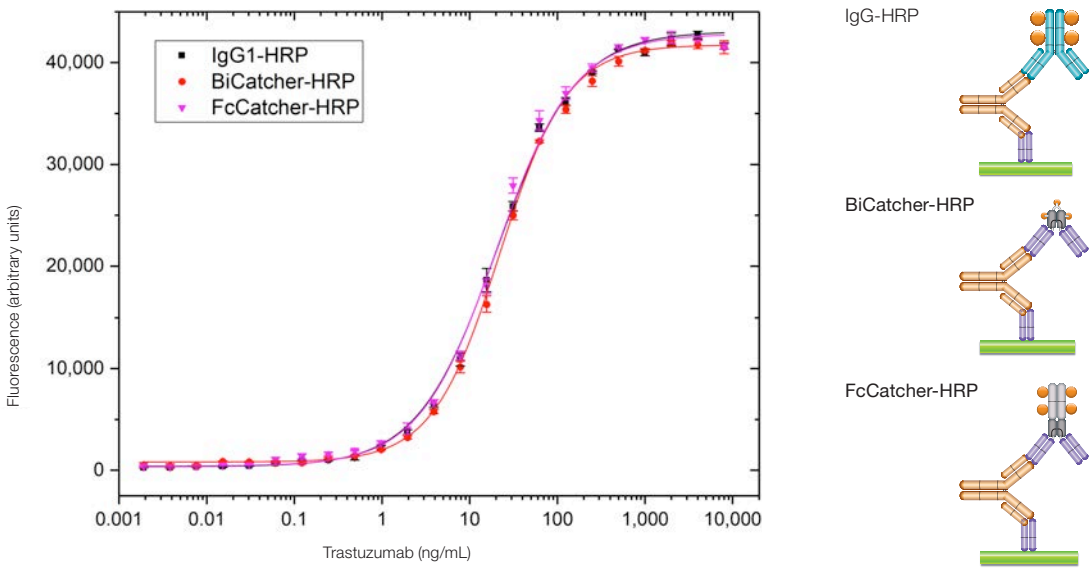
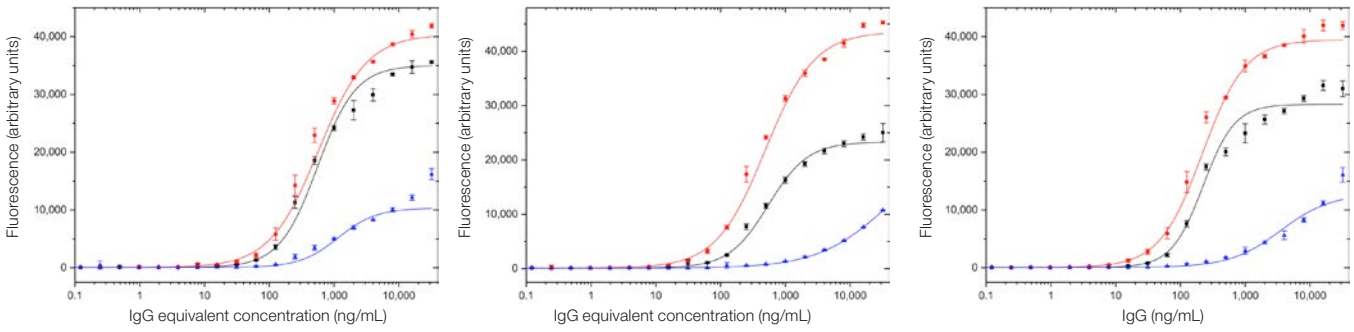


Fig. 8. Performance of SpyTag antibody formats compared with full immunoglobulin in a bridging ELISA. The three curves were generated each with the same capture antibody and the same detection antibody, the latter being prepared in different formats. A microtiter plate was coated overnight with human anti-trastuzumab antibody (clone AbD35758) at a concentration of 1 µg/ml in PBS. After washing and blocking with PBST + 5% milk, 10% human serum was added spiked with increasing concentrations of trastuzumab. Detection was performed using HRP conjugated human anti-trastuzumab, either clone AbD18018_hIgG1 (black square) or AbD18018-FSpy2H coupled to FcCatcher3-HRP (pink triangle) or BiCatcher2-HRP (red circle) at a concentration of 2 µg/ml IgG1, or 0.5 µg/ml FcCatcher and BiCatcher, in HISPEC Assay Diluent and QuantaBlu Fluorogenic Peroxidase Substrate. Data are shown as the mean of three measurements.

Quick Comparison of Performance in ADA Assay with Ig-Like Format

A set of antibodies in monovalent Fab-SpyTag format can be rapidly converted to Ig-like format using the FcCatcher for comparison as a reference standard in an anti-drug antibody (ADA) assay. This rapid modelling can help the user select the appropriate candidates for conversion to fully human IgG for the clinical assays (Figure 9). In instances where ADA isotyping is required, the FcCatchers with different human IgG isotypes offer a quick and cost effective alternative to full conversion to multiple antibody products.



A. Antibody + BiCatcher B. Antibody + FcCatcher C. Antibody in full IgG1 format

Fig. 9. Performance of high, medium, and low affinity anti-adalimumab antibodies compares well across BiCatcher, FcCatcher, and IgG formats. Antibody1, high affinity (red circle) KD 0.06 nM, antibody 2, medium affinity (black square) KD 0.2 nM, antibody 3, low affinity (blue triangle) KD 11 nM; the three different antibodies were compared in an ADA assay, coupled to **A,** BiCatcher, **B,** FcCatcher, and **C,** in full length IgG1 format.

Additional Benefits of Using Non-Animal-Derived Antibodies

Our recombinant monoclonal antibodies are selected from a synthetic, naïve antibody phage display library (HuCAL), and therefore benefit from the all the advantages of non-animal-derived antibodies, including precision, reproducibility, and flexibility.

The recently published recommendations of the European Commission Joint Research Centre (JRC) shine a spotlight on the versatility and scientific benefits of universal antibody libraries in combination with selection technologies, such as phage display, in generating precision antibodies for biomedical and scientific use (Barroso et al. 2020).

Antibodies generated through phage display libraries are developmentally, functionally, and structurally equivalent to those produced by animals, and improvements can be made to elevate quality further by refining or adapting the resulting gene sequences (Gray et al. 2016).

What Difference Could SpyTag Technology Make to Your Research Projects?

We have incorporated the option of SpyTag technology into our custom HuCAL antibody generation services. Monovalent Fabs generated from a new antibody project can be produced with a SpyTag from the outset, and existing HuCAL antibody clones can be easily converted to SpyTag format on request. A variety of Catchers is available for fast conversion from monovalent to bivalent Fab format and synthetic Ig-like construct, with and without labels. These products and services offer the user an enormous degree of flexibility to test different assay designs on different platforms, to achieve optimum results.

The degree of labeling of Catchers is measured and controlled between batches, and coupling Fab to Catcher is site-specific at a 1:1 ratio using SpyTag-SpyCatcher technology. This results in consistency of labeling between batches and avoids conjugation occurring at the active site.

Take Advantage of TrailBlazer Antibody Custom Services

- Rapid generation of highly specific Fab antibodies in as little as 8 weeks
- Isolation of antibodies against virtually any type of antigen, for instance proteins, peptides, biologics, low immunogenic antigens, and toxins
- Fast, flexible format conversion using SpyTag technology
- Site-directed conjugation with consistent DOL using SpyTag technology
- Improvement of binding affinity by molecular evolution, if needed
- Sequenced — antibody identity known, long-term secure supply guaranteed

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