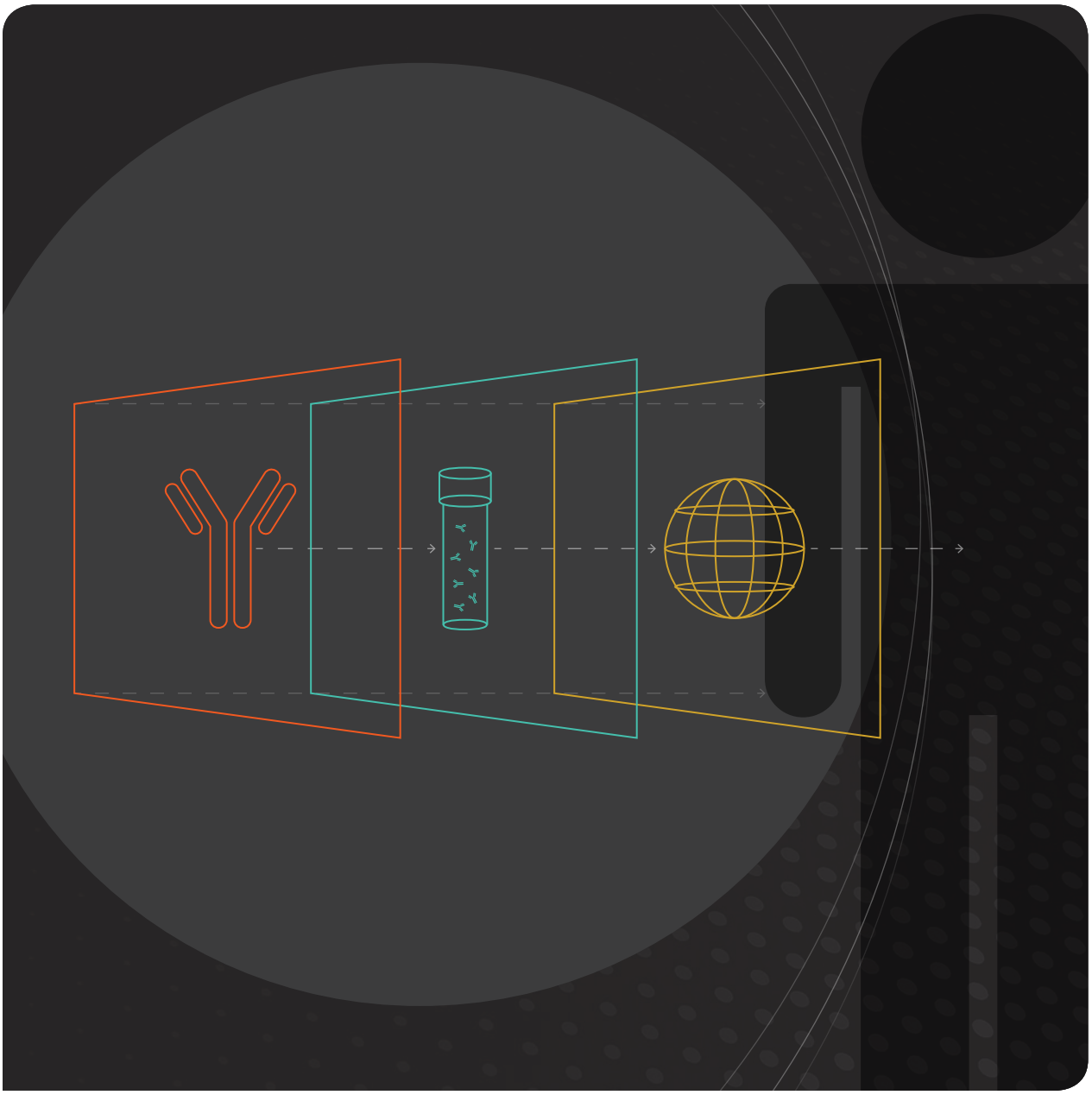


A Biosimilar Workflow: From Purification to Characterization

BIO-RAD

FLEXIBLE AND COST-EFFECTIVE

Biologics are medicines produced using living cells and highly complex manufacturing processes. As the number of biologics with expired patents increases, biosimilar medicines promise to be an attractive, cost-effective alternative. To bring a biosimilar to market, developers are required to demonstrate high similarity to the approved biological originator product, which includes evidence that demonstrates no clinically meaningful differences between the biosimilar and the reference biologic. This is provided as part of the totality of evidence reported to regulatory agencies.

We present a workflow that guides you through four of the main steps of biosimilar development: purification, impurity analysis, comparability studies, and bioanalysis. We designed a three-step, cost-effective purification strategy that includes impurity analysis for adalimumab, a HUMIRA-like biosimilar. A downstream cell-based assay was designed to assess comparability and efficacy of the biosimilar. We also developed a series of immunoassays to measure other characteristics such as immunogenicity and pharmacokinetics (PK), which are important considerations for the development of any biologic or biosimilar.

BIO-RAD'S BIOSIMILAR WORKFLOW: FROM PURIFICATION TO CHARACTERIZATION

1 PURIFICATION

STAIN-FREE GELS

NGC™ CHROMATOGRAPHY SYSTEM, CHROMATOGRAPHY COLUMNS AND RESINS

2 IMPURITIES

HOST CELL PROTEIN WORKFLOW

QX200™ DROPLET DIGITAL™ PCR SYSTEM AND ddPCR™ KITS FOR RESIDUAL DNA QUANTIFICATION

3 COMPARABILITY

ZE5™ CELL ANALYZER

S3e™ CELL SORTER

4 CHARACTERIZATION

HuCAL® CUSTOM ANTIBODY TECHNOLOGY

BIOANALYTICAL ANTIBODIES

PURIFICATION

FLEXIBLE AND COST-EFFECTIVE

NGC™ CHROMATOGRAPHY SYSTEM

RESINS

PLATES

STAIN-FREE GELS

A challenge for the biosimilar developer is to produce a product at a cost lower than that of the original biologic. Identifying the best purification strategy and migrating it from lab to process scale requires a reliable stream of workflow components from instruments to resins and columns. Costs must be controlled by designing a purification strategy with the least number of steps possible while retaining the purity, efficacy, and structure of the biosimilar.

The NGC Chromatography System along with Bio-Rad chromatography resins enable fast and easy screening of methods and components when designing a purification strategy for biosimilars. The modular NGC System allows addition of new capabilities as you move from discovery to scale up. Software tools for scouting and multidimensional (Multi-D) chromatography automate both discovery and scale-up processes and improve reproducibility.

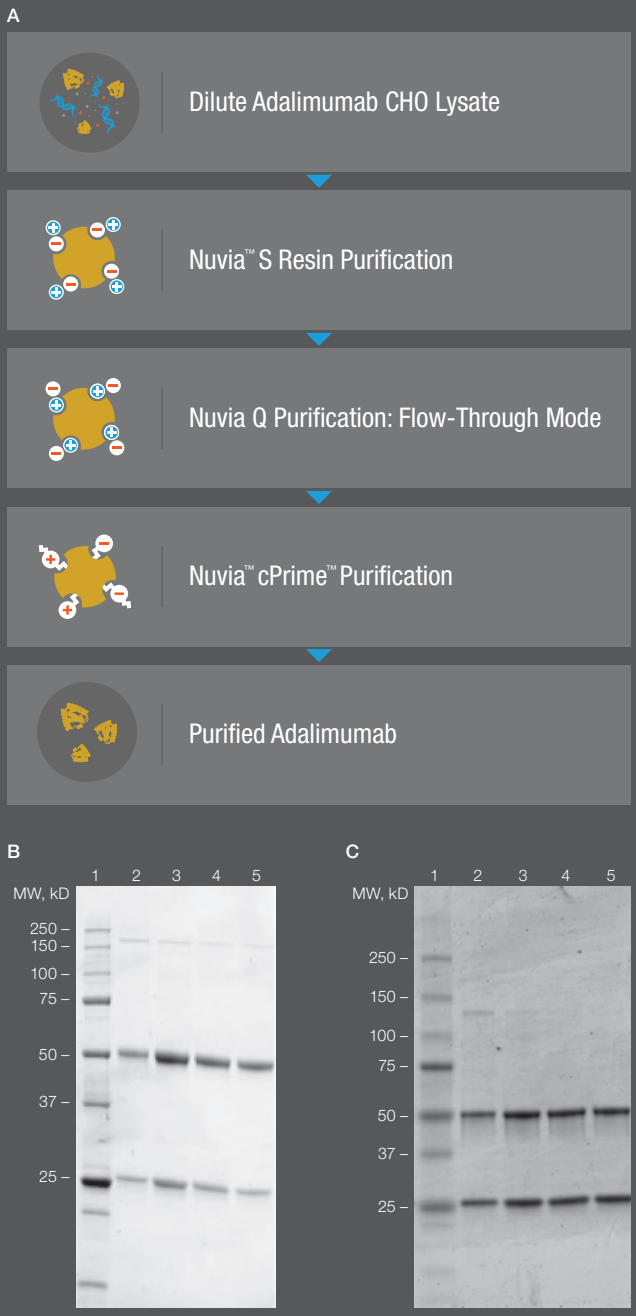
Bio-Rad chromatography resins can be utilized in a variety of applications, including size exclusion, ion exchange, HIC, mixed-mode, and affinity chromatography. Resins are available in multiple formats, including plates, columns, and in bulk and are designed to facilitate small- to commercial-scale purifications.

We describe a novel three-step workflow that uses the NGC System to purify adalimumab. This workflow reduces the costs associated with employing a Protein A capture strategy.

SUCCESS STORY Lončar et al. describe single-step downstream processing of an important industrial enzyme by using the mixed-mode resin Nuvia™ cPrime™. This resin combines hydrophobic interactions with cation exchange groups. These interactions can happen either individually or simultaneously, leading to enhanced selectivity.

Lončar N et al. (2015). Mixed-mode resins: taking shortcut in downstream processing of raw-starch digesting α-amylases. Sci Rep 5, 15772.

RAPID PROCESS DEVELOPMENT FOR BIOSIMILAR PURIFICATION



Rapid development of a manufacturing process for adalimumab. Screening of resins for downstream purification was carried out using 96-well filter plates containing multiple resins. The final optimized process contained three chromatography steps: **A**, capture with Nuvia S Cation Exchange Resin, intermediate purification with Nuvia Q Anion Exchange Resin, and polishing with Nuvia cPrime Mixed-Mode Media. Adalimumab was harvested from Chinese hamster ovary (CHO) cells and the lysate was adjusted to pH 4.5 with 1 M hydrochloric acid (HCl). This was then applied to Bio-Rad Nuvia S Resin equilibrated with 25 mM sodium acetate (pH 4.5), 150 mM NaCl (buffer A). The resin was washed several times with buffer A and adalimumab was eluted with 25 mM sodium acetate (pH 5.0). Eluted adalimumab was concentrated and the buffer was exchanged into 25 mM HEPES (pH 8.25), 5 mM NaCl (buffer B) using an Amicon Ultra-4 Centrifugal Filter Unit with a 50 kD molecular weight cutoff. Bio-Rad Nuvia Q Resin, equilibrated in buffer B, was used to purify adalimumab in flow-through mode. Resulting adalimumab was concentrated and buffer exchanged into 25 mM sodium acetate (pH 5.0), 250 mM NaCl (buffer C) and applied to Bio-Rad Nuvia cPrime Media equilibrated with buffer C. The media was washed several times before adalimumab was eluted with 25 mM Tris (pH 8.0), 800 mM NaCl. **B**, resulting protein was analyzed by native gel electrophoresis. **C**, this plate method was scaled up tenfold to 1 ml columns and showed comparable results by native gel. Lane 1, Precision Plus Protein™ Unstained Protein Standard; Lane 2, CHO supernatant; Lane 3, Nuvia S Resin eluate; Lane 4, Nuvia Q Resin eluate; Lane 5, Nuvia cPrime Media eluate.

IMPURITIES

VALIDATED ABSOLUTE QUANTIFICATION

ChemiDoc™ MP
IMAGING SYSTEM

TRANS-BLOT® TURBO™
TRANSFER SYSTEM

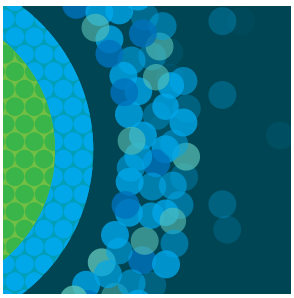
QX200™ DROPLET DIGITAL™
PCR SYSTEM

ddPCR™ SUPERMIX FOR RESIDUAL
DNA QUANTIFICATION

Expression systems used to generate biosimilars contain many components that can compromise the safety and efficacy of a biopharmaceutical, even when present at low levels in the final product. Contaminants such as host cell proteins (HCPs) and host cell DNA (hcDNA) can be reduced to safe levels by employing a robust purification strategy. To meet regulatory requirements, drug manufacturers employ sensitive and reliable detection methods.

Antibodies are the most commonly used tool for detecting and assessing HCPs because they can be used for identification, detection, and quantification. Evaluation and validation of anti-HCP antibodies are streamlined by Bio-Rad's HCP validation workflow, which provides fast, precise, and cost-effective evaluation of antibodies. Highly sensitive, reliable detection on western blots and subsequent analysis are provided in a workflow that takes less than two days.

Quantifying residual hcDNA by Droplet Digital PCR (ddPCR) provides the most sensitive absolute quantification. Traditionally, qPCR has been used for hcDNA quantification, but this technique can be susceptible to PCR inhibitors found in complex matrices, such as biological material, and thus often requires a DNA extraction step. Bio-Rad's ddPCR technology eliminates the need for DNA extraction and allows direct quantification of residual DNA from multiple species without compromising accuracy, sensitivity, or precision.



SUCCESS STORY Hussain et al. developed a Droplet Digital PCR based method for hcDNA detection that increases sensitivity and the linear range of determination while retaining high precision and accuracy. This method simplifies residual hcDNA quantification by eliminating DNA extraction, protease digestion, and the need for DNA standards.

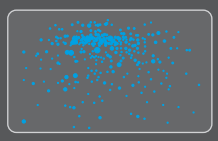
Hussain M et al. (2016). A direct Droplet Digital PCR method for quantification of residual DNA in protein drugs produced in yeast cells. J Pharm Biomed Anal 10, 128–131.

MONITORING HOST CELL PROTEIN AND HOST CELL DNA

A

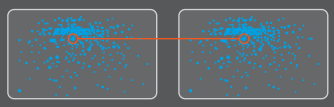
HCP VALIDATION WORKFLOW

16 HOURS — PREPARE SAMPLE



6.5 HOURS — PERFORM 2-D ELECTROPHORESIS

10 MINUTES — TRANSFER PROTEIN



4 HOURS — IMAGE BLOT AND ANALYZE RESULTS

HCP ELISA (3 HOURS)

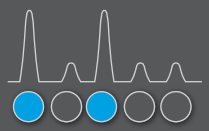
C

hcDNA WORKFLOW

30 MINUTES — PREPARE ddPCR REACTION MIX

30 MINUTES — GENERATE DROPLETS

2 HOURS — PERFORM PCR WITH EvaGreen® OR HYDROLYSIS PROBES



2.5 HOURS — READ DROPLETS AND ANALYZE RESULTS

B

	Overall HCP Reduction, %	
	Plate Method	Scale-Up
Raw feedstock	—	—
After resin 1	93.91	90.56
After resin 2	99.93	99.65
After resin 3	100.00	99.99

D

Host Cell dsDNA, pg per mg of adalimumab	
Raw feedstock	2.84×10^{11}
After resin 1	2.63×10^{7}
After resin 2	12.6
After resin 3	5.3

HCP and hcDNA analysis. HCP analysis was performed prior to, during, and after three-step purification using an HCP Detection ELISA Kit (Cygnus Technologies) (**A**). Details of our HCP validation workflow can be found in Bio-Rad bulletin 6493. In both the plate-based purification and the scale-up column purification more than 90% of HCPs were removed in step 1 of the purification. Up to 99% reduction was found after step 2 and 99.99% after step 3 in the final purified product, with 6 ppm HCP for the plate purification and 5 ppm HCP for the column scale-up. (**B**). To assess residual hcDNA, eluate at each stage of our three-step adalimumab purification protocol was analyzed using the ddPCR Host Cell Residual DNA Quantitation Kit (**C**). Residual hcDNA was reduced by almost 100% by using the three-step purification strategy (**D**).

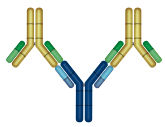
COMPARABILITY

CELL-BASED CONFIDENCE

ZE5™ CELL ANALYZER

S3e™ CELL SORTER

ZOE™ CELL IMAGER



ANTIBODIES

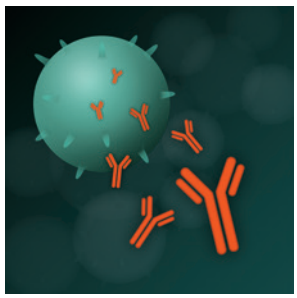
Biosimilars must show comparability to their originator biologic across multiple factors, including structure, function, and efficacy. Designing a cell-based assay to measure comparability requires an understanding of the underlying mechanism of action of the originator and the biosimilar. The data that are produced must be precise, accurate, and reproducible in order to satisfy regulatory requirements for comparability studies.

Cell-based assays are valuable at this stage as they provide multifaceted data that can inform downstream preclinical and clinical phases of development. The ZE5 Cell Analyzer supports this by enabling analysis of up to 30 parameters from a single sample, greatly expanding the number of biomarkers that can be monitored by a given cell-based assay. This is important when gathering data to provide as part of the totality of evidence required by regulatory agencies. The ZE5 Cell Analyzer is also well-suited for measuring rare or transient cell populations due to its fast analysis speed.

If a particular cell type is required for cell-based assays, then the benchtop S3e Cell Sorter allows sorting of those populations at your bench, without the need to schedule time at a core facility.

The ZOE Fluorescent Cell Imager lets you easily image cells at your bench, allowing you to monitor the health and activity of your cells throughout your experiment.

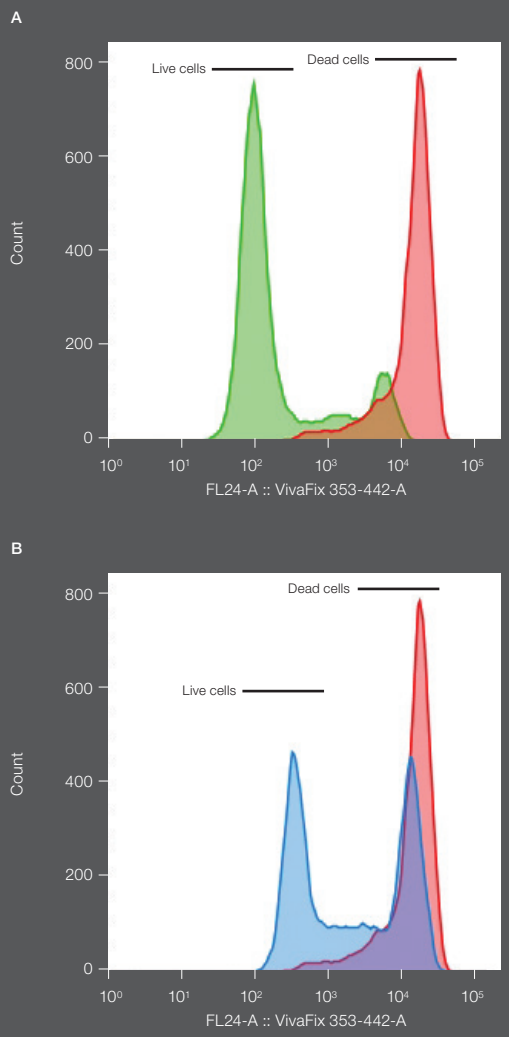
We designed a cell-based assay that uses the ZE5 Cell Analyzer to monitor the activity of the purified HUMIRA biosimilar adalimumab.



SUCCESS STORY The S3e Cell Analyzer was used to sort fluorescently labeled transfected cells prior to seeding and development into a 3-D cell culture early lung model. This lung model may be used to test the effectiveness of potential treatments, such as chemotherapy and small and large molecule drugs, against lung disease.

Chen YW et al. (2017). A three-dimensional model of human lung development and disease from pluripotent stem cells. Nat Cell Biol 19, 542–549.

CELL-BASED ASSAY VALIDATION OF IN VITRO BIOSIMILAR ACTIVITY

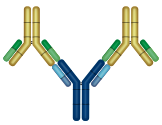


Measuring live vs. dead cells using the VivaFix™ Cell Viability Assay. A, comparison of cells treated with TNF-α only (■) and cells treated with TNF-α and the anti-TNF-α antibody adalimumab purified from lysate (■). **B**, comparison of cells treated with TNF-α only (■) and cells treated with TNF-α and control anti-TNF-α antibody (purchased control adalimumab) (■). Both adalimumab biosimilars show a protective effect on cells after treatment with TNF-α. To carry out these experiments, murine L929 cells were plated on growth media (MEM, 2 mM glutamine, 10% FBS) at 37°C, 5% CO_2. Anti-TNF-α antibody (either control adalimumab or purified adalimumab) was prepared in serum-free media and added to cells at a final concentration of 300 ng/ml. Following a 2 hour incubation with the antibody, TNF-α was added at a final concentration of 5 ng/ml and the cells were further incubated for 36 hours at 37°C, 5% CO_2. VivaFix Viability Assays fluoresce more brightly in dead cells than in live cells. In live cells they bind only to cell surface primary amines. In dead cells the compromised cell membrane allows additional binding to intracellular primary amines. Cells were stained using VivaFix Viability Assays according to manufacturer's instructions. Treated cells were washed with PBS, treated with trypsin, neutralized, and cell suspensions were added to microcentrifuge tubes. Cells were counted, spun at 300 rcf, and then resuspended in PBS. VivaFix 353/442 Viability Assay was reconstituted in DMSO and added to each treated sample. One sample was left untreated and unstained for instrument setup. Samples were incubated at RT in the dark before washing in 1 ml PBS. Samples were resuspended in PBS at 2 x 10^6 cells per ml. Cell viability was assessed using Bio-Rad VivaFix 353/442 Cell Viability Assay and the ZE5 Cell Analyzer. VivaFix Dye was excited with the 355 nm laser and detected in the 447/60 nm channel. Data were analyzed using FlowJo 10 Software. 5 ng/ml TNF-α (■); 5 ng/ml TNF-α + 300 ng/ml purified adalimumab (■); 5 ng/ml TNF-α + 300 ng/ml control adalimumab (■).

CHARACTERIZATION

CUSTOMIZED AND SPECIFIC

HuCAL® ANTIBODY GENERATION TECHNOLOGY



BIOANALYTICAL ANTIBODIES

As part of the drug development program, the biosimilar is compared to the reference product to ensure that there are no clinically meaningful differences in terms of safety, efficacy, and immunogenicity, including the titre and reactivity of anti-drug antibodies (ADA) produced in vivo. The results form part of the totality of evidence required by regulatory agencies. These data must be robust; therefore the reagents used must meet high standards.

Bio-Rad's antibiotherapeutic antibody reagents for ADA and PK assay development are selected in vitro from the synthetic Human Combinatorial Antibody Libraries (HuCAL). Guided selection strategies enable the generation of fully human, highly specific inhibitory and noninhibitory anti-idiotypic antibodies and specialized drug-target complex binders. These different types of antibodies enable development of PK assays to detect free or total drug, or drug bound to its target. Additionally, the inhibitory antibodies can be used as reference standards in an ADA assay, replacing the need to source antibodies raised in animals.

Bio-Rad provides an expanding portfolio of antibodies against marketed monoclonal antibody drugs and a custom service for additional specificities. Our QC process incorporates several relevant published best practices, helping you with critical reagent life cycle management and characterization and the maintenance of optimal assay performance over time.

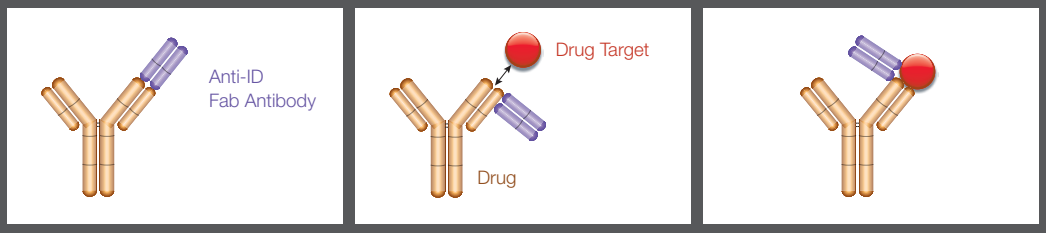
Here we designed a series of ELISA experiments to show the precision of our antibodies in PK and ADA assays.

SUCCESS STORY Bio-Rad's Characterization of Critical Reagents for Ligand Binding Assays article provides recommendations and best practices used by bioanalytical laboratories in the development of ligand binding assays for the successful characterization of critical reagents in drug development and manufacturing.

Visit **bio-rad.com/biosimilar1** to learn more.

PK AND ADA BIOANALYSIS FOR BIOSIMILAR DEVELOPMENT

A.



TYPE 1

- Anti-idiotypic (Anti-ID) antibody
- Paratope-specific
- Inhibitory
- Neutralizing
- Detects free drug

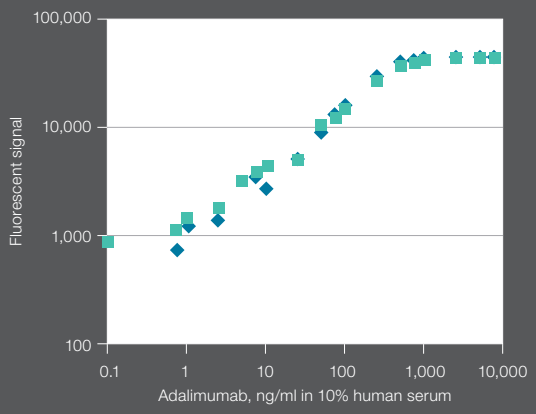
TYPE 2

- Anti-idiotypic antibody
- Paratope-specific
- Not inhibitory
- Detects total drug (free, partially bound, fully bound

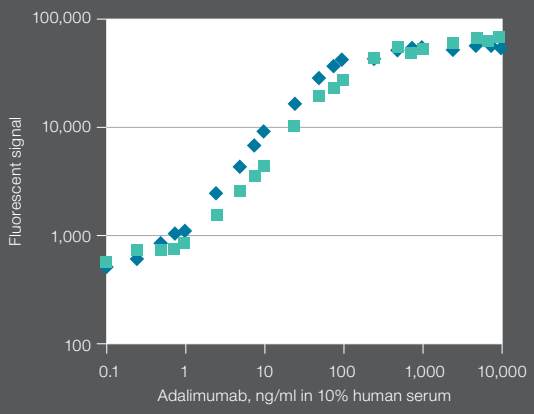
TYPE 3

- Drug-target complex specific
- Not inhibitory
- Detects bound drug exclusively

B. Capture ELISA



C. Bridging ELISA



Multiple PK assay orientations for the biosimilar adalimumab using highly specific anti-idiotypic antibodies. A, three main types of antibody bioassay orientation are shown. **B**, data from PK Antigen Capture ELISA. A 96-well plate was coated with 100 µl of 5 µg/ml TNF-α overnight at 4°C and washed five times with PBST. Each well was blocked with 300 µl 5% BSA in PBST for 1 hour at room temperature (RT). The plate was washed five times with PBST and 100 µl of each sample was added in triplicate. The plate was incubated for 1 hour at RT followed by washing with PBST. Anti-adalimumab conjugated with HRP (catalog #HCA232P; clone AbD20349_hIgG1) was diluted to 2 µg/ml in HISPEC buffer (catalog #BUF049) and 100 µl of the detection antibody was added to each well. The plate was incubated at RT for 1 hour before being washed with PBST. One hundred microliters of QuantaBlu (Thermo Fisher Scientific) was added to each well and the fluorescence was measured after 30 minutes. The concentration of purified adalimumab was calculated from a standard curve of adalimumab prepared in 10% human serum in PBST. **C**, data from PK bridging ELISA. The anti-adalimumab capture antibody (catalog #HCA202; clone AbD18654) was diluted to 1 µg/ml in PBS. One hundred microliters of the antibody was added to each well of a 96-well plate and incubated overnight at 4°C. The plate was washed with PBST followed by blocking with 300 µl 5% BSA in PBST. The plate was incubated at RT for 1 hour before washing with PBST. One hundred microliters of each sample were added in triplicate and incubated for 1 hour at RT. The plate was washed with PBST. The HRP conjugated detection antibody (catalog #HCA204P; clone AbD18655_hIgG1) was diluted to 2 µg/ml in HISPEC buffer and 100 µl of the antibody was added to each well. The plate was incubated at RT 1 hour and then washed with PBST. QuantaBlu (100 µl) was added to each well and fluorescence was measured after 30 minutes. The concentration of purified adalimumab was calculated from a standard curve of adalimumab prepared in 10% human serum in PBST. purified adalimumab (■); adalimumab control (◆).

WHY BIO-RAD?

Bio-Rad has over 60 years of experience supporting life science research and clinical diagnostics. We are the leader in digital PCR, transfection, and in gene and protein expression analysis technologies. Our innovative products are available as stand-alone solutions or combined into workflows that are optimized to minimize downtime and to provide the right answer the first time, every time. The Biosimilar Workflow is only one of many Bio-Rad workflows designed to shorten discovery and development time lines for therapeutics.

As part of our commitment to your research, we provide:

- Products and reagents that are reliable and validated
- Responsive, trusted global service and technical support teams
- Technical expertise across multiple workflows and applications

	Purification	Impurities	Comparability	Characterization
Cells				
S3e Cell Sorter			•	•
ZOE Fluorescent Cell Imager			•	•
ZE5 Cell Analyzer			•	•
TC20™ Automated Cell Counter			•	•
Antibodies for Flow Cytometry and Immunohistochemistry			•	•
Genes				
QX200 Droplet Digital PCR System		•	•	•
C1000 Touch™ Thermal Cycler		•		
PrimePCR™ ddPCR Assays Residual DNA		•		•
PrimePCR ddPCR Assays			•	•
CFX384 Touch™ Real-Time PCR Detection System			•	•
PrimePCR Assays			•	•
qPCR Reagents and Consumables			•	•
Proteins				
Bio-Plex® Multiplex Systems and Immunoassays			•	•
NGC Chromatography Systems	•			
Chromatography Resins	•			
Stain-Free Mini and Midi Protein Gels	•		•	•
Trans-Blot Turbo Transfer System	•		•	•
ChemiDoc MP Touch Imaging System	•		•	•
PrecisionAb™ Validated Western Blotting Antibodies	•		•	•
ReadyPrep™ 2-D Cleanup Kit		•		
ReadyPrep 2-D Starter Kit		•		
PROTEAN® i12™ IEF Systems and Consumables		•		
Criterion™ Dodeca™ Cell		•		
Criterion™ TGX™ Precast Gels		•		
SYPRO Ruby Protein Gel Stain		•		
HuCAL Generated Custom Antibodies		•	•	•
Anti-idiotypic Antibodies			•	•

Visit **bio-rad.com/biosimilarinfo** to learn more about our solutions to support your biosimilar research and development.

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TGX Stain-Free Precast Gels are covered by U.S. Patent Numbers 7,569,130 and 8,007,646. Bio-Rad Laboratories, Inc. is licensed by Life Technologies Corporation to sell SYPRO products for research use only under U.S. Patent Number 5,616,502.

Bio-Rad's thermal cyclers and real-time thermal cyclers are covered by one or more of the following U.S. patents or their foreign counterparts owned by Eppendorf AG: U.S. Patent Numbers 6,767,512 and 7,074,367.

Precision Plus Protein Standards are sold under license from Life Technologies Corporation, Carlsbad, CA for use only by the buyer of the product. The buyer is not authorized to sell or resell this product or its components.

The QX200 Droplet Digital PCR System is covered by claims of U.S. patents, and/or pending U.S. and non-U.S. patent applications owned by or under license to Bio-Rad Laboratories, Inc. Purchase of the product includes a limited, non-transferable right under such intellectual property for use of the product for internal research purposes only. No rights are granted for diagnostic uses. No rights are granted for use of the product for commercial applications of any kind, including but not limited to manufacturing, quality control, or commercial services, such as contract services or fee for services. Information concerning a license for such uses can be obtained from Bio-Rad Laboratories. It is the responsibility of the purchaser/end user to acquire any additional intellectual property rights that may be required.

The Bio-Plex Suspension Array System includes fluorescently labeled microspheres and instrumentation licensed to Bio-Rad Laboratories, Inc. by the Luminex Corporation.

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Life Science Group

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Bulletin 7038 Ver A US/EG *17-0979 0218 Sig 0118*