From Discovery to Manufacturing: A Workflow for Protein-Based Immunotherapies

BIO-RAD

INTRODUCTION TO IMMUNOTHERAPY

Every year, 14 million people receive a new cancer diagnosis. Though progress has been made in detection and treatment, common cancers such as lung, breast, colorectal, and prostate remain deadly. In fact, cancer is the leading cause of death worldwide, responsible for one in every seven deaths. That leads to one big question: can we improve prognoses with better science?

Immunotherapy is the next evolution of cancer treatment. Unlike radiation and chemotherapy, immunotherapy combats disease by either boosting the native immune system or introducing modified components to enhance its defense. Many new therapeutic drugs have already been given breakthrough status.

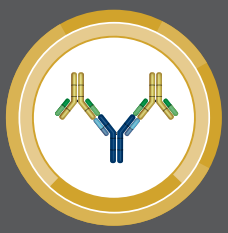
Tracking the effects of immunotherapeutics across cells, genes, and proteins is complex — numerous pathways and cell types must be accounted for. Success is often the combination of choosing appropriate tools and selecting the right biomarkers.

We have created a workflow to guide you through cellular, proteomic, and genomic analyses to better understand the effects of an immunotherapeutic at the cellular level. We also provide key tools for purifying and controlling the quality of your manufactured protein-based immunotherapy with confidence.

THE BIO-RAD IMMUNOTHERAPY WORKFLOW: DEVELOPMENT, MANUFACTURING, AND QC

1 IMMUNE CELL ANALYSIS

ZE5 CELL ANALYZER



ANTIBODIES

2 IMMUNE PROTEIN MONITORING

BIO-PLEX 200 SYSTEM AND ASSAYS

ANTIBODIES

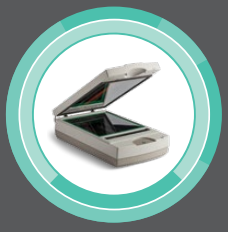
3 GENOMIC ANALYSIS AND BIOMARKER DISCOVERY

CFX OPUS REAL-TIME PCR SYSTEMS

PrimePCR ASSAYS

4 MANUFACTURING AND QC

CHROMATOGRAPHIC RESINS



GS-900 CALIBRATED DENSITOMETRY SYSTEM

CELLULAR ANALYSIS

SEE MORE THAN BEFORE

ZE5 CELL ANALYZER

S3e CELL SORTER

GENE PULSER Xcell ELECTROPORATOR

In order to fully understand the effects of an immunotherapeutic, cell-based assays should take into account as many pathways and cell types as you need. The right analytical tools support both automation and multiparameter experiments so you can be confident that you are progressing therapies that have the most potential in the clinic.

Our comprehensive, easy-to-use suite of cell development and analysis products includes the ZE5 Cell Analyzer, the S3e Cell Sorter, and a variety of other tools.

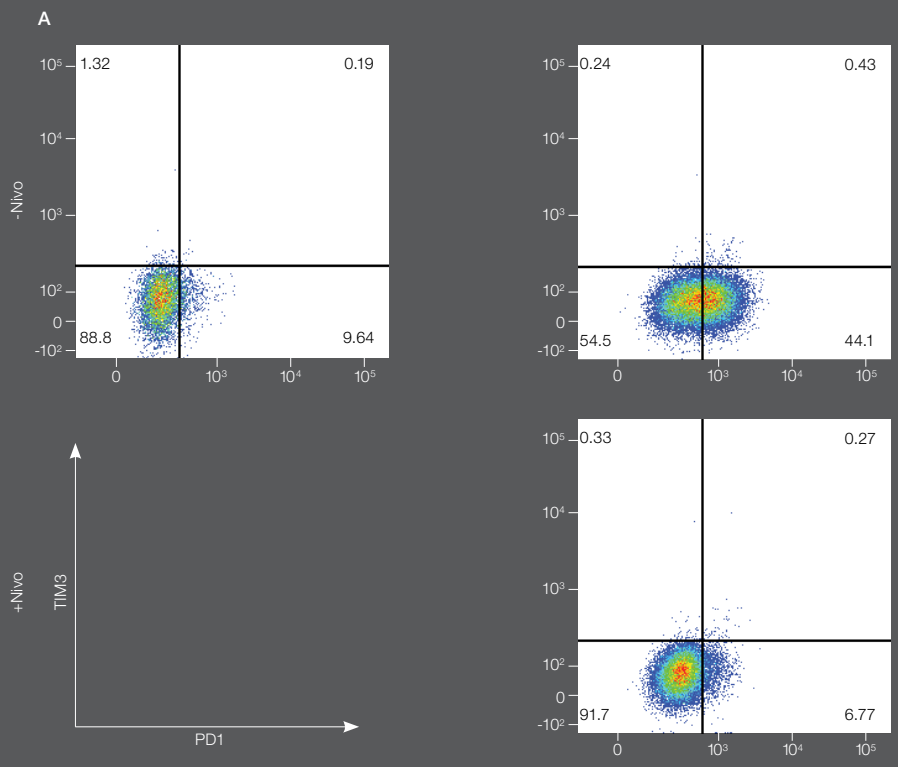
- Maximize data by using up to 30 experimental parameters simultaneously with the ZE5 Cell Analyzer
- Leverage the automation-readiness, high-throughput capabilities, and flexible sample handling of the ZE5 Cell Analyzer
- Sort cells in your own laboratory with the S3e Cell Sorter
- Create stable cell lines with the reliable Gene Pulser Xcell Transfection System

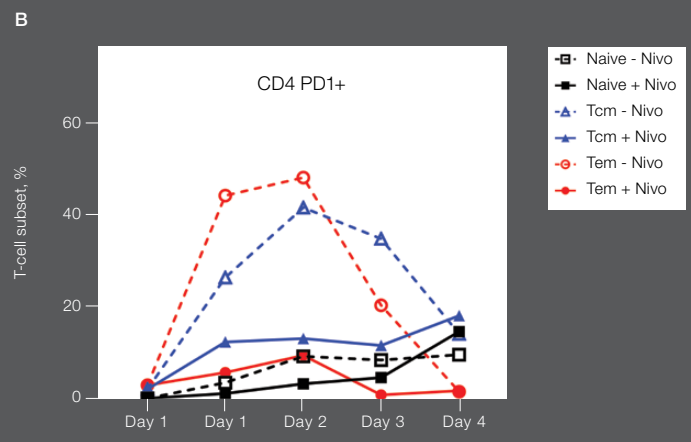
To demonstrate the capabilities of the ZE5 Cell Analyzer, we used it to take a multiparameter look at the effects of a PD1 blocker, nivolumab, against CD4 T cells in vitro across a broad range of checkpoint inhibitors over the course of 4 days.

SUCCESS STORY Reliable cellular analysis is crucial in the early developmental phases of immunotherapies. Bio-Rad **bulletin 7125 highlights the multiparameter capabilities of the ZE5 Cell Analyzer** to monitor T-cell exhaustion and phenotype as well as effects from an immunotherapy.

For more details, see **bulletin 7125**.

EXPLORING IMMUNE CELL RESPONSE





Monitoring checkpoint inhibition in T cells treated with nivolumab. Pan T cells from healthy donors (AllCells, LLC) were stimulated with Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific Inc.) and treated with or without the anti-PD1 antibody nivolumab (Selleck Chemicals), for up to 4 days. On the day of collection, cells were treated with 1x Brefeldin A (Bio-Rad) for 5 hours and Dynabeads were removed via magnet prior to staining. Surface staining was performed for 30 min in the presence of Human BD Fc Block (BD Biosciences) (**A**) followed by intracellular cytokine staining using the eBioscience Intracellular Fixation and Permeabilization Reagents (Thermo Fisher Scientific) (**B**). Cells were stained daily for various checkpoint receptors and intracellular cytokines; they were analyzed using the ZE5 Cell Analyzer and FlowJo Software. Single-stain controls were generated using AbC Total Antibody Compensation Beads (Thermo Fisher Scientific). Single-stain controls and fluorescence minus one (FMO) controls were run for each time point. CD4+ and CD8+ T-cell subsets were separated into naïve (CD45RAhiCCR7hi), TCM (CD45RAloCCR7hi), and TEM (CD45RAloCCR7lo) populations prior to analysis. A total of 16 fluorochromes were used in conjunction with the ZE5 Cell Analyzer for this panel. Data demonstrated that while nivolumab inhibited PD1 expression in this donor, there was minimal effect on other checkpoint receptors or cytokines. For further details, see **bulletin 7125**.

CYTOKINE ANALYSIS

MULTIPLEX YOUR CYTOKINE ANALYSIS

BIO-PLEX 200 SYSTEM

BIO-PLEX MULTIPLEX ASSAYS

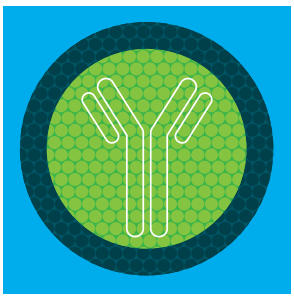
STAIN-FREE WESTERN BLOTTING

HuCAL ANTIBODY
GENERATION TECHNOLOGY

Gaining insight into the roles proteins such as cytokines play in the immune system and how they are modified by a potential immunotherapeutic drives the development of the most effective treatments. Tools that offer multiplexing, high accuracy, and specificity allow you to pursue complete answers.

- The Bio-Plex Multiplex Assays and Immunoassay Systems analyze a multitude of cytokines and other critical proteins in a single experiment, using either predesigned panels or custom assays
- Stain-Free western blotting is a validated, streamlined workflow that quantitates protein expression faster and more accurately than other western blotting procedures. With the ChemiDoc MP Imaging System, this workflow analyzes up to three proteins at once via multiplex fluorescent western blotting
- Our anti-idiotype antibody range provides off-the-shelf solutions for many key bioassays; our HuCAL Custom Antibody Technology can generate specific, custom human antibodies in 8 weeks for a variety of applications such as flow cytometry, ELISA, and western blotting

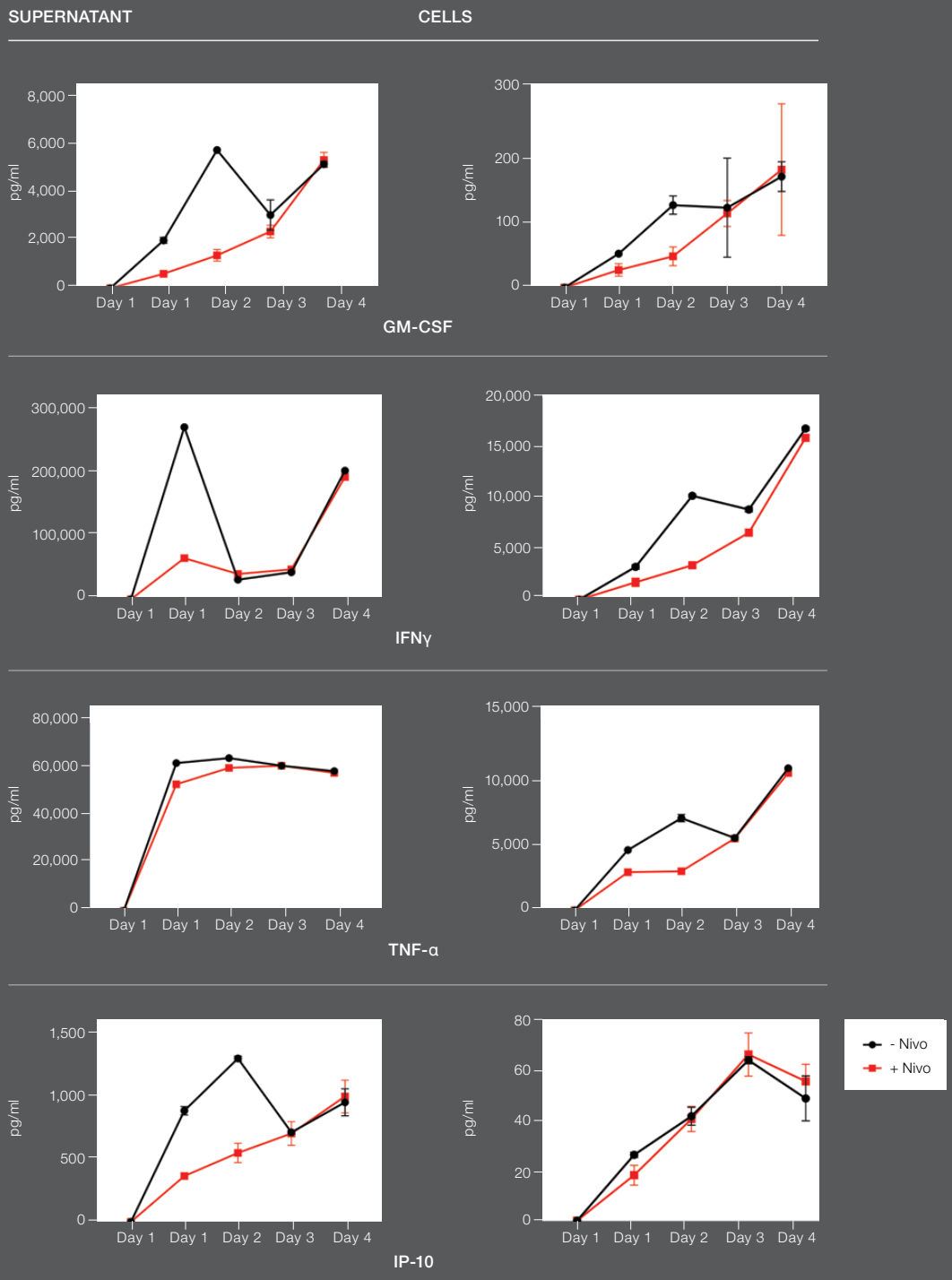
To explore the effects of nivolumab on T-cell cytokine expression, we used the Bio-Plex Multiplex Immunoassay System and were able to show that long-term cytokine expression is not always impaired by the anti-PD1 antibody.



SUCCESS STORY Multiple myeloma, considered to be incurable, may see treatment progress in the realm of immunotherapy. A study published in 2015 used Bio-Plex Cytokine Assays and System to test whether blocking checkpoint receptors in lymphodepletion could boost tumor resistance.

See Jing W et al. (2015). **Combined immune checkpoint protein blockade and low dose whole body irradiation as immunotherapy for myeloma**. J Immunother Cancer 3, 2 for more.

MULTIPLEX MEASUREMENT OF CYTOKINE EXPRESSION



Measuring cytokine expression in T cells with the Bio-Plex Immunotherapy Panel. For each respective time point, supernatants were collected prior to Brefeldin A treatment. After Brefeldin A treatment, CD3/CD28 Dynabeads were removed with a magnet and 5 x 10^4 cells were collected. Supernatants and cells were kept at –80°C until analysis was ready to be performed. Cells were thawed on ice and lysed with Bio-Plex Pro Cell Signaling Cell Lysis Buffer (Bio-Rad). Supernatants and cell lysates were analyzed for the presence of various inflammatory cytokines using the Bio-Plex Human Immunotherapy Panel (Bio-Rad). Results were visualized with GraphPad Prism. The graphs represent about a quarter of the total number of targets detected by the 20-plex kit. The data show that while T cells treated with nivolumab had reduced expression of PD1, expression of some of the inflammatory cytokines was initially impaired. However, given some time, expression of these cytokines returns to levels comparable to that of untreated T cells. Our data may explain why most cancer patients are not responsive to anti-PD1 monotherapy — even with decreased PD1 expression and, consequently, signaling, T-cell function is not necessarily increased. Thus, the data presented above are in agreement with published literature. These experiments also show that the Bio-Plex Immunotherapy Panel is capable of analyzing many targets, in different types of sample matrices and over short incubation times.

GENOMIC ANALYSIS

EXPLORING GENOMIC RESPONSE TO IMMUNOTHERAPY

QX200 DROPLET DIGITAL PCR SYSTEM

CFX FAMILY OF REAL-TIME PCR SYSTEMS

Even after development, it remains critical to understand how a patient's immune system will affect and be affected by a therapeutic drug.

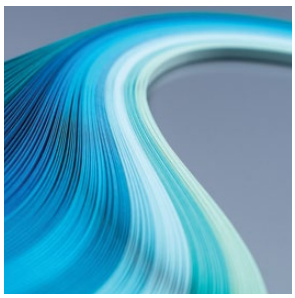
Genomic analysis tools need to provide answers in a high-throughput, robust way. The ability to explore all aspects of any genome, including long noncoding RNA (lncRNA), is key to identifying potential biomarkers.

- The family of CFX Real-Time PCR Systems, when used with custom-built or predesigned PrimePCR Assays, measures multiple genes from multiple samples at once, be they liquid biopsy, tissue, or cell-based materials
- When the CFX Automation System II* is coupled with the Reliance One-Step Multiplex RT-qPCR Supermix, 48 plates can be prepared, left at room temperature, and read automatically
- For increased sensitivity, such as is needed for the detection of rare cancer mutations, the QX200 Droplet Digital PCR (ddPCR) System detects low-abundance genetic biomarkers

Both systems come equipped with IQ/OQ workflows and tools for U.S. FDA 21 CFR Part 11 compliance.

In addition, the SEQuoia Complete Stranded RNA Library Prep Kit enables the capture of long and short ncRNA, and everything in between, in a single step, saving significant time in library preparation for next-generation sequencing. The simple workflow is also compatible with unpurified samples.

To examine both gene and lncRNA expression in Pan T cells, we designed an experiment using PrimePCR Panels for detection and the CFX Automation System II and CFX Maestro Software for analysis.

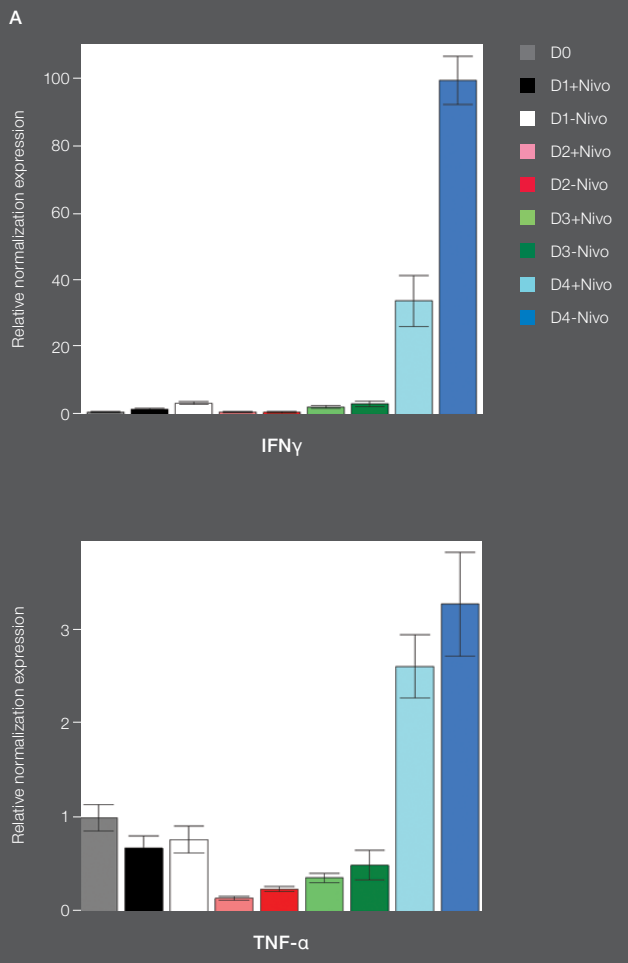


SUCCESS STORY Using PrimePCR Assays, Traci Lyons at the CU Anschutz Medical Campus and her students identified a protein that functions downstream of COX-2, promoting metastasis in breast cancer. When they knocked down the gene encoding for the protein, tumors transplanted into mice were less likely to progress from stage 0.

Visit **bio-rad.com/BreastCancer** for the story.

* The CAS II System works with the CFX Touch and CFX Connect Systems.

FINDING ANSWERS WITH qPCR AND lncRNA



Quantitative PCR (qPCR) and lncRNA analysis of patient immune response to an immunotherapeutic. Pan T cells (5 x 10^4) were collected at each time point and stored at –80°C until RNA isolation. RNA was isolated using the Aurum Total RNA Mini Kit (Bio-Rad) and quantified using the Agilent RNA 6000 Nano Kit workflow (Agilent Technologies, Inc.). Equal amounts of RNA were used to generate cDNA using the iScript Explore One-Step RT and PreAmp Kit (Bio-Rad) with genomic clearance and containing the Inflammation lncRNA PreAmp Pool (Bio-Rad) or a PreAmp Pool for the 20 cytokine targets. Each sample was diluted 1:10 in water before proceeding with lncRNA or qPCR detection. Samples for lncRNA analysis were run on the Inflammation lncRNA PrimePCR Pathway Plates (Bio-Rad) containing lncRNA targets. Each target for each sample was run in quadruplicate (qPCR) or triplicate (lncRNA). A CFX384 Touch Real-Time PCR System (Bio-Rad) was used in conjunction with the CFX Automation System II and CFX Maestro Software (Bio-Rad). Data from qPCR analysis show gene expression correlated with analysis using the Bio-Plex System — T cells treated with nivolumab showed a lower level of target RNA expression when compared to untreated T cells (**A**). Conversely, no change was observed in the majority of inflammation-related lncRNA targets following treatment with nivolumab (**B**). **Note:** not all of the 96 targets in the lnRNA PrimePCR Pathway Plates are shown. There are 85 lncRNA targets in the panel, with three reference genes and seven controls.

MANUFACTURING AND QC

FROM THE BENCH TO SCALE

QX200 DROPLET DIGITAL PCR SYSTEM

RESINS

ddPCR RESIDUAL DNA QUANTIFICATION KITS

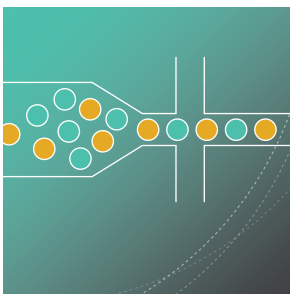
Arriving at a final therapeutic product with the desired stability, efficacy, and purity requires a robust purification strategy. For protein-based immunotherapeutics built using novel scaffolds or with modifications such as the conjugation of a chemotropic agent, identifying the best resin for the process may be more complicated due to unique binding properties.

Our chromatography resins are designed for a wide range of protein-based therapeutics, allowing you to target purity, efficacy, and stability; many of our resins also offer improved process economics. Most importantly, they're available in multiple formats, including plates, columns, and bulk, and are designed for commercial scale.

Testing purity through scale-up and into commercial processes requires trusted, compliance-friendly tools.

- The GS-900 Calibrated Densitometry System delivers protein-based purification electrophoresis data within a regulatory framework, with IQ/OQ and U.S. FDA 21 CFR Part 11–compliant software tools
- The ddPCR Residual DNA Quantification Kits provide the most sensitive, robust method for residual DNA quantification

We designed a thorough and economical mixed-mode purification strategy to purify the biosimilar rituximab. We quantified residual DNA throughout the process using Droplet Digital PCR; host cell DNA (hcDNA) was reduced to below the level of detection. We also used the GS-900 Calibrated Densitometer to validate an increase in purity from 7% to greater than 96%.

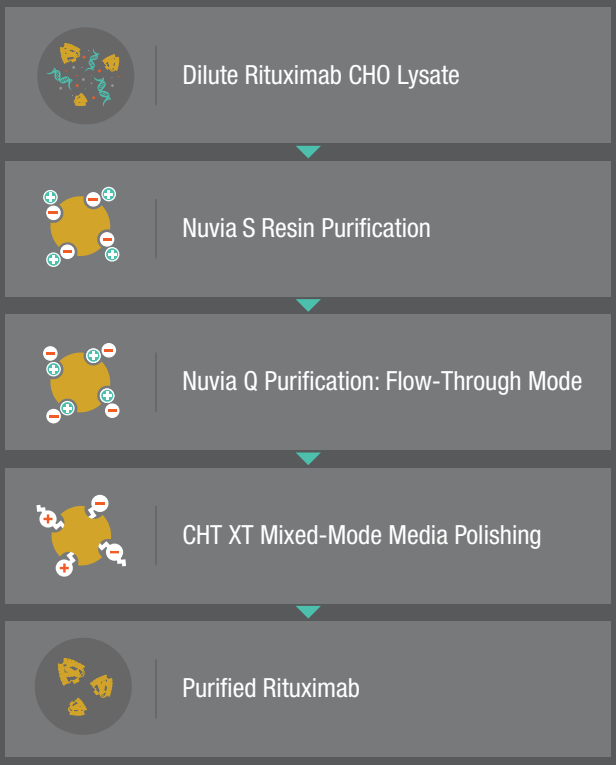


SUCCESS STORY Hussain and Bowers describe a sensitive and precise method for quantifying host residual DNA — without DNA extraction steps — employed in the development of biologic drugs developed by Merck.

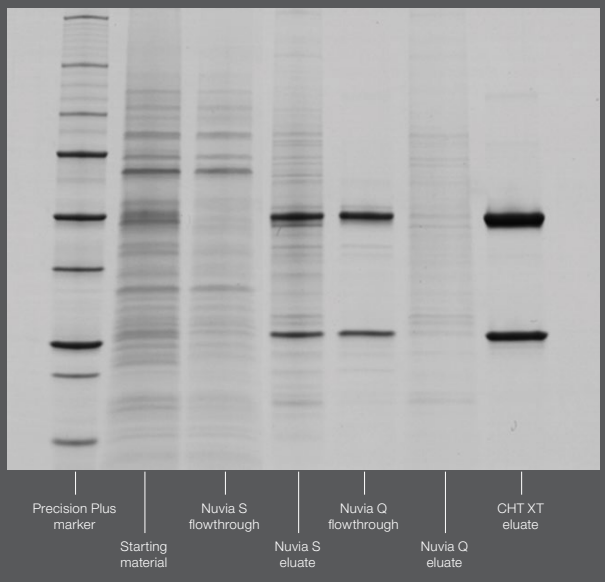
See Hussain M. and Bowers J. (2017). **A droplet digital PCR method for CHO host residual DNA quantification in biologic drugs**. J Anal Pharm Res 4, 00107 for more.

IMMUNOTHERAPY MANUFACTURING AND QC

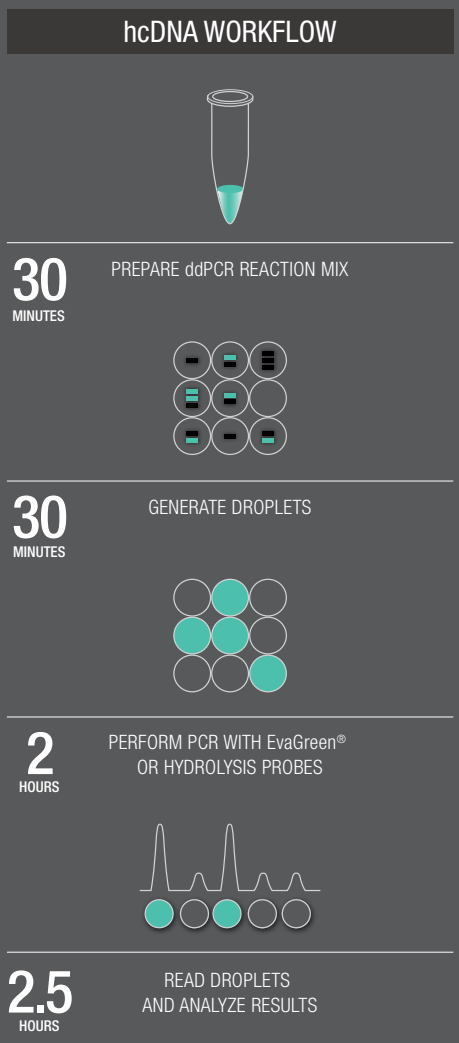
A



B



C



D

Picogram of hcDNA per milligram of Rituximab	
Raw feedstock	1,200
After resin 1	400
After resin 2	7
After resin 3	Not detected

Rapid development of a manufacturing process for rituximab. 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 CHT XT Mixed-Mode Media. **B**, resulting protein was analyzed by reduced gel electrophoresis. **C**, to assess residual hcDNA, eluate at each stage of our three-step purification protocol was analyzed using the ddPCR Supermix for Residual DNA Quantification. **D**, residual hcDNA was reduced to below the limit of detection by using the three-step purification strategy.

WHY BIO-RAD?

Bio-Rad has more than 60 years' experience supporting life science research and clinical diagnostics. As an industry leader in digital PCR, transfection, and 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. This workflow supports immunotherapy development, manufacturing, and quality control (QC). It is just one of the many workflows Bio-Rad has designed to help you bring your therapeutics to market.

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

	Discovery and Development	Manufacturing and QC
Cellular Analysis		
S3e Cell Sorter	•	
Gene Pulser Xcell Electroporation System	•	
TC20 Cell Counter	•	
ZE5 Cell Analyzer	•	•
Antibodies for Flow Cytometry and Immunohistochemistry	•	•
Genomic Analysis		
QX200 Droplet Digital PCR System	•	•
ddPCR Supermix for Residual DNA Quantification	•	•
CFX Real-Time PCR Systems	•	•
Hard-Shell PCR Plates	•	•
Reliance One-Step Multiplex RT-qPCR Supermix	•	
iProof High-Fidelity PCR Reagents	•	
ddSEQ Single-Cell Isolator	•	
PrimePCR lncRNA Assays	•	
SEQuoia Complete Stranded RNA Library Prep Kit	•	
Proteomic Analysis		
Bio-Plex Multiplex Immunoassay Systems	•	
Bio-Plex Multiplex Immunoassays	•	
Human Combinatorial Antibody Library (HuCAL) Technology	•	•
Anti-Idiotypic Antibodies	•	•
GS-900 Calibrated Densitometry System		•
Chromatography Resins	•	•

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

BIO-RAD

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Life Science Group

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