APPLICATION NOTE

Bring Product Quality Assessment into Early Clone Selection with the Opto® Assure Aggregation Assay

In this Application Note we outline:

- Screen for both yield and product quality at the single-cell cloning stage
- Identify CHO cell lines with favorable manufacturability profiles earlier in cell line development
- Reduce costs, shorten timelines, and improve probability of success by selecting fewer, better clones for scale-up

View More Application Notes

BRUKER

www.brukercellularanalysis.com

Introduction

Product aggregation is a major stability, efficacy, and safety concern in the manufacturing of antibody therapeutics, particularly for highly engineered molecules such as bispecifics. As therapeutic molecules become more complex and pressure to shorten development timelines increases, the ability to screen and select production cell lines for critical quality attributes like aggregation as early as possible has become a critical challenge in 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 product that meets target quality profiles, potentially leading to costly processing inefficiencies downstream. 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.

Opto Assure Assays Provide Early Information on Product Quality

The Opto Assure assay series for CHO cell line development is an enhancement to the Opto CLD workflow on the Beacon® system that provides product quality information at the earliest stages of cell line development. Here we introduce the Opto Assure Aggregation assay, the first in the series, which enables detection of product aggregates within days of single cell cloning (**Figure 1**). Early elimination of clones susceptible to aggregation not only increases likelihood of identifying more optimal production cell lines, but also helps speed development by reducing the number of clones that must be selected and processed.

Transformative Clone Selection Technology

Opto Assure assays are integrated into Bruker Cellular Analysis' end-to-end automated Opto CLD workflow for rapid cloning and selection in mammalian cell line development. With capacity to evaluate up to 6,000 cells in a single run, Opto CLD enables the user to get from transfected pool to top-producing clones in less than one week with minimal hands-on time (**Figure 2**).

Cells are cloned, cultured, and assayed on OptoSelect® nanofluidic chips, which contain thousands of tiny NanoPen® chambers ("pens") connected to a continuous fluidic path (**Figure 3A**). Single cells from one or more transfected pools are sorted into individual pens by opto-electropositioning (OEP™) technology. OEP uses light to capture and gently manipulate hundreds of individual cells in parallel, guiding them from the fluidic channel into the pens ("penning", **Figure 3B**). With Selective Cell Cloning, cells with desired phenotypes can be specifically targeted for penning based on product expression, viability, or other characteristics.

A Unique Micro-Environment for Cell Expansion and Evaluation

A constant flow of fresh media through the channel creates an environment similar to that of a perfusion bioreactor, maintaining cell viability and enabling onchip

Figure 1 | Early, multi-parameter assessment of clones with Opto Assure enables early elimination of clones that are susceptible to quality issues like aggregation. Only top producers with best quality profiles are selected for initial scale-up, minimizing process risk and saving valuable development time.

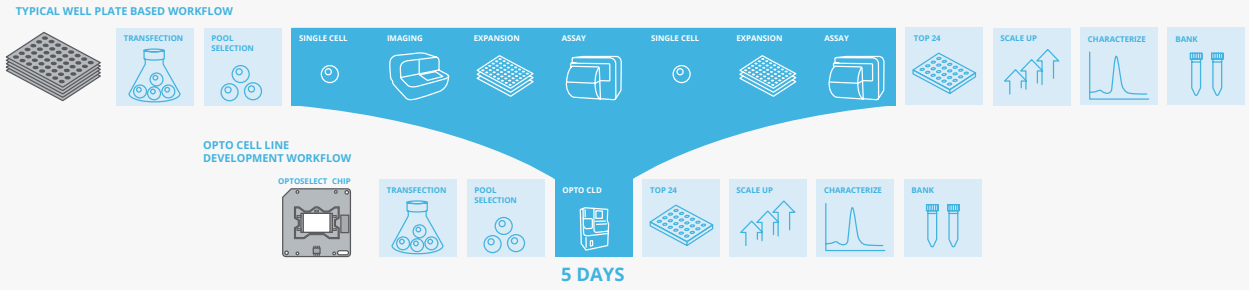

Figure 2 | Cell line development timeline reduction with Opto CLD workflow on the Beacon system. Compared to conventional methods that require expansion in well plates and multiple rounds of cloning, the accelerated Opto CLD workflow transforms clone selection with significant time and labor savings and cost-effective scalability.

culture over several days. The small dimensions of the pens reduce clone expansion and analysis down to a microscale level, meaning relevant information about a clone can be obtained after just a few generations.

Fluorescence-based assays are performed at multiple time points to assess growth, productivity, and other attributes such as quality metrics like aggregation. After about 5 days of on-chip culture and assays, topranking clones are exported into microwell plates for expansion.

Automated imaging and in-process controls ensure each clone is visually tracked throughout the workflow enabling recovery of top clones with validated, FDA-accepted >99% monoclonality[1].

Product Aggregates are Detected and Quantified On-chip

The Opto Assure aggregation assay is performed in conjunction with on-chip antibody secretion assays

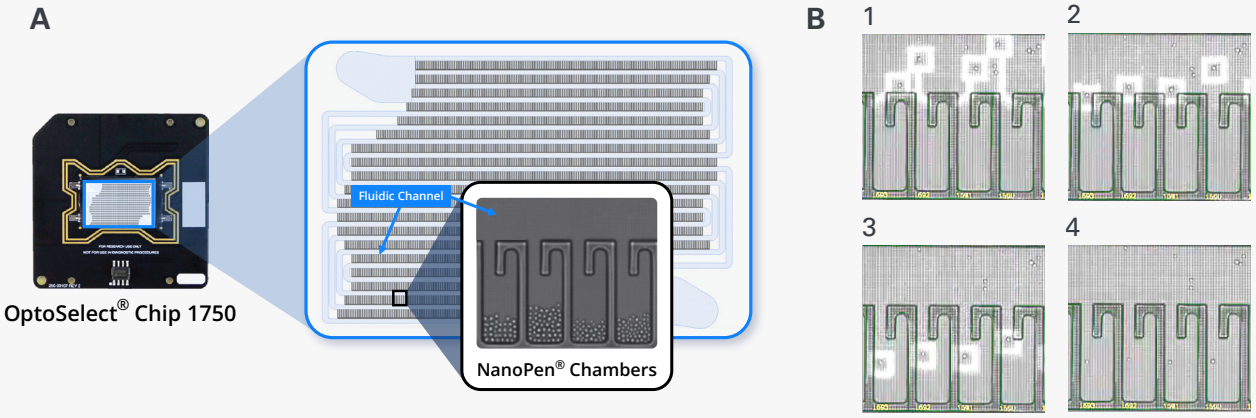

Figure 3 | Cells are cloned, cultured, and assayed on OptoSelect chips. A) Cells are cloned, cultured, and assayed in individual NanoPen chambers connected to a fluidic channel. Each pen is less than 2 nanoliters in volume with an imaging area >700X smaller than a microtiter plate well. **B)** Selective penning of single cells with OEP technology: Light is used to capture single cells with desired phenotypes and gently guide them into individual NanoPen chambers (1-3). Single cells are easily visualized in pens after cloning (4).

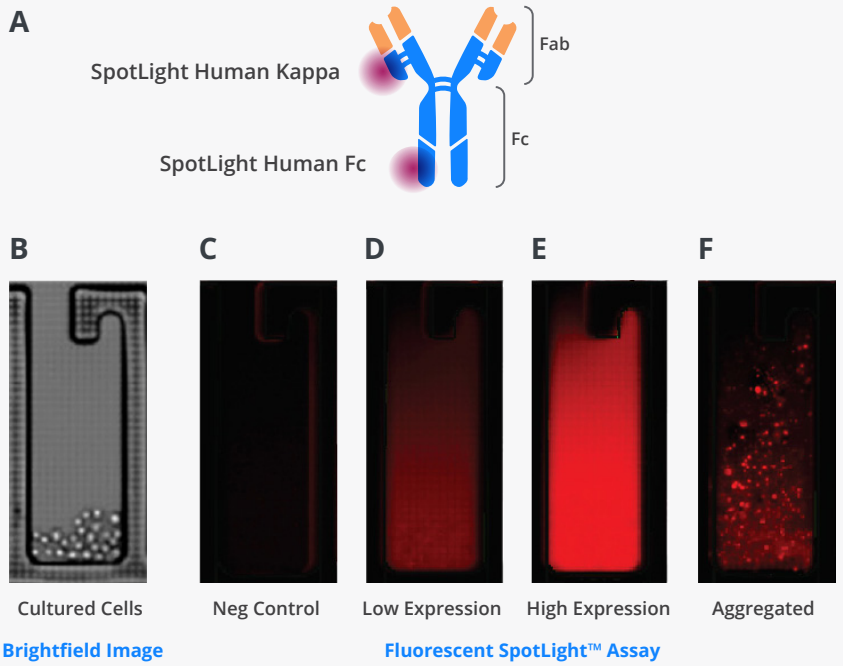

Figure 4 | **The diffusion gradient fluorescence assay for productivity assesses both product yield and quality. A)** SpotLight Human Kappa and SpotLight Human Fc reagents enable detection of monoclonal antibodies and antibody fragments. **B-E)** Typically, a productive clone will show a diffuse fluorescent signal gradient in the pen where fluorescence intensity correlates to level of expression. **F)** Product aggregates, however, result in the appearance of spotted patterns.

specifically developed for the Opto CLD workflow. At 3–4 days post cell penning, a fluorescently-labeled binding molecule that targets a monoclonal antibody, antibody fragment, or bispecific (SpotLight™ Human Fc, Bruker Cellular Analysis #520-00024 or Human Kappa reagent, Bruker Cellular Analysis #520-08018), is introduced into the chip fluidic channel and allowed to diffuse into the NanoPen chambers where it binds to secreted product (**Figure 4A**). The measured level of fluorescence correlates to the relative productivity of the cultured cells in the pen.

Typically, a producing clone will demonstrate a diffuse fluorescent signal gradient during product secretion assays (**Figure 4**, **B-E**). However, the appearance of a punctate fluorescent pattern is sometimes observed in the pens (**Figure 4F**). Characterization of these fluorescent spots, described in the next section, has shown that they primarily represent insoluble aggregates of secreted product and that the aggregate phenotype is predictive of growth and quality issues that persist through expansion of the clones.

Characterization of Fluorescent Aggregates

1 Fluorescent aggregates are not an artifact of detection molecule

To verify that the spots represent product aggregates we first confirmed that they are not an artifact of the fluorescent assay detection reagent. To demonstrate this, SpotLight Human Fc (Bruker Cellular Analysis) and a second fluorescently labeled antibody-binding molecule (Alexa Fluor anti-human Fc FAb, Jackson

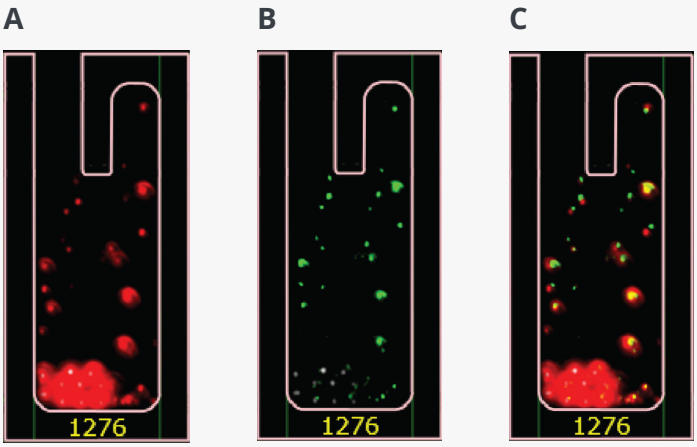

Figure 5 | **Co-localization of two independent fluorescent antibody binding reagents to aggregate spots in the NanoPen chambers. A)** SpotLight Human Fc staining, **B)** anti-human Fc FAb staining, **C)** Overlay in which yellow color indicates where both molecules have bound. The white dots indicate CHO cells cultured in the pens.

ImmunoResearch #109-546-170) were simultaneously injected onto OptoSelect chips containing cultured CHO cell clones expressing a human monoclonal antibody. An overlay of fluorescent images shows that the two reagents co-localize to the same spots in the pens (**Figure 5**). This replicate binding by a second reagent confirms the aggregates are not formed by SpotLight reagent alone.

2 Co-localization of anti-human Fc detection reagents is specific

To establish specificity of reagent localization to the spots, a FITC labeled anti-canine Ig antibody with no cross reactivity with human IgG was injected with the two anti-human Fc detection molecules described previously. Imaging showed no detectable FITC signal in pens where aggregates were illuminated with SpotLight Human FC reagent and anti-human Fc FAb (**Figure 6AC**), further confirming the fluorescent aggregates contain secreted human antibody product.

3 Spots do not contain cell debris

A lipophilic fluorescent dye typically used for cell membrane staining (ThermoFisher, #A-47) was used to assess whether spots were potentially formed by membrane fragments from damaged or dead cells. The ANS dye stained the membranes of live cells growing in the pens, but did not stain aggregates that were visible using antibody-specific binding molecules Spotlight Human Fc and anti-human Fc FAb (**Figure 6D**). This result suggests aggregate spots do not contain cell membrane fragments.

4 Fluorescent punctate pattern can be reproduced with aggregated purified human IgG

Purified human IgG was subjected to heat stress at 60°C for 48 hours to generate protein aggregates. Several dilutions of the stressed IgG sample were first incubated with with SpotLight Human Fc reagent and then injected into individual channels of OptoSelect chips. The image in **Figure 7A** shows the stressed IgG/SpotLight samples in the fluidic channel display a similar pattern to the aggregate spots observed in the pens. Automated image-based counting verified that the number of aggregates detected in the channels increased in a concentration-dependent manner (**Figure 7B**).

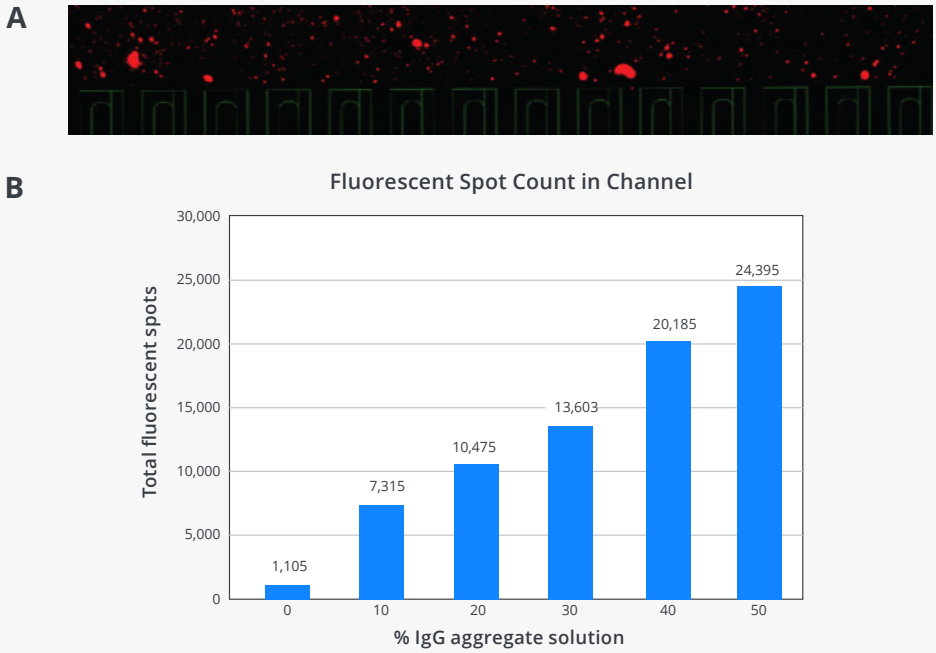

Figure 7 | **Fluorescent punctate pattern can be reproduced with aggregated purified human igg. A)** Heat stressed IgG forms spotting pattern in the chip fluidic channel when stained with SpotLight Human Fc reagent. **B)** A dilution series of aggregated IgG sample shows number of spots counted in the channel increases with concentration of IgG aggregates loaded, confirming the spots represent the aggregated protein.

Automated Detection and Scoring of Aggregates for Clone Selection

We next implemented an aggregate detection algorithm in Bruker Cellular Analysis' Assay Analyzer software to enable ranking of clones based on the presence of detectable aggregates. The aggregate particles are identified in images generated during fluorescence secretion assays in the Opto CLD workflow. The fluorescence signal within the contours of the spots is quantified in an area of the pen that excludes the live cells by image masking (**Figure 8A)**. An aggregation score is then assigned to each pen based on the count and intensity of the detected spots. Clones can then be filtered and sorted in Assay Analyzer software based on their aggregation score along with ranking by productivity, cell growth, and other user-defined parameters to identify and select the optimal ones for export (**Figure 8B**).

High Opto Assure Aggregation Scores are Predictive of Poor Clone Quality

To establish relevance of the aggregation scores assigned with the Opto Assure aggregation assay to scaled up clone behavior, we partnered with Opto CLD customer Daiichi Sankyo. Several antibody-expressing clones with a range of aggregation scores from an Opto CLD workflow were exported from the chip into 96-well plates for scale-up and analysis. The clones were expanded to 125 mL shake flask culture, and viable cell density and titer were assessed. Cell supernatants were harvested for subsequent purification and analysis by size-exclusion chromatography (SE-UPLC) and quantitative laser diffraction (qLD).

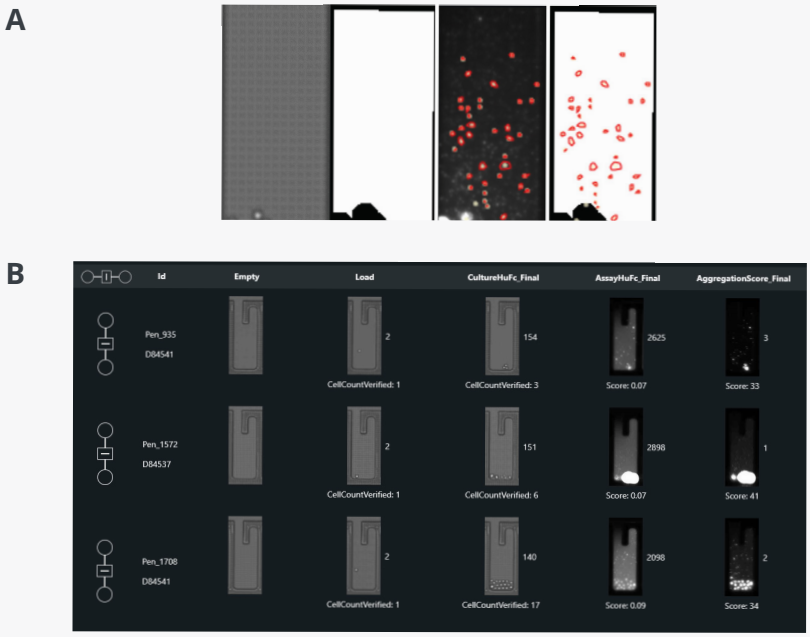

Figure 8 | **Opto Assure Aggregation assay analysis in the Opto CLD workflow. A)** Detection of fluorescent aggregate contours outside expanded cell mask so that cells are excluded from scoring. **B)** Aggregation scores in Assay Analyzer software, with counts and productivity assay scores. Multiple filters can be used to pinpoint the best clones for export.

Clones With High Aggregation Scores Tend to Have Reduced Densities and Titers

We first compared relative titers and viable cell densities from shake flask cultures in clones with high versus low aggregation scores (**Figure 9**). The distributions clearly show a lower average cell density and lower average titer for clones with aggregation scores >=10. These clones also demonstrate more variability, with wider, less predictable density and titer distribution.

More Aggregates and Larger Particles Are Detected in Clones With High Aggregation Scores

qLD particle analysis was performed on shake flask supernatants using Shimadzu nano particle analyzer, which quantifies insoluble aggregate particles by size in the subvisible to visible range. The total measurement of particles for each sample was integrated and normalized by the cell density in the flask at time

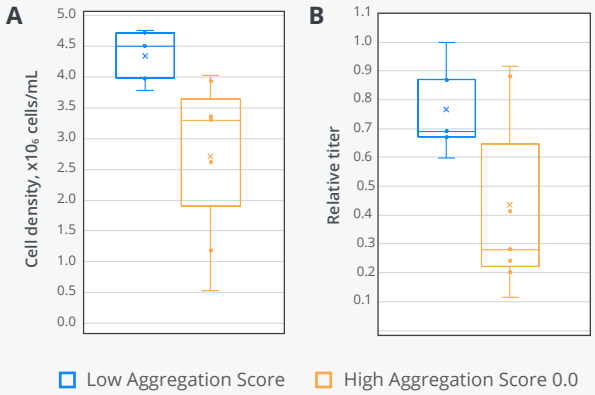

Figure 9 | **On-chip aggregation scores correlate with lower growth and titer.** Comparison of distribution of **A)** viable cell density and **B)** relative titer of clones with high versus low aggregation scores. High aggregation score is defined as >/=10.

of harvest. The chart in **Figure 10A** shows the total concentration of aggregates separated by low and high aggregation scores. High Opto Assure aggregation scores successfully predicted the highest levels of total aggregates.

Figure 10B shows qLD aggregate size profiles for the supernatant samples as overlaid histogram plots for high versus low Opto Assure aggregation scores. qLD profiles of supernatants from clones with higher scores showed more significant percentage of largersized particles than those from lower scoring clones. This observation further validates the approach of measuring fluorescent spots in NanoPen chambers to indicate presence of large, insoluble aggregate particles.

Clones With High Aggregation Scores Are More Likely to Demonstrate Poor Yield and Sec Profiles

To assess Opto Assure aggregation score correlation to product yield and presence of soluble protein aggregates, shake flask supernatant samples were filtered with 0.2 µm pore size filter and subjected to two rounds of Protein A purification, followed by SE-UPLC analysis of the purified product using Nexera X2 HPLC/ UPLC system (Shimadzu).

The heat map chart in **Figure 11** visually summarizes purification and SEC results alongside growth, titer, and qLD data according to Opto Assure aggregation score rank. While a clear correlation of percent soluble high molecular weight species (HMW) was not established

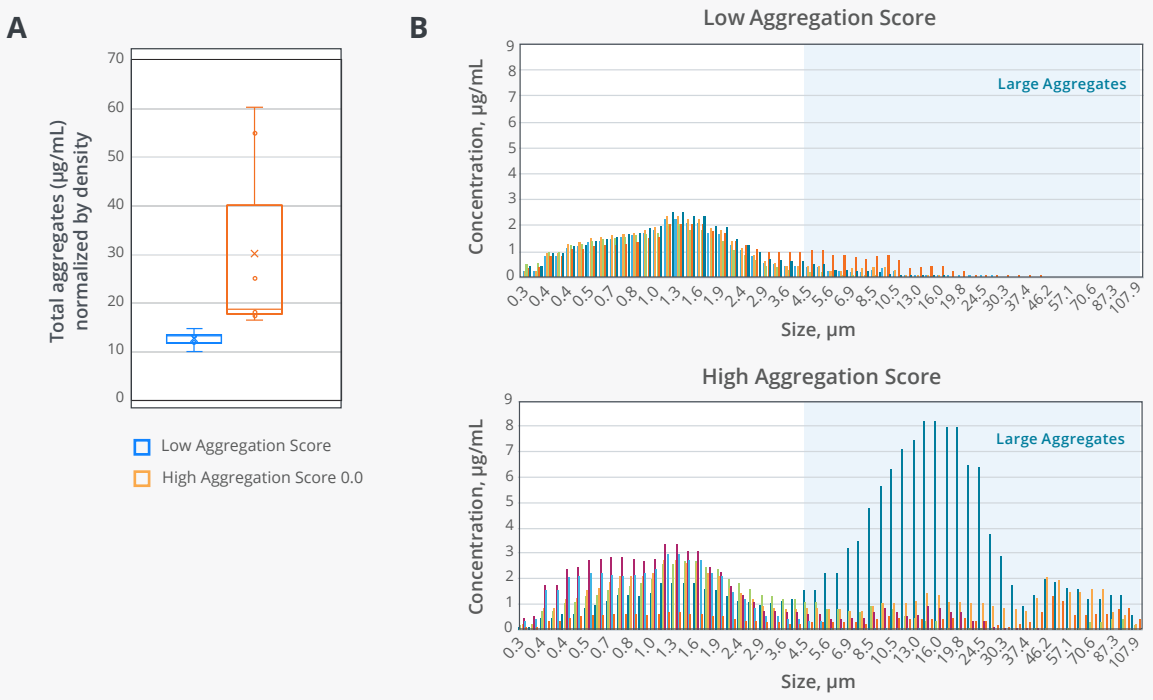

Figure 10 | Comparison of qLD profiles of shake flask supernatants from high and low scoring clones. **A)** Distributions of total aggregate concentrations for clones with high and low Opto Assure aggregation scores. Aggregate concentrations were determined by integration of qLD particle concentration curves and normalized to cell density at harvest. **B)** qLD histogram plots of particle concentrations in shake flask supernatants binned by size. When viewed by high versus low Opto Assure aggregation score, larger aggregate particles are observed primarily in supernatants from high scoring clones.

AGGREGATION SCORE RANK		DENSITY AT HARVEST (cells/mL)	ViABiLiTY (%)	RELATIVE TITER	qLD (µg/mL)	PROTEIN A PURIFICATION (µg/mL)	HMW BY Se-UPLC (area, %)
Low	1	4.73e+06	99.7	0.9	56.0	1,006.1	2.0
	2	3.79e+06	99.6	0.7	56.4	736.4	1.8
	3	3.98e+06	99.7	1.0	53.0	1,181.5	2.2
	4	4.49e+06	99.2	0.7	60.6	721.2	1.6
	5	4.70e+06	98.9	0.6	47.0	703.3	1.9
High	6	6.32e+05	89.4	0.3	34.8	210.5	1.4
	7	2.66e+06	99.4	0.1	160.4	ND	ND
	8	1.26e+06	96.3	0.2	20.8	108.9	3.9
	9	3.93e+06	99.6	0.9	68.1	1,048.5	2.1
	10	3.32e+06	98.2	0.4	62.2	411.3	1.5
	11	3.38e+06	97.8	0.2	85.2	198.2	1.3
	12	4.03e+06	99.6	0.9	72.9	797.8	2.3

Figure 11 | **Heat map chart of clones sorted by Opto Assure aggregation score rank.** Lighter color intensity corresponds to more desirable attribute at shake flask scale (eg. higher purification yield, lower aggregate concentration). When assessed over multiple criteria, higher Opto Assure aggregation scores clearly associate with less optimal clone performance.

with SE-UPLC, the clones with lowest purification yield and/or highest percent HMW also had high Opto Assure aggregation scores. One high-scoring sample was unable to be purified. Taken as a whole, these data suggest that high Opto Assure aggregation score can be predictive of sub-optimal cell line characteristics, including greater levels of aggregation at scale-up and more difficulty obtaining high yield of quality product.

Conclusion

Minimizing product aggregates improves process efficiency, at a time when flexibility and speed to market are increasingly important. We have demonstrated that with the Opto Assure Aggregation assay, clones with potential aggregation liabilities can be identified and bypassed at the early screening stage to ensure that high producing, high quality cell lines are brought forward into production. Using this new assay along with additional Opto Assure assays currently in development, Opto CLD workflow users can identify CHO cell lines with favorable manufacturability profiles earlier in development, reduce overall bioprocess costs, improve the probability of success, and shorten timelines by selecting fewer, better clones for scale-up.

References

1. FDA-Accepted monoclonality assurance on the Beacon optofluidic system for cell line development. Bruker Cellular Analysis. (2019).