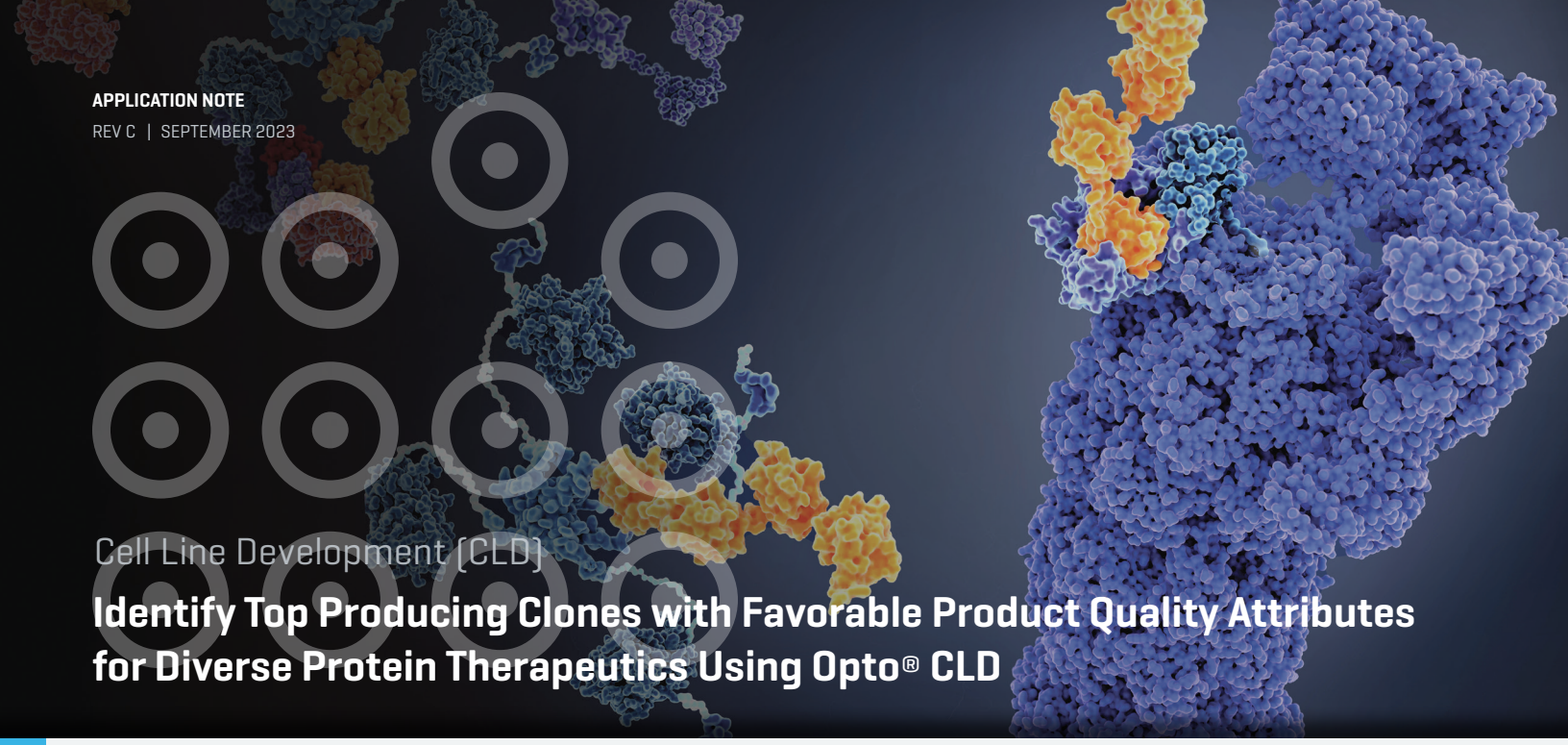


APPLICATION NOTE
REV C | SEPTEMBER 2023

Cell Line Development (CLD)

Identify Top Producing Clones with Favorable Product Quality Attributes for Diverse Protein Therapeutics Using Opto® CLD

KEY HIGHLIGHTS

- Identify clones with favorable growth, titer, and product quality attributes prior to scale up
- Go from transfected pools to top clones in under one week for a wide variety of protein therapeutics, including protein-based vaccines, enzymes and bispecific molecules
- Cloning efficiencies 5x greater than FACS and 10x greater than limiting dilution, and >99% monoclonality assurance

INTRODUCTION

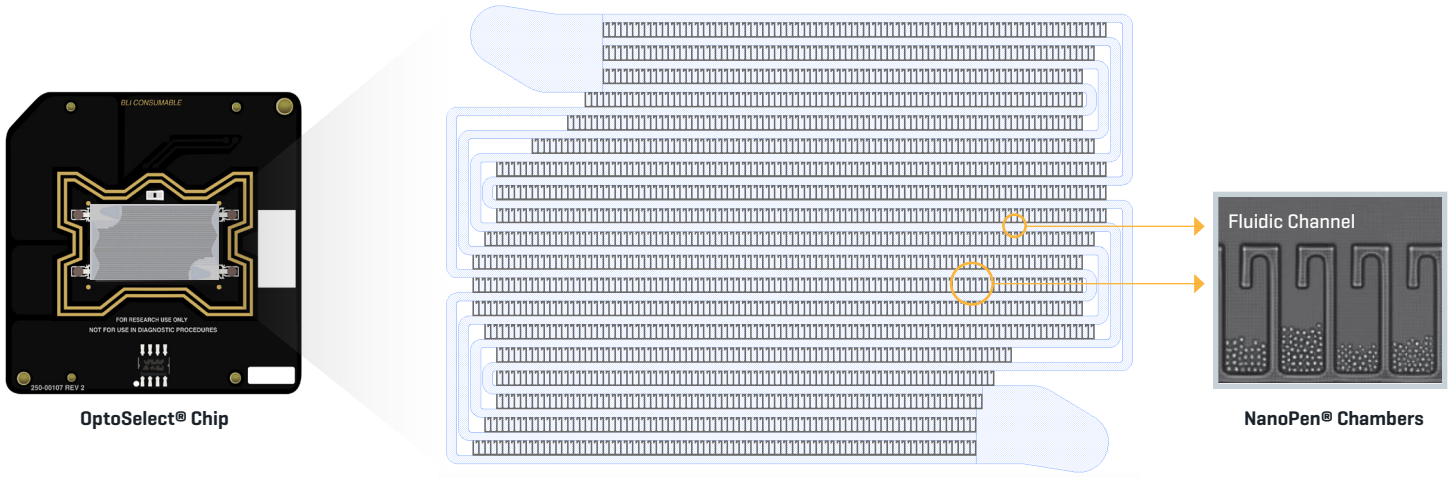
Protein therapeutics have emerged as a highly effective class of drugs over the last three decades.[1] As of 2019, 8 out of the top 10 drugs sold in the United States were biologics.[2] An increasingly diverse range of novel and complex protein therapeutics such as vaccines, viral antigens, bispecifics, tri-specifics, and fusion proteins are currently entering industry bioproduction pipelines. These biomolecules are often difficult to express and purify at desired yields, driving a need for improved solutions in early bioprocess development.

Despite a growing need for earlier information on quality and manufacturability, initial clone screening in mammalian cell line development continues to focus on selection for growth and titer. Yet the fastest-growing and highest-producing clones may not secrete a product with the appropriate quality attributes. As a consequence, large numbers of clones must be expanded and characterized through repeated rounds of selection in order to maximize the probability of finding a cell line that makes high titers of manufacturable product.

At Bruker, we are adapting and improving our Opto® CLD workflow to meet the challenges of a post-monoclonal-antibody world. Opto CLD on the Beacon® Optofluidic System reduces FTE and resource costs for cell line development with a fully integrated, automated high-throughput workflow for rapid cell screening. In addition, pools can be cloned 2.5 weeks post-transfection[3] further reducing overall timelines. The powerful combination of OptoSelect® nanofluidic chip technology, single cell cloning, customizable fluorescent assays, and sophisticated imaging enables our customers to clone, culture, and profile thousands of CHO clones in parallel. Top clones are identified and exported with >99% monoclonality assurance[4] (see also **Application Note: FDA Accepted IND**) within days of cloning, eliminating weeks of costly and labor-intensive well plate processing (**FIGURE 1**).

FIGURE 1: Cell Line Development Timeline Reduction is Achievable with Opto® CLD Workflow. Compared to conventional methods that require stable pool screening and selection before cloning, the accelerated Opto CLD workflow provides superior access to pool diversity by allowing for high-throughput screening and cloning 2.5 weeks post-transfection. Top producing clones with favorable product quality attributes can then be identified within 5 days of cloning.

OptoSelect® Chips



OptoSelect® Chip **NanoPen® Chambers**

FIGURE 2: The OptoSelect® 1750 Chip. Cloning, culture, assays and recovery is performed on OptoSelect chips, which contain 1,758 NanoPen® chambers. Cells are cloned into NanoPen chambers, where they are then monitored during culture and screened using high-throughput automated assays. The chip design allows for importing of cells, media, and assay reagents without disrupting the clones.

Beyond Monoclonal Antibodies

Bruker's fluorescent SpotLight™ reagents bind specifically to the Fc region or the Kappa region of human IgGs to enable on-chip productivity measurements and ranking of antibody-secreting CHO clones. The Opto® CLD workflow goes beyond traditional antibodies by using the Custom Productivity Assay to select clones secreting a diverse range of protein therapeutics. The use of custom product-specific assay reagents expands the benefits of Opto CLD to the development of non-antibody therapeutics, such as protein-based vaccines and enzymes [FIGURE 3].

The Custom Productivity Assay measures the amount of detection molecule bound to secreted protein and can be performed with user-provided detection molecules, ranging from small peptides to monoclonal antibodies, as long as these molecules bind specifically to the secreted protein of interest. Refer to the Custom Productivity Assay on the Beacon® Optofluidic System Protocol [MAN-08173] for detection reagent requirements. The molecule must be labeled with a compatible fluorophore and quantified using common well-established methods (see Info Sheet: Detection Reagents for Custom Productivity Assay on the Beacon Optofluidic System).

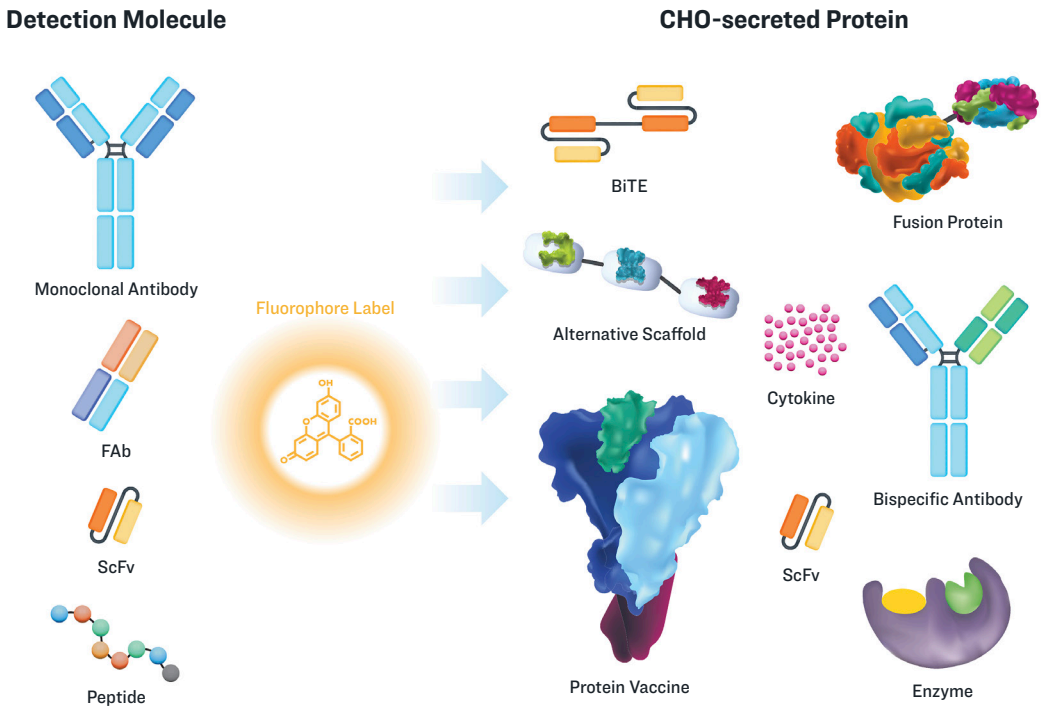


FIGURE 3: The Custom Productivity Assay Enables Selection of Clones Secreting a Wide Variety of Non-Antibody Molecules. The Custom Productivity Assay uses labeled detection molecules to measure a range of secreted proteins, including antibodies, protein-based vaccines, enzymes, fusion proteins, antibody fragments, and cytokines.

Some advantages of the Custom Productivity Assay include:

- **Flexibility to use your own detection reagent to validate that the expressed protein is properly folded and functional**
- **Works for a wide variety of secreted proteins**
- **Robust, easily optimized assay with a wide range of detection molecules from peptides to mAbs** (molecular weight less than ~150 kDa)

Bruker's customers at the University of Queensland successfully used this custom assay format and the Opto® CLD to select a cell line expressing a 600 kDa molecular clamp COVID-19 vaccine. In just two weeks, the team optimized the assay and identified clones with 10 times higher titer than their standard CLD workflow. The vaccine produced by a clone selected using the Custom Productivity Assay was approved for human clinical trials.[5]

Calibrated Pen Scores

Calibrated assay measurements are automatically generated as part of the Custom Productivity Assay. To achieve this, reference images combined with the assay image generate a two-point standard curve (FIGURE 4). The first point (0,0) on this standard curve comes from the background-corrected reference image, resulting in an intensity value of 0 A.U. at 0 nanomolar concentration of the detection reagent. The second point (250 nM, 5000 A.U.) is measured from the channel signal in the assay image using the concentration of the detection reagent, which is measured off-instrument before the assay is run. The pen signal is then used to quantify the concentration of the detection reagent from the unbound and bound complex in the NanoPen. The signal from the bound complex is reported as the calibrated pen score.

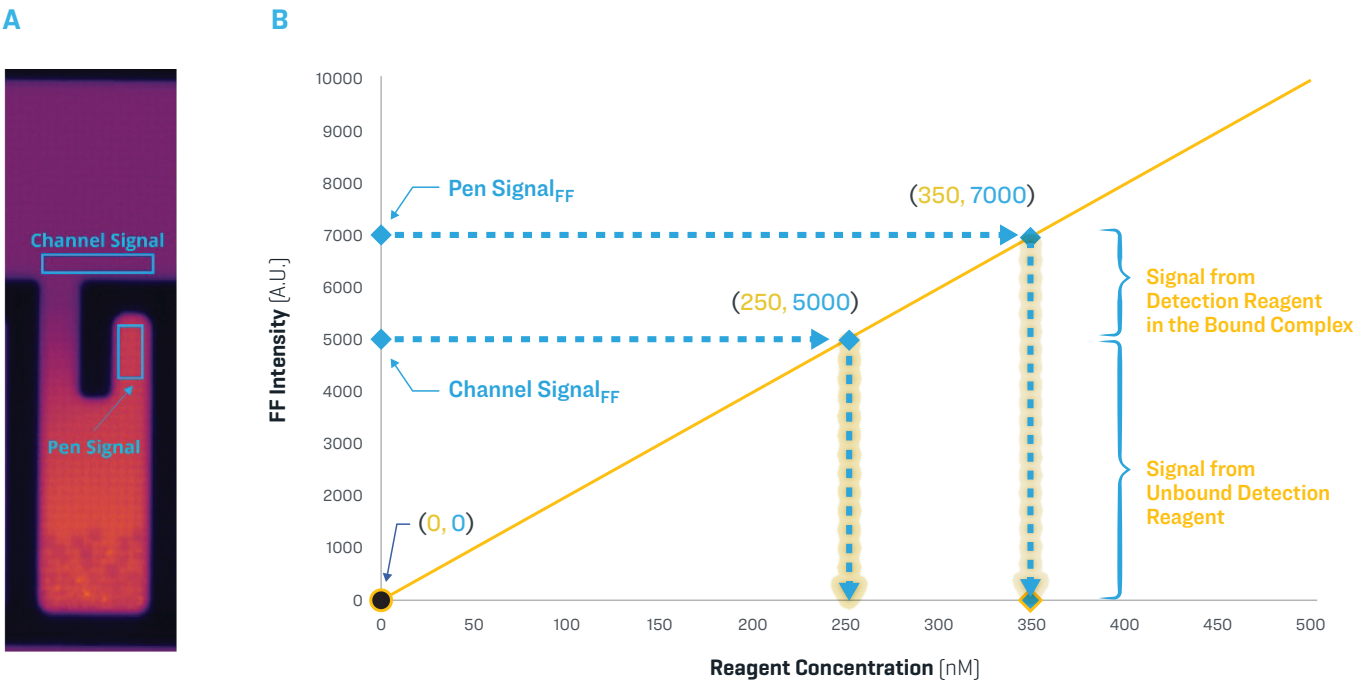


FIGURE 4: Calibrated Pen Scores in the Custom Productivity Assay. **A)** A NanoPen® is shown with the channel signal and pen signal regions highlighted. Both the channel signal and the pen signal are corrected for background fluorescence and flat fielded using the reference images. **B)** A two-point standard curve is generated using the flat-fielded (FF) channel signal from the corrected background image. The first point on this curve is (0,0) as the channel signal from the background reference image reduces to 0 after flat fielding and is generated in the absence of fluorescently labeled detection reagent. The second point on the standard curve (in this example, (250, 5000)) is generated from the flat-fielded channel signal from the assay image and a known concentration, which is determined off instrument. This standard curve can then be used to convert the arbitrary units from the pen signal to the concentration of the detection reagent in the bound complex, reported as the calibrated pen score.

The calibrated pen score can then be used to rank clones in different NanoPen chambers. For instance, in **FIGURE 5**, four NanoPen chambers are shown: an empty pen with a calibrated pen score close to 0, and three pens with similar cell counts but varying levels of secretion. A custom-built software analysis tool can be used to filter NanoPen chambers based on a variety of characteristics such as monoclonality, growth, and secretion. Secretion levels can be ranked using the calibrated pen score [**FIGURE 5B**] or the calibrated pen productivity, which is the amount of protein secreted per cell (specifically, the calibrated pen score divided by the number of cells), equivalent to a relative specific productivity [rQp] [**FIGURE 5C**]. Further, using sequential assays, the relative stoichiometry of different binding sites can be measured and used to select high quality products, such as high purity bispecific heterodimers [see **Bispecific Heterodimer Assay** below].

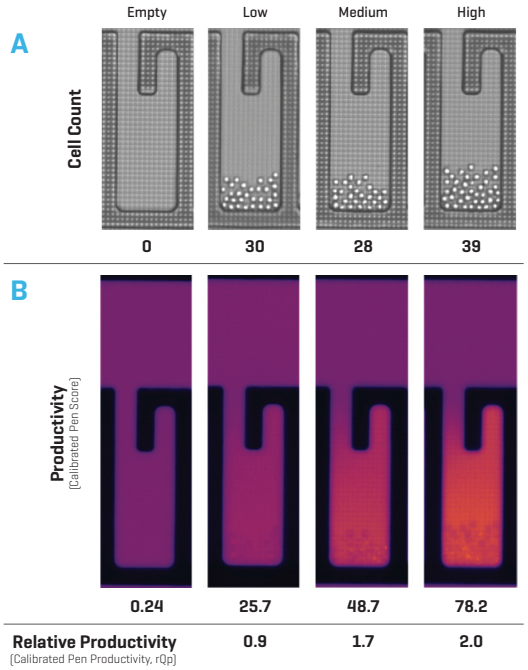


FIGURE 5: The Calibrated Pen Score and the Calibrated Pen Productivity Score Allow Clones to be Ranked. The Custom Productivity Assay measures thousands of clones in parallel allowing for characterization of the productivity (measured with a fluorescent image). **A)** Growth is monitored using brightfield time lapse imaging and cell counting with AI algorithms. **B)** Secretion levels are quantified using calibrated pen scores. The relative specific productivity (rQp) or secretion per cell is calculated and reported as the calibrated pen productivity.

Custom Productivity Assays Work for a Wide Range of Molecules

Multiple Custom Productivity Assays can be performed during the course of a 5-day Opto CLD workflow. Calibrated pen scores and calibrated pen productivity scores allow for comparison of results between different assays with different detection molecules – regardless of molecular weight, binding affinity, average number of fluorophores per molecules, or concentration.

By comparing calibrated pen scores from sequential assays using multiple detection reagents (*e.g.* by a scatter plot or a calculated ratio), the relative stoichiometry can be measured allowing the identification of clones secreting secreted protein with the desired product quality.

To demonstrate that top clones can robustly and consistently be selected using the Custom Productivity Assay, we compared the ranking from two sequential assays using detection reagents targeting the same epitope. The expected ratio of scores between sequential assays should be equal to 1, indicating that the clones are ranked identically between assays. A secreted monoclonal antibody (mAb) was measured with four different detection reagents of varying molecular weights (3 kDa, 15 kDa, 50 kDa, and 150 kDa) and binding affinities (all ≤50 nM). All four reagents bind to the conserved FC region, providing two binding sites on each mAb. Using sequential assays, more than 1000 clones of varying secretion levels were measured and compared [**FIGURE 6**]. The measurements demonstrated strong correlation (R^2 values ≥ 0.98) and the same top clones were identified regardless of the detection reagent used.

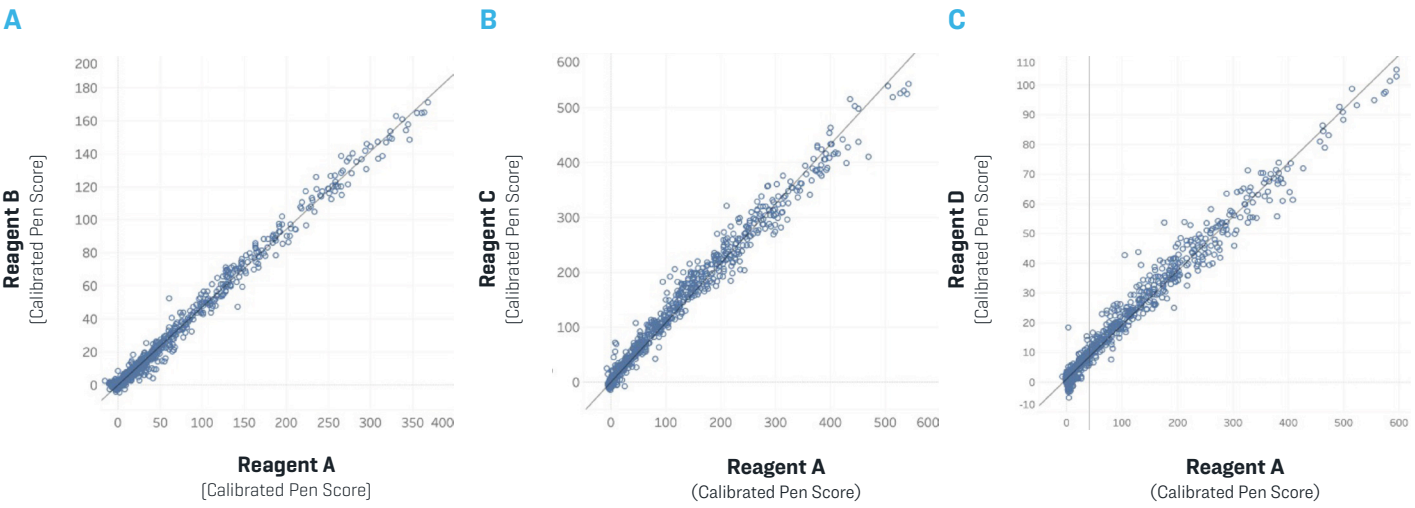


FIGURE 6: Evaluation of Custom Productivity Assay with Four Different Detection Reagents. Scatter plots show the correlation between assays over the linear dynamic range of the assays using two sequential Custom Productivity Assays run on a population of clones on Day 3 of an Opto CLD workflow. **A)** A scatter plot of Reagent A : Reagent B with a strong 1:1 correlation, with an R^2 of 0.99. **B)** A scatter plot of Reagent A : Reagent C with a strong 1:1 correlation, with an R^2 of 0.98 C) A scatter plot of Reagent A : Reagent D with a strong 1:1 correlation, with an R^2 of 0.98.

The Bispecific Heterodimer Assay

A significant challenge in bispecific antibody production is the formation of byproducts – improperly or incompletely assembled versions of the desired heterodimer form [FIGURE 7]. These byproducts are difficult to remove in bioprocessing and are prone to issues such as aggregation, which can impact product yield and immunogenicity. The Bispecific Heterodimer Assay measures the relative stoichiometry ratio of different binding sites using the calibrated pen scores or calibrated pen productivity from sequential Custom Productivity Assays run with fluorescently labeled antigens [FIGURE 8A].

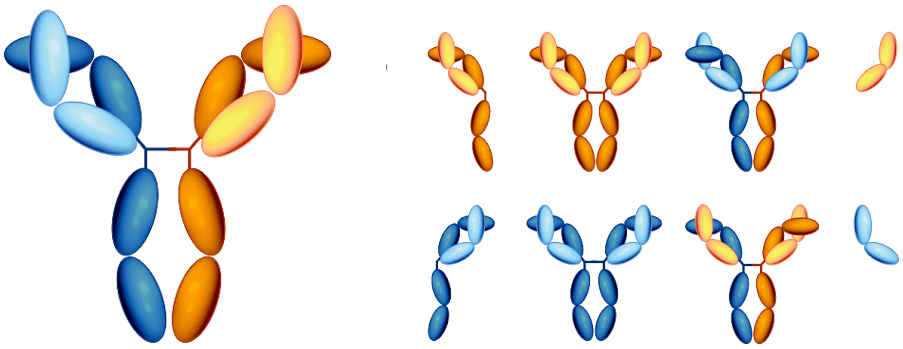


FIGURE 7: Bispecific Desired Species and Undesirable Variants. Contaminating byproducts pose challenges in downstream processing of bispecifics and can significantly impact product yield.

Each arm of a correctly assembled heterodimer binds to its respective target antigen, giving a relative stoichiometric ratio of 1. With the presence of undesirable variants, that for example bind only one of the antigens, this will bias the measured stoichiometry. Thus, the apparent stoichiometry of antigen binding can indicate whether a clone secretes product with a high percentage of bispecific heterodimer.

To determine the relative stoichiometric ratio, the assays are accurately calibrated using a well characterized cell line or pool during the assay. For accurate calibration the pool is characterized ahead of the Beacon workflow, and the average percentage of each species of secreted product [*e.g.* heterodimer, homodimer/halfmer] is used to calibrate the assays.

To demonstrate the ability to identify high quality clones, a set of pools were screened using sequential assays with customer labeled antigens as the detection reagents (Antigen 1 and Antigen 2). The antigen detection reagents were monovalent with a molecular weight of 15 and 17 kDa and a binding affinity of 4.2 nM and 40 pM, respectively. Plotting and calculating the ratio of the Calibrated Pen Scores for Antigen 1 and Antigen 2, three distinct groups were identified [**FIGURE 8B**], clones with no measured binding to Antigen 1 (orange line), clones with the relative stoichiometric ratio of ~1 (green line), and clones secreting molecules that preferentially bound Antigen 1 with a ratio around ~0.5 (blue line).

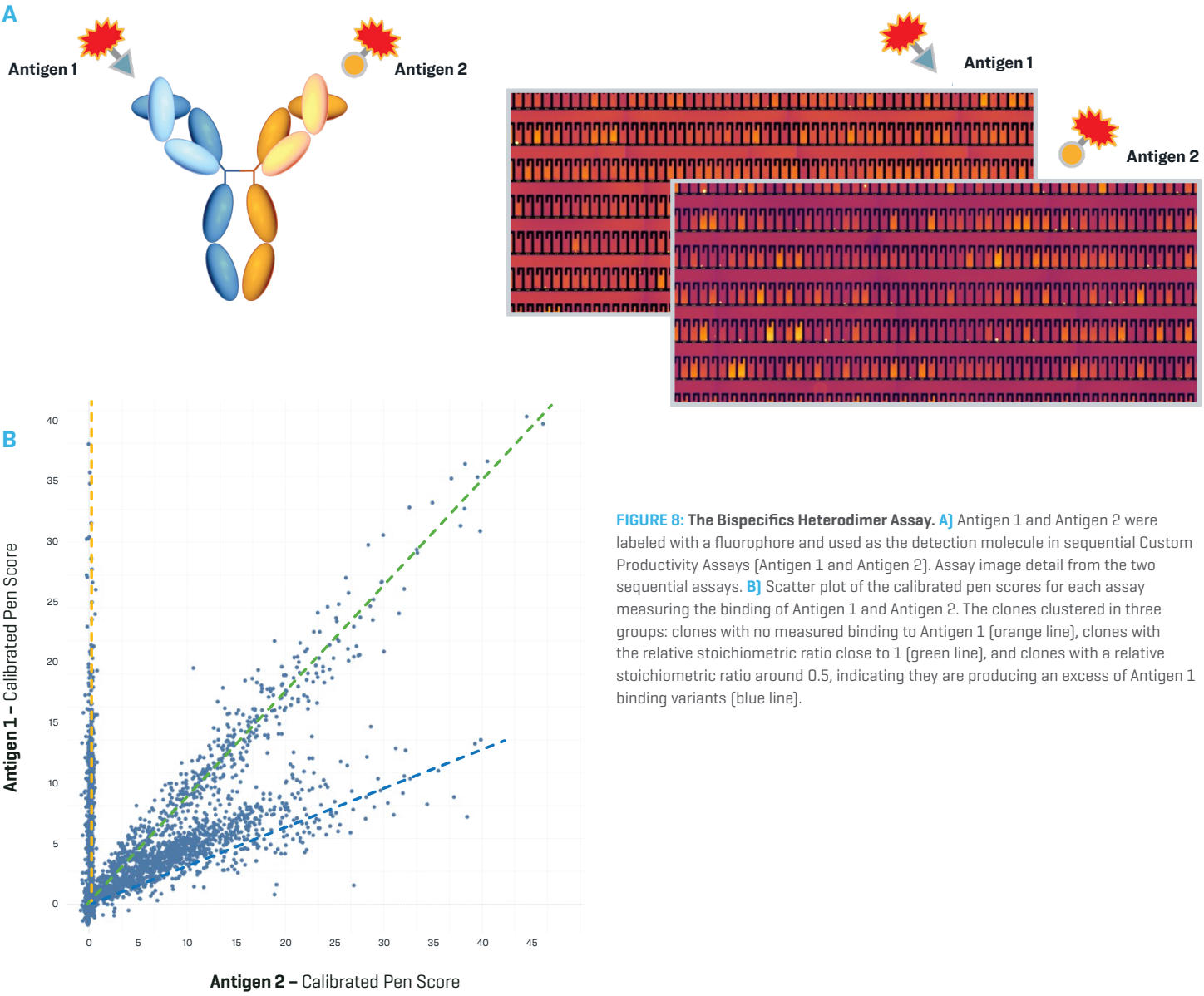
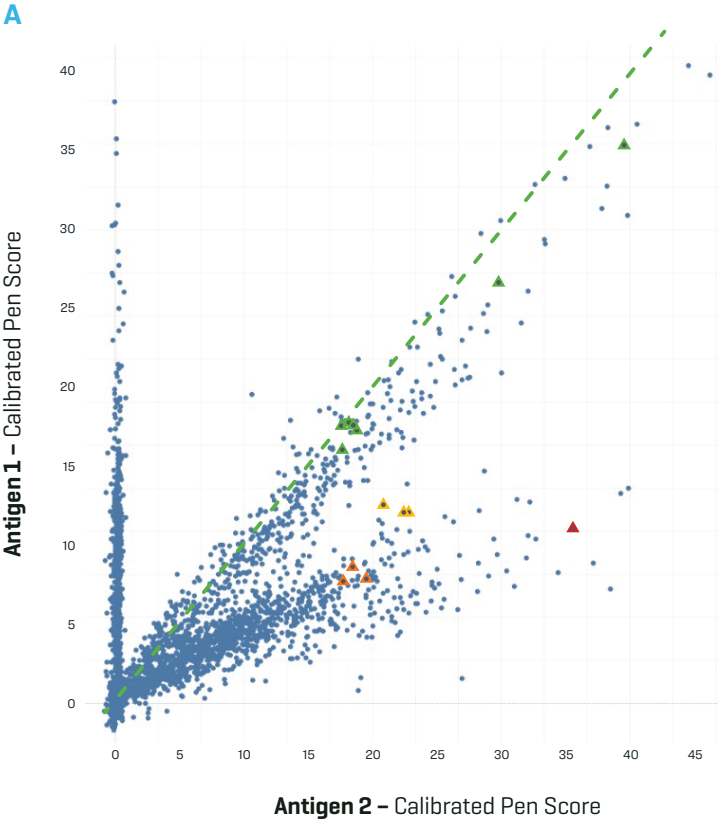


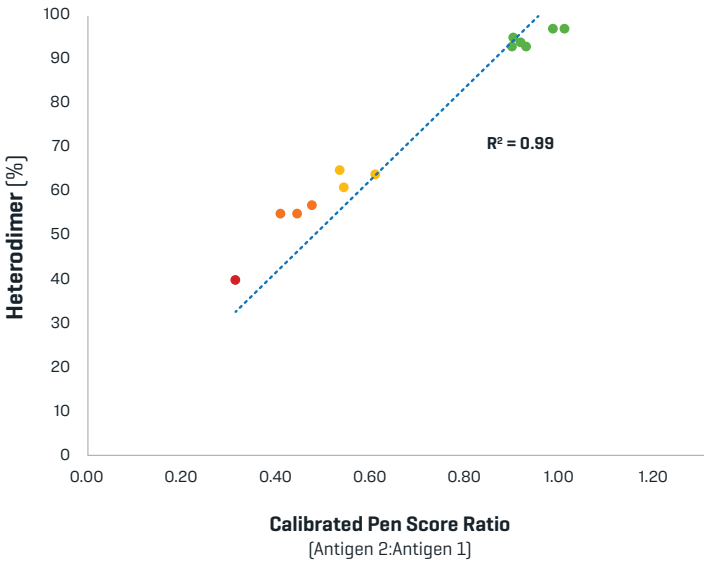
FIGURE 8: The Bispecifics Heterodimer Assay. A) Antigen 1 and Antigen 2 were labeled with a fluorophore and used as the detection molecule in sequential Custom Productivity Assays (Antigen 1 and Antigen 2). Assay image detail from the two sequential assays. **B)** Scatter plot of the calibrated pen scores for each assay measuring the binding of Antigen 1 and Antigen 2. The clones clustered in three groups: clones with no measured binding to Antigen 1 (orange line), clones with the relative stoichiometric ratio close to 1 (green line), and clones with a relative stoichiometric ratio around 0.5, indicating they are producing an excess of Antigen 1 binding variants (blue line).

A distribution of clones that bound both antigens were selected of varying stoichiometries (Figure 9A, divided into 4 sub-groups denoted by color) and scaled-up and characterized after fermentation in an AMBR fermentation system. For scale up, the clones were incubated in a 96-well plate after unload from the Beacon system for 1-2 weeks and then transferred and cultured progressively up to a 125-mL shake flask at which point the clones were frozen to create a cell bank. After fermentation, the percent heterodimer was measured using an HPLC method for each clone and compared to the Bispecific Heterodimer Assay measurement (Figure 9B and 9C). The Beacon measurement correlated strongly with the percent heterodimer with an R^2 of 0.99. Further, the assay was able to correctly rank the quality of the clones, matching the measured quality of the high purity clones with a high percent heterodimer within 5% of the fermentation product purity. The Bispecific Heterodimer Assay allows for the identification of clones producing high quality clones during the initial screen, eliminating clones that are producing undesirable variants (non-green clones).

A



B



C

Clone #	Calibrated Pen Score Ratio	% Heterodimer
1	1.01	97%
2	0.99	97%
3	0.90	95%
4	0.92	94%
5	0.90	93%
6	0.93	93%
7	0.53	65%
8	0.61	64%
9	0.54	61%
10	0.48	57%
11	0.41	55%
12	0.44	55%
13	0.31	40%

FIGURE 9: HPLC Analysis Confirms That Clones Selected Using the Bispecific Heterodimer Assay Secrete the Expected Percentage Heterodimer. The ratio of the assays was calibrated using the average percent heterodimer measured from one of the pools used in the screen. Clones are grouped by color into four clusters, representing varying purities of bispecific heterodimer molecules. **A)** Scatter plot of the antigen assays highlighting the distribution of clones selected for AMBR fermentation (triangles). Green dotted line indicates a calibrated pen score ratio of 1. **B)** Scatterplot of the ratio of the calibrated pen scores with the measured percent heterodimer of the fermentation cultures. **C)** Table showing the values for each characterized clone.

Custom Productivity Assays in combination with ratiometric analysis can be a powerful tool for eliminating process liabilities early on in cell line development. Opto® CLD on the Beacon system enables multi-parametric selection of clones based on growth, productivity, and product quality within days of single cell cloning. The ability to perform quality screens at the microscale eliminates the need to expand and characterization of a large number of clones that are not secreting manufacturable product.

The Custom Productivity Assay is robust for a wide variety of protein therapeutics, including protein-based vaccines, enzymes and bispecific molecules. This capability greatly expands the type of projects that can be completed with the Opto CLD workflow, meaning a variety of projects can be completed on a single instrument. This workflow thereby provides a reduction in overall bioprocess costs, shortened timelines, improved cloning efficiencies and >99% monoclonality assurance.

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1 Tejwani, V, *et al.* High-throughput and automation advances for accelerating single-cell cloning, monoclonality and early phase clone screening steps in mammalian cell line development for biologics production. *Biotechnology Progress*, 2021 37(6), e3208. **https://doi.org/10.1002/btpr.3208**

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4 Le, Kim, *et al.* Assuring clonality on the Beacon® digital cell line development platform. *Biotechnology Journal*. 2020 15.1: 1900247. **https://doi.org/10.1002/** biot.201900247

5 Watterson, D, *et al.* Preclinical development of a molecular clamp-stabilised subunit vaccine for severe acute respiratory syndrome coronavirus 2. *Clin Transl Immunology*. 2021 5;10(4):e1269. **https://doi.org/10.1002/cti2.1269**

ORDERING INFORMATION

Opto® CLD Kit, Human Fc 750-08126

Part No.	Product Name	Quantity
750-00018	OptoSelect® 1750b Chip*	4
520-00024	SpotLight™ Human Fc Reagent	
500-00030	Beacon Plastic Flush Chips	4
n/a	Wetting Additive	

** Includes Wetting Solution*

Opto® CLD Kit, Human Kappa 750-08127

Part No.	Product Name	Quantity
750-00018	OptoSelect® 1750b Chip*	4
520-08018	SpotLight™ Human Kappa Reagent	
500-00030	Beacon Plastic Flush Chips	4
n/a	Wetting Additive	

** Includes Wetting Solution*

Opto® CLD Kit, Human Fc + Kappa 750-08128

Part No.	Product Name	Quantity
750-00018	OptoSelect® 1750b Chip*	4
520-08018	SpotLight™ Human Kappa Reagent	
520-00024	SpotLight™ Human Fc Reagent	
500-00030	Beacon Plastic Flush Chips	4
n/a	Wetting Additive	

** Includes Wetting Solution*

Other Consumables and Reagents

Part No.	Product Name	Quantity
520-08018	SpotLight™ Human Kappa Reagent	
520-00024	SpotLight™ Human Fc Reagent	
750-00018	OptoSelect® 1750b Chip	
500-00030	Beacon Plastic Flush Chips	
750-08091	Wetting Additive Kit	

Services

Part No.	Product Name	Quantity
870-02008	Service to enable Bubble Export*	

** This service is only required to access the OEP + Bubble Dislodge Unload method*

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