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RESEARCH ARTICLE

BIOTECHNOLOGY PROGRESS

Development of a novel, high-throughput screening tool for efficient perfusion-based cell culture process development

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Abstract

Perfusion technology has been successfully used for the commercial production of biotherapeutics, in particular unstable recombinant proteins, for more than a decade. However, there has been a general lack of high-throughput cell culture tools specifically for perfusion-based cell culture processes. Here, we have developed a high-throughput cell retention operation for use with the ambr® 15 bioreactor system. Experiments were run in both 24 and 48 reactor configurations for comparing perfusion mimic models, media development, and clone screening. Employing offline centrifugation for cell retention and a variable volume model developed with MATLAB computational software, the established screening model has demonstrated cell culture performance, productivity, and product quality were comparable to bench scale bioreactors. The automated, single use, high-throughput perfusion mimic is a powerful tool that enables us to have rapid and efficient process development of perfusion-based cell culture processes.

KEYWORDS
cell culture, high throughput, perfusion, process development

1 | INTRODUCTION

The use of perfusion in the upstream manufacture of biologics has seen increased utilization recently, with most biopharmaceutical manufacturers employing it at some level.[1,2] The rising costs associated with bringing drugs to market, coupled with lower success rates, have resulted in companies placing greater emphasis on efficiency and flexibility for drug production, as well as drug development.[3,4] With the incorporation of QbD methodologies, there is a greater need for better identification of critical quality attributes, and control of the process conditions that affect them.[4,5] For production, efficiency and flexibility improvements are often attained through continuous processing and disposable technology, with perfusion and single use bioreactors (SUBs) being utilized for the upstream portion of the process. In development, high-throughput (HT) technologies and techniques are leveraged to improve optima selection through an increase in experimental parameter design space, reduced level granularity, and shortened selection timelines. This creates a dilemma, as there are currently no HT perfusion systems that have proven to accurately mimic bench and large scale perfusion bioreactor systems.

Cell culture process development is a combination of science and engineering. Therefore, its implementation often covers a broad range of approaches; however, overall conceptual flows often follow a similar path and order of operations, and the opportunities to implement high throughput technologies are mirrored across organizations and philosophies. Screening investigations for new or next generation processes are conducted at milliliter scales, using various experimental methods. These experiments look for process starting points and determine what conditions are important to the overall process, process parameters, or product qualities in question. Results are used to apply Design of Experiments or univariate approaches to bioreactors and fermenters at benchtop scales to further define the process. This knowledge is combined with scale-up information to determine the final bioreactor operational specifications, and confirmed at pilot scale. With a lifecycle approach, after final parameter setting, the cycle continues, to improve scale-down models, troubleshoot process

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issues, and increase process knowledge. In application, these procedures are not necessarily rigid, well defined steps, as the screening studies often utilize DOE and univariate methods, and benchtop reactors can be used for screening experiments when needed. They represent a general summary of cell culture process development stages and methodologies. Depending on specific process or project requirements, concerns, stage in lifecycle, resource availability, and current process knowledge, steps may be skipped, reversed, or augmented. Of these steps, screening has the greatest propensity for improvement through high throughput techniques, with benchtop DOE experiments also amendable to HT usage with the wide scale utilization of microbioreactors in the biopharmaceutical industry.[6–10]

Perfusion is primarily accomplished by retaining cells within a bioreactor vessel, while exchanging the liquid medium used to sustain the culture. With all currently marketed cell retention devices targeting bench scale bioreactors, a perfusion mimic is necessary for an approximation of cell culture performance in a high throughput system. Current approaches generally make use of one of two systems: shaken tubes that can be centrifuged daily (tubespin), but lack any DO and pH control or monitoring[4,6,11,12]; or ambr15 systems with a cell settling step, either using adherent cultures that can settle quickly after the discontinuation of agitation, or the natural settling of biomass with an extended period of static culture.[13–15] The lack or loss of in-line DO and pH monitoring and/or control may have significant impact on the usability of these systems. Screenings involving production scale densities are unlikely to be predictive of bioreactor scale performance,[11] and could imply effects that do not translate to larger scale processes. The lack of an easily applicable perfusion system or process for the ambr15 has meant that perfusion development has not been as widely impacted by the high throughput trend. Using an ambr15 system and employing a centrifugation step, similar to tubespin operation, eliminates these issues, but still represents several challenges and unknowns. Three theoretical models were tested and are described in subsequent sections. The final model was applied in two case studies: media optimization, and clone selection.

Here, we show that a rationally developed procedure, using an external centrifuge with a variable volume process, allows for maintaining the high density cultures associated with perfusion processes at high viability. This process matches the cellular microenvironment of true perfusion systems, and gives excellent cell culture performance fidelity across scales, with minimum full time employee (FTE) requirements.

2 | MATERIALS AND METHODS

2.1 | ambr15 perfusion

A Thermo Sorval Legend centrifuge was used for centrifugation of the ambr15 disposable bioreactors. These reactors are of a proprietary geometry, and a custom holder was fabricated to allow for centrifugation in an efficient manner. Centrifugation experiments were conducted to ensure that an adequate level of separation was achieved, without over-packing the cells and hindering the resuspension of the culture, postmedia removal. Cultures exposed to 30,000*g*-seconds retained >95% of their viable cell density (VCD) while being entirely resuspended within 90 s when agitated at 1,000 RPM (data not shown).

Utilization of the automation included with ambr15 systems allows for a more continuous perfusion mimic model than is possible with standard tubespin operation. In a standard system, the volume of the culture within the vessel is considered a constant, and known as the working volume of the system. A simple, variable volume model, where the instantaneous volume in the reactor, or volume at time *t*, varied through a 24 hr period, was derived for idealized operation of the ambr15 mimic. This model's volume was only equal to a baseline working volume for a short time between media doses. To decouple cell number changes from changes in volume, the cell concentration measurements at time *t* were converted to a cell concentration based on the working volume by:

$$\frac{\text{Cells}}{\text{Volume at time }(t)}\cdot\frac{\text{Volume at time }(t)}{\text{WV}}=\frac{\text{Cells}}{\text{WV}}$$

Consumption of substrates and generation of metabolites can both be considered on a per cell basis. Assuming zero order kinetics, so that:

$$\frac{\text{Cells}}{\text{WV}}\cdot\frac{\text{Rate}}{\text{Cells}}=\frac{\text{Rate}}{\text{WV}}=R_{S\,or\,M}$$

With $R_{S\text{ or }M}$ equal to the rate of substrate consumption or metabolite generation in g/Day/mL.

Using simple step changes in generation and consumption rates (or cell concentration) as an estimate to calculate concentrations, with the rates of consumption or generation, and substrate added in this per-volume form, and with a theoretical constant working volume as the rate basis, the equations for substrates and metabolites become:

Consumption:

$$\begin{gathered}\frac{dS}{dt}=F\cdot S_{\text{in}}-R_{\text{s}}\cdot(\text{WV})\\ S=S_0+F\cdot S_{\text{in}}\cdot T-R_{\text{S}}\cdot(\text{WV})\cdot T\end{gathered}\tag{1}$$

where:

S = substrate (g).
S_{in} = substrate concentration of media (g/mL).
S_0 = initial substrate (g).
F = flow rate of media (mL/Day).
R_{S} = rate of substrate consumption (g/Day/mL).
T = time (Day).
WV = working volume (mL) of reactor.

Generation:

$$\begin{gathered}\frac{dM}{dt}=R_{\text{m}}\cdot(\text{WV})\\ M=M_0+R_{\text{M}}\cdot(\text{WV})\cdot T\end{gathered}\tag{2}$$

M = metabolite (g).

M_0 = initial metabolite (g).

R_M = rate of metabolite generation (g/Day/mL).

Volume:

$$\frac{dV}{dt} = F \qquad V = V_0 + F \cdot T \tag{3}$$

V = vessel volume (mL).

V_0 = initial vessel volume (mL).

For concentration of consumed substrate, combining Equations (1) and (3):

$$C_{(T,S)} = (S_0 + F \cdot S_{in} \cdot T - R_S \cdot (WV) \cdot T)/(V_0 + F \cdot T) \tag{4}$$

For concentration of generated metabolites, combining Equations (2) and (3):

$$C_{(T,M)} = (M_0 + R_M \cdot (WV) \cdot T)/(V_0 + F \cdot T) \tag{5}$$

Comparing to a step change in typical perfusion operation, with the general equation taking the form

$$V\frac{dC_j}{dt} = F\left(C_{j,in} - C_j\right) + R_j V$$

where the j subscript = S or M. Solving, rearranging and substituting $D = F/V$, the general perfusion equation becomes:

$$C_{(T)} = \left(C_0 - \left(C_{j,in} + \frac{R_j}{D}\right)\right) \cdot e^{-Dt} + \left(C_{j,in} + \frac{R_j}{D}\right) \tag{6}$$

With R_j being negative for consumption, $C_{j,in}$ being zero for metabolites not found in media, and j being the component of interest (S for substrate and M for metabolite). If the culture is to be bled, a steady-state is expected sometime after the bleed is initiated. With this assumption, $t \rightarrow \infty$ in the true perfusion model and $C_{(t-1)} = C_{(t)}$ in the daily variable volume model, so Equation (6) simplifies to:

for consumption

$$C_{(T,S)} = C_{S,in} + \frac{R_s}{D}$$

for generation

$$C_{(T,M)} = \frac{R_M}{D}$$

and for variable volume:

Consumption:

$$C_{(T,S)} = \left(S_0 + F \cdot C_{S,in} \cdot T + R_S \cdot (WV) \cdot T\right)/(V_0 + F \cdot T) = S_0/V_0$$

Generation:

$$C_{(T,M)} = (M_0 + R_S \cdot (WV) \cdot T)/(V_0 + F \cdot T) = M_0/V_0$$

rearranging, eliminating time, and simplifying:

Consumption:

$$S_0/V_0 = C_{S,in} + R_S \cdot (WV)/F = C_{S,in} + R_S/D = C_{(T,S)}$$

Generation:

$$M_0/V_0 = R_M \cdot (WV)/F = R_M/D = C_{(T,M)}$$

So that dilution ratios are the same for both the general perfusion and variable volume models at steady state. However, the dynamic portions of the concentration curves do not reduce to equivalence, and the real-world automation is not capable of continuous additions. This necessitated model exploration, which was conducted using MATLAB simulations comparing various addition intervals and exchange rates to addition rate ratios using Equations (4) and (5) and comparing to Equation (6) (see results).

Centrifugation and media removal was performed at 24 hr intervals, and ambr15 sample scripts were modified to regulate aspiration level height. Samples heights were made to match minimum volume levels to prevent over-aspiration and cell pellet perturbation. For the 24 reactor system, robot arm speed was sufficient to make the necessary exchanges when decks were operated so that exchanges did not occur at the same times. However, the arm's need to use 1 mL pipettes for removal of liquid from each vessel precludes the operation of all 48 reactors in perfusion mode on the 48 way system, due to time constraints. For complete utilization of the system in perfusion mode, a manual procedure was developed for removing the required volumes, using an adjustable, six-way micro-pipettor.

2.2 | Cell lines

For media development: CHO (K) line producing an antibody-enzyme was screened across multiple, CD media.

For clone selection: A pooled transfection product of a media adapted CHO (K) clone was subcloned and compared by growth and titer in microplates. Twenty clones were selected for screening in perfusion mode in duplicate. Seven clones from this group were selected for assessment in a 10 L perfusion bioreactor and compared to the previous adapted clone pool as a control. Selection and comparison criteria consisted of cell culture performance, productivity, and product quality.

2.3 | Media

Off the shelf media formulations were used for basal media preparation of the clone screening experiments. For the media development work, four custom formulations were screened, based on vendor recommendations.

2.4 | Bioreactor systems

2.4.1 | ambr15™

The ambr15™ system was a mammalian culture model using standard mammalian ambr15 reactors. The system houses 12-reactors in a block, using optical pH and DO control, with air heating only.[16] Agitation settings were initially based on vendor recommendations, but modified due to suspected interactions with liquid level heights.

Agitation settings of 1,000 rpm in the initial proof of concept (POC) experiment showed limited growth and lower viability than similar conditions at larger scales. Growth rates and viabilities rebounded for some conditions when agitation rates were reduced to 600 RPM at minimum volume levels, post-exchange. Subsequent cultures were run with a step-increase from 600 to 800 RPM daily, as volumes increased, and showed similar growth and viability to large scale runs, as well as sufficient kLa for high density cultures. Gassing was composed of oxygen on demand through the dip tube, along with CO_2 as needed for pH control. A minimum flow of N_2 was also used to ensure no liquid backflow occurred when CO_2 and O_2 demand was low. pH was controlled at the low end by sodium carbonate addition using the ambr15 liquid handler. pH deadbands were set low (0.05) to attempt to mimic typical large scale bioreactor performance. Substrate additions consisted of bolus glucose and glutamine additions as needed.

2.5 | Bench scale bioreactor system

The Bench scale bioreactor system is comprised of 10 L glass vessels from Sartorius, agitated with axial flow, pitch blade impellers, and operated at 5.3 L working volume. Dissolved oxygen (DO) levels were monitored using optical probes, and were controlled by a combination of micro and drilled-hole spargers, using air and O_2 as supply gases. pH was monitored using electrochemical, glass probes and was adjusted using either CO_2 or sodium carbonate. Overall operation was supervised and maintained using a DeltaV based control system developed by Finesse. An alternating tangential flow filtration (ATF) system was used for perfusion. Flowrates were set within vendor recommended ranges and previously optimized to minimize protein retention.

2.6 | Viable cell density control

Target VCD's were maintained by removing cell culture to waste, often referred to as bleeding. In the 10 L system, this is accomplished by a continuous pump system that removes culture at a constant flow rate. The ambr system was bled either by a bolus cell culture removal step at the time of centrifugation for the media development work, or multiple bleed steps spaced evenly throughout the day for the clone screening. Initial bleed calculations were done manually, creating a high strain on available FTEs. Automating the bleed and incorporating it into the data acquisition macro was accomplished during the clone screening study. This greatly reduced necessary manpower requirements. Three bleeds were used for the automated procedure, with the bleed calculator reducing the VCD to the target value by the second bleed step, and using the final bleed at sample time to maintain VCD, based only on growth. The resulting equation, factoring in growth:

$$B_n = 1 - \left[\frac{\mathrm{VCD}_{\mathrm{target}}}{\mathrm{VCD}_t \times (1 - B_{n-1})} \times e^{-\mathrm{grt}}\right]^{1/(N_B - 1)} \quad (7)$$

B_n = New Bleed Value (%/100).

$\mathrm{VCD}_{\mathrm{target}}$ = VCD target value (VC/mL).

VCD_t = Current VCD (VC/mL).

B_{n-1} = Previous Bleed, equal to previous projected growth rate (%/100).

grt = Projected Specific Growth Rate (dimensionless).

N_B = Number of Bleeds per Day (dimensionless).

2.7 | Offline samples

2.7.1 | Cell counting

Cell counting was performed on a Trypan Blue based NOVA® Biomedical BioProfile FLEX analyzer with the appropriate internal dilution levels to prevent densities above levels recommended by NOVA. External dilutions were also done to lower required sample volumes and prevent unnecessary cell loss.

2.7.2 | Metabolites

The NOVA® FLEX was also used for metabolite analysis on the supernatant removed during the centrifugation stage. No dilution was needed as the levels were within NOVA specs and sample volume was not a concern.

2.7.3 | pH

An offline pH sample was taken three times a week, using a Siemens blood gas analyzer (BGA), to ensure accurate, equivalent pH among all conditions. Currently, the pH check has been automated using Sartorius's available analyzer.

2.7.4 | Titer

Sample retains were obtained along with metabolites during the centrifugation step. Titer was determined using a sandwich ELISA method,[17] and all plates were run with both a standard of drug-substance equivalence purified protein and an in-process control sample from an early reactor run.

2.7.5 | Product quality

The product quality was established using a multiattribute monitoring peptide mapping method. This method is similar to previous published examples.[18,19] Protein samples (62 μg) are dried by centrifugal evaporation and then denatured with 75 μL solution of 6M Guanidine hydrochloride, 500 mM Tris, 1 mM EDTA at pH 8.2. Samples are reduced by addition of DTT (Thermofisher) to a final concentration of 5 mM and incubated at room temperature for 30 min. The sample is then alkylated at room temperature for 30 min after by addition of 5 μL of 500 mM Iodoacetamide (Thermofisher). Prior to digestion, samples are buffer exchanged using Zebaspin filters (Thermofisher) into 50 mM pH 8.0 Tris buffer. Samples are then digested by addition of 6.25 μL of 100 μg tryspin (Proteomics Grade, Roche, CH).

Digestion takes place in a CEM Discoverer microwave (CEM) set to 30°C, 100 W, 15 min digestion time.

Liquid chromatography of the digested sample was conducted using a Acuity UPLC system (Waters) coupled with online ESI-MS/MS on a Q-Exactive (Thermofisher). Chromatography took place using a 2.1 × 150 mm HSS T3 column with 1.8 μm 130 Å particles (Waters). Mobile phases were A (0.1% TFA in LC–MS Grade Water) and B (0.085% TFA in LC–MS Grade ACN). The sample inject volume was 30 μL. Chromatography takes place at 0.4 mL/min, starting at 2% B, with a 50 min linear gradient to 55% B, a 5 min wash at 95% and then a 5 min re-equilibration at initial conditions. The Q-Exactive is operated using an electrospray source at 3,500 V, 300°C. The full MS is performed using the Orbitrap at 70,000 resolution and MS/MS selection is based on top five ions with a dynamic exclusion list. MS/MS analysis for confirmation and investigations of the automatically selected ion uses stepped normalized collision energy set at 25, 30, and 35 V and a resolution of 17,000. Quantification was determined based on the MS data using the Byolgic software (Protein Metrics).

2.8 | Data acquisition and operation

Due to the large number of data points and the need to make adjustments based on offline analyses' (i.e., bleed rates, glucose and additional feeds, etc.), macros were written to extract offline and online data (such as Bioreactor pH, pCO_2) from either CSV files, or from an online Shire data network. The script would then create CSV files that could be uploaded to the ambr15 system as biological values or actionable variables to control feeds, bleeds, sampling, and any other necessary parameters.

2.9 | Statistical analysis

All statistics were calculated using SAS's JMP software package.

3 | RESULTS AND DISCUSSION

3.1 | Model development

As previously discussed, the dynamic portion of perfusion cell culture processes cannot be mimicked absolutely with a noncontinuous system. Idealized MATLAB models were developed to help select operational parameters that would likely mimic bench scale perfusion systems. These simulations were used to model a step change in perfusion and pseudo-perfusion systems, with model comparisons and selections based on proximity to an idealized concentration in true perfusion bioreactor, concentration variation during steady state, degree of overshoot or undershoot during the dynamic phase, and real-world feasibility. Base and glucose additions, along with evaporation loses, were expected to be minimal, and not included in the perfusion calculations. Additionally, the ambr15 reactors have minimum and maximum operating volumes of 8 mL and 15 mL, respectively. This limits the ratio of starting volume to volume added; however, it was found that reactors could be operated at a low volume of 7.75 mL and a high volume of 16 mL, if sufficient antifoam is added. These constraints were included in the simulations. A target perfusion rate of 1 vessel volume of reactor exchanged per day (1 VVD) was used for the comparisons. From these models, two gave the closest proximity to true perfusion and were selected for screening, with a third being used as a control; a more traditional, tubespin-like approach, with all media recovery and resupply done in a single bolus exchange at the time of centrifugation; a volume doubling model, where the volume would double in a 24 hr period by way of equivalent, periodic media additions. The cells would be spun down, and half the volume would be removed, so that the addition process could begin again; and a hybrid model, that would have a specified ratio of bolus versus periodic media exchange and addition.

For the variable volume mimics, Equations (5) and (6) were used for both volume doubling and hybrid systems between centrifugation exchanges, assuming discrete, step changes in volumes with continuous changes in metabolites and substrates. Periodic media volumes were assumed to be added in 10 equivalent, bolus additions. Post-exchange concentrations were modeled and calculated as follows:

3.2 | Volume doubling

For the volume doubling model, the initial volume is 8 mL at day 0, with equally spaced doses of 800 uL done until the reactor volume reaches 16 mL on day 1. Fifty percentage of the total volume/metabolite is removed at the centrifugation step, and the periodic doses begin immediately. The working volume is considered 8 mL at a perfusion rate of 1 VVD, and the concentration is changed by only the addition of 800 uL, or 8 mL/10 doses, after the centrifugation. This is repeated daily.

3.3 | Hybrid

For the hybrid model, the initial volume is 9.8 mL at day 0, with 6.2 mL added over 24 hr in equal doses, giving a volume of 16 mL on day 1. About 9.8 mL is removed after centrifugation, leaving 6.2 mL. This volume is resuspended with 1.55 mL fresh media, and the first periodic dose of media is delayed by 3 hr and 48 min, or 1.55/9.8 days. Media (8.25 mL) is then added in 10 doses, or 825 uL per dose. The final volume is 16 mL before centrifugation, with 9.8 mL considered the working volume, and 9.8 mL removed daily to target 1 VVD.

3.4 | Model controls

Equation (6) was used to represent a true perfusion, bench scale system. The tubespin model is a single, 12 mL exchange by removing as much of the 12 mL as possible and resuspending with 12 mL of fresh media after centrifugation.

The model trends (Figure 1) show volume doubling models gave the least amount of variation, but diverged from true perfusion during the dynamic phase, while a hybrid approach of bolus exchange and periodic addition trended well with all phases of true perfusion, but

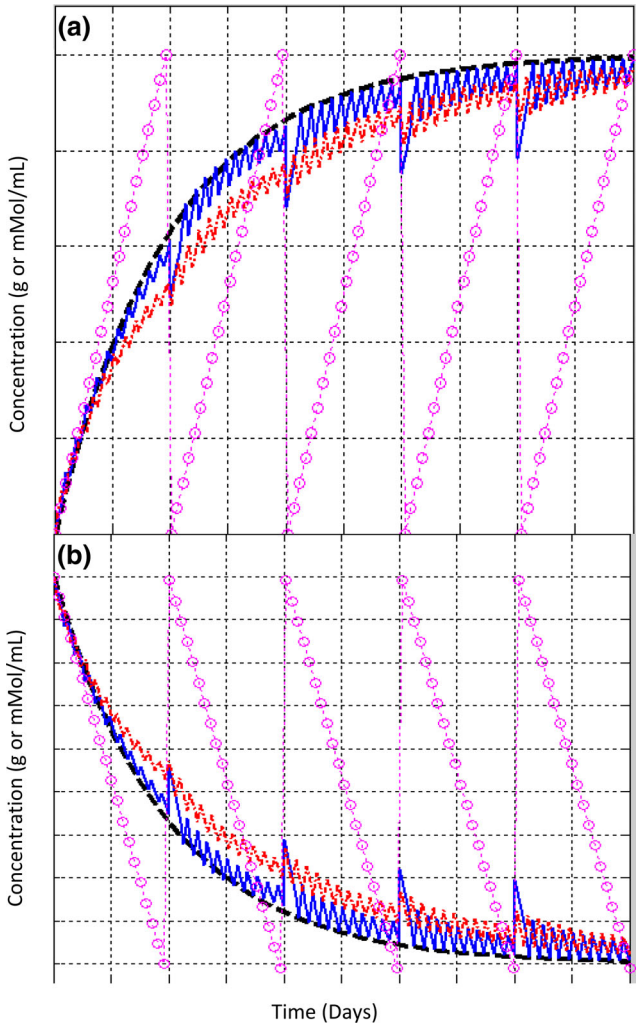

FIGURE 1 Theoretical step changes in (a) generation of metabolites and (b) consumption of substrates in various perfusion models. MATLAB rendered profiles for metabolite and substrates in four perfusion systems: (– ■ –) volume doubling, (o) bolus exchange, (—) hybrid, and (– –) true perfusion. Initial concentrations and generation rates were varied and compared to true perfusion levels. The volume doubling, hybrid, and tubespin models used Equation (5) between centrifugation steps, but used different values for flow rate of media, working volumes. The true perfusion model used Equation (6) throughout. Profiles show the hybrid approach more closely matches the true perfusion trend, but gives large swings in concentrations after centrifugation/resuspension. These spikes are not seen in the volume doubling process

showed greater concentration perturbation due to lower initial volumes after exchanges. For POC runs, both models were employed and compared to the more traditional bolus exchange approach.

3.5 | Model operation

Operation consisted of initial seed, followed by five daily steps performed in sequence. The order of operation was as follows: (a) Begin periodic media additions. For the volume doubling model and the hybrid model, 10 periodic media additions were scheduled using ambr's liquid handler. (b) 24 hr from media start, centrifuge reactors to retain cells. (c) Remove spent media, either manually, or using a liquid handler. (d) Add bolus media. For single bolus models, this was the entire exchange amount. For the volume doubling model, this was equal to the periodic media addition volume, and for the hybrid, it was equal to a volume sufficient to ensure that the liquid level was above the top of the impeller. (e) Resuspend culture.

3.6 | Model comparison

Control culture was subjected to the original three models (volume doubling, single bolus, and hybrid) to test suitability and confirm the best method moving forward. Conditions were run in duplicate and compared to 10 L control reactors data. Both variable volume models showed a saw tooth pattern in the pH profile, due to periodic media additions. This generally did not exceed ±0.05 of setpoint for the majority of the run, once cells were resuspended. DO control was also affected, but values were less than ±20% DO of setpoint, and was not expected to have an effect on culture performance. All systems saw spiking or crashing during the centrifugation of the cells, but control returned within minutes of resuspension. The hybrid model correlated well with 10 L data, and consistently mirrored cell growth and health (Figure 2). The single bolus model was difficult to maintain, due to issues removing >90% of volume without disturbing the pelletized cell mass. This could feasibly be corrected by doing multiple centrifugations, but would result in an increase in necessary FTEs to operate the system. The volume doubling model performed similar to both the true perfusion 10 L scale and the hybrid perfusion mimic early on. However, the culture growth slowed near day 7, and viability began dipping on day 9. The slow washout performance of this model when the system is in dynamic flux may have resulted in prolonged exposure to inhibitory metabolites, or extended time without needed nutrients, while the culture was transitioning to a more steady state condition. While this may not be the case with all cell lines or processes, for the sake of robustness and brevity, the hybrid model was confirmed as the optimum methodology, moving forward.

3.7 | Media development

The hybrid model was used for work concerning perfusion media development for future Shire projects. Four variations of a previous high preforming formulation were run in triplicate (Figure 3). Due to previous experiences with the system, a lower agitation rate was selected for immediately postmedia exchange times, with the intent to limit interactions with surface agitation effects. As cell densities increase, agitation may be stepped up with daily volume increases, as needed. RPMs from 600 to 900 were used and set to maintain a power to volume ratio (P/V) of 44–72 W/m^3.[20,21] This is on the low end of what the vendor recommends, but is about threefold higher than values we typically see with other reactor systems. This is likely required due to the lack of a microsparger, and the low residence times of the bubbles generated by the open pipe, meaning that most of the oxygen transfer is coming from the surface area and headspace.

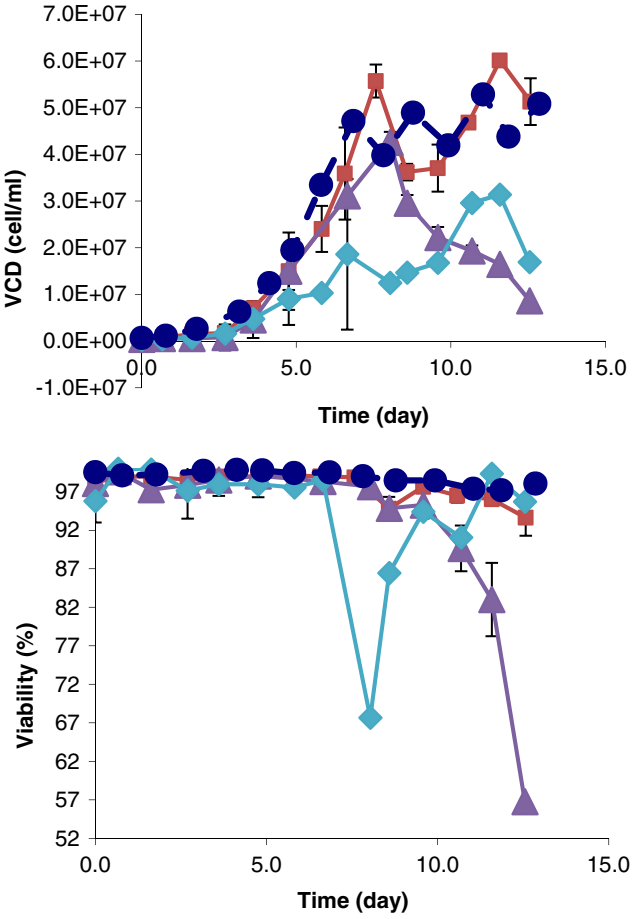

FIGURE 2 Model comparisons. Results of culture performance for the four perfusion systems. (◁) Volume doubling, (♦) bolus, (■) hybrid, (●) true perfusion. A 50E6 cell density was targeted using automated bleed. Only the hybrid model required continued bleeding after bleed initiation. The viability and VCD drops in the tubespin model were likely due to issues encountered during media aspiration. The cell pellet for the bolus model was subjected to high shear and loss at this step, due to the low volumes that were reached during exchange. VCD, viable cell density

For very high densities, the nitrogen mass flow controller on the ambr15 system can be supplied with oxygen, to increase the overall O_2 gas flow rate.

All other POC parameters were repeated. Bleeding was accomplished by a single, bolus removal of culture at the time of sample.

Cell densities >90E6 were attained for some conditions, with viabilities generally >90%, indicating that the perfusion mimic method used here was a significant improvement over previously reported high throughput methods.[22,23] Current perfusion cell culture process development is trending to higher cell culture densities,[24,25] and these results indicate that the ambr15 perfusion centrifugation model could handle densities in this range.

3.8 | Clone screening

Twenty clones were run in duplicate, compared as described above, and summarized in Table 1. Rankings were assigned based on equally weighted results from specific productivity in picograms per cell per day (PCD), growth rates, and ending viability. Due to the number of conditions and maximum speed at which the ambr15's liquid handler can operate, manual media removal was needed to prevent culture from being left without control for extended periods of time. Culture was run for 12 days to ensure steady state was reached, with 10 doses of media during the periodic media addition step. The hybrid approach was chosen for screening, due to theoretical proximity to true perfusion, and slight growth differences from reactor conditions observed during the demo run (data not shown). Controls were run using the volume doubling and single bolus model as well, to ensure these differences could not be attributed to other issues.

Vials were thawed into the 250 mL shake flask scale and passaged once to ensure no thaw effects translated to bioreactor cultures. Cultures were seeded at 5e5 and perfusion was started on day 2. Most cultures reached >20E6 by day 6 and a bleed was initiated on day 7, using a bleed strategy based on Equation (7) to target 50E6 VC/mL, as described above. Several clones maintained viabilities >95% for the duration of the runs. Cell counts were done daily, and retains and metabolite data was collected from supernatant samples after centrifugation. NOVA Flex counts were conducted on three machines due to the number of cell counts needed (48 per day) and the time the

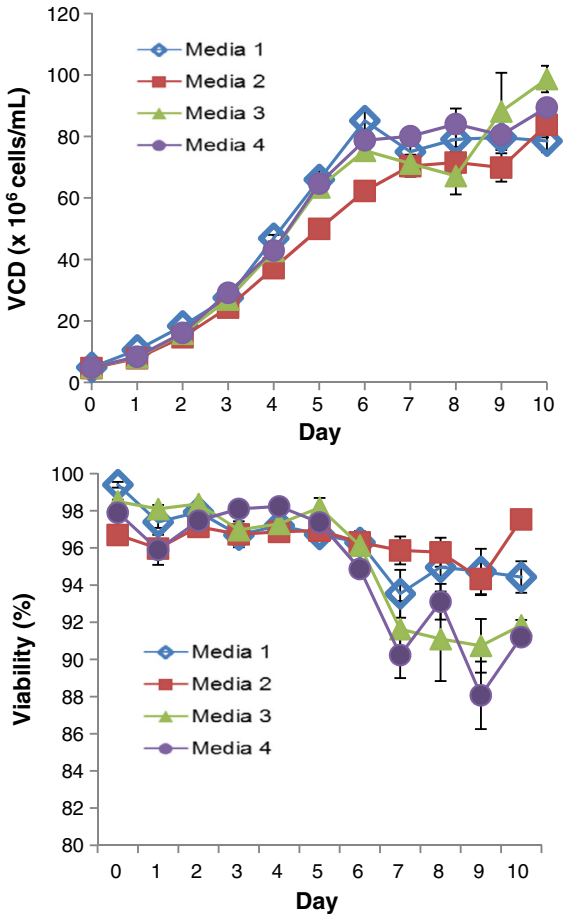

FIGURE 3 Media study. Multiple media were compared for use as a platform. Cell densities of >95E6 were attained, with best conditions ending in >95% viability

TABLE 1 Clone comparison

Clone	Avg SGR	PCD	Average ending viability	Rank score	Rank
Control	0.84 ± 0.11	1.4	95.4 ± 0.25	1.13	7
Bioreactor control	**0.68**	**1.7**	**97.6**	**1.14**	-
F1	0.67 ± 0.02	1.1	91.4 ± 0.93	0.67	17
F10	0.6 ± 0.04	3.2	88.8 ± 8.61	1.74	2
F11	0.72 ± 0.04	0.9	94.4 ± 1.06	0.59	20
F12	0.7 ± 0.05	1.8	90.5 ± 3.95	1.12	8
F14	0.73 ± 0.04	1.1	94.1 ± 2.92	0.73	14
F15	0.83 ± 0.11	1.6	90.7 ± 0.42	1.22	6
F16	0.64 ± 0.02	2.9	87.8 ± 2.09	1.66	3
F17	0.72 ± 0.08	1.0	97.8 ± 0.12	0.68	16
F18	0.61 ± 0.06	1.3	95.1 ± 1.41	0.77	12
F19	0.73 ± 0.01	1.2	90.8 ± 0.85	0.79	11
Bioreactor F19	**0.65**	**1.6**	**85.8**	**0.87**	-
F20	0.66 ± 0.04	0.9	95.8 ± 0.36	0.58	21
F21	0.61 ± 0.06	1.3	95.7 ± 0.61	0.74	13
Bioreactor F21	**0.59**	**1.4**	**90.4**	**0.73**	-
F23	0.57 ± 0.02	1.6	93.5 ± 1.45	0.85	9
Bioreactor F23	**0.54**	**1.6**	**91.3**	**0.81**	-
F24	0.69 ± 0.04	1.2	93.8 ± 2.85	0.79	10
F28	0.64 ± 0.01	2.3	92.4 ± 1.45	1.33	5
Bioreactor F28	**0.6**	**1.7**	**83**	**0.84**	-
F48	0.76 ± 0	0.9	98.6 ± 0.53	0.70	15
Bioreactor F48	**0.74**	**1.2**	**99.7**	**0.89**	-
F5	0.64 ± 0.03	1.1	95.9 ± 0.33	0.66	18
F6	0.7 ± 0.08	2.6	87.4 ± 4.72	1.60	4
Bioreactor F6	**0.54**	**3.1**	**94.5**	**1.57**	-
F7	0.6 ± 0.08	3.1	96.6 ± 3.03	1.77	1
Bioreactor F7	**0.65**	**3.0**	**99.5**	**1.97**	-
F9	0.66 ± 0.06	1.1	90.6 ± 4.33	0.66	19

Abbreviation: PCD, picograms per cell per day.
Bold values indicate Bench scale bioreactors.

Flex system requires per count (7 min). A simple ranking assessment was conducted using equally weighted results for PCD, average ending viability, and average specific growth rate. Table 1 and Figures 4 and 5 give a detailed summary of clone performance and ranking. This data was used to determine best performers.

Bench scale bioreactors were compared to ambr15 profiles for cell growth, viability, PCD, and Titer, helping to establish scale reproducibility and validate the model.[26] All reactors were targeted for 50E6 for control. Bleeds were performed by a continuous pump in 10 L, bench scale, while ambr15 reactors were bled using three bulk samples according to Equation (7). Titer was run on only one of the two duplicates to prevent undue burden on analytical development resources. VCD, viability, metabolites, and titer were similar across scales and gave good confidence in the ambr15 model. Viability trends matched particular well, with viability declines evident at equivalent times across multiple clones (see Figure 6). Some cell count variation and anomalies occurred on days 7 and 8, likely due to the manner of manual sample handling and dilution, along with some machine to machine variability. This was corrected in subsequent experiments by using automated dilutions and dedicated equipment. There also appeared to be a delay in the growth of clone 7 in comparison to the 10 L scale. The growth rate was observed to increase proportionally to culture duration, so the increased number of expansion steps, from vial thaw to reactor inoculation, that are needed to seed a 10 L reactor may have had an effect. The growth delay also impacted metabolites and titer, with trends shifting to the right while profile slopes remained equivalent. Glucose levels were somewhat higher in the ambr conditions (Figure 7), likely due to a delay between sample and addition (as much as 8 hr). This may have had an effect on lactate levels as well, as peak levels appear to be lower on the ambr; however, all other metabolites and titer appear to overlap from 10 L to ambr15 scale (Figures 7 and 8).

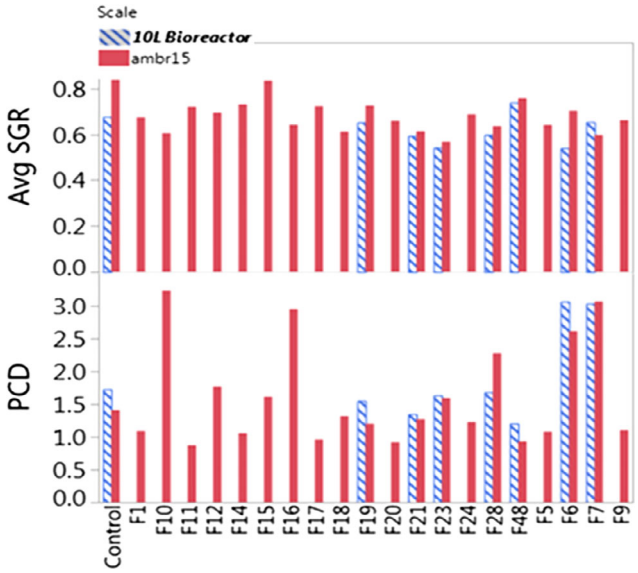

FIGURE 4 Comparison of clone performance. Average specific growth rate of the first 7 days was compared across clones and scale. Most clones performed similarly in bench scale and the perfusion mimic. This was also true for specific productivity in picograms per cell per day (PCD)

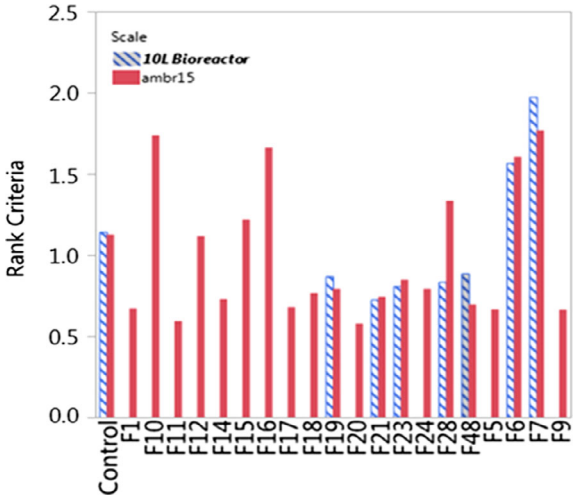

FIGURE 5 Clone ranking. A simple ranking of clones, as discussed below, was used to compare the performance of clones in the perfusion mimic to bench scale

Product quality was assessed from two clones and evaluated for two sensitive critical quality glycosylation attributes of the conjugate on a mol/mol basis (Figure 9). Product quality 2 was especially susceptible to process conditions, and requires micro environmental homology between corresponding runs for accurate interpretation of screening results. This had previously lead to concerns over data generated by other perfusion mimics and screening tools that did not actively control critical parameters, and had more operational discrepancies with bench scale perfusion systems. Both the 10 L and ambr15 mimic showed clonal differences in product quality, but varied by <5% between scales, affirming the use of this system for product quality assessments.

A comparison of average specific growth rate for the first 7 days, along with PCD, show that using deep well plates for clone selection can result in elimination of possible high performing clones (Figures 4 and 5). Neither F10, nor F16 was selected for 10 L reactor runs due to lower performance without pH control. Comparing clones using a crude ranking as discussed above, F28 appeared to perform better in the ambr mimic than at bench scale due to higher PCD. This is in contrast to the overall results, however, as clones run at bench scale

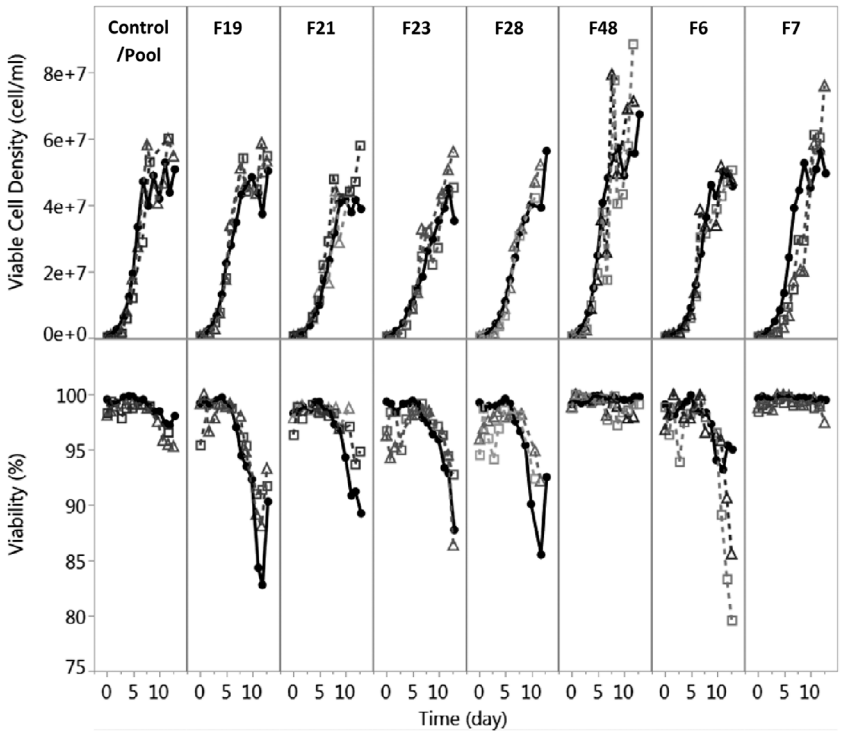

FIGURE 6 VCD and viability profiles. (●) Bioreactor, (-□-) ambr15 duplicate A, (-Δ-) ambr15 duplicate B. Bioreactor trends matched ambr15 perfusion mimic results. There was a growth delay in clone F7, associated with a lag that dissipated proportional to cell generations, and not attributed to the perfusion mimic. VCD, viable cell density

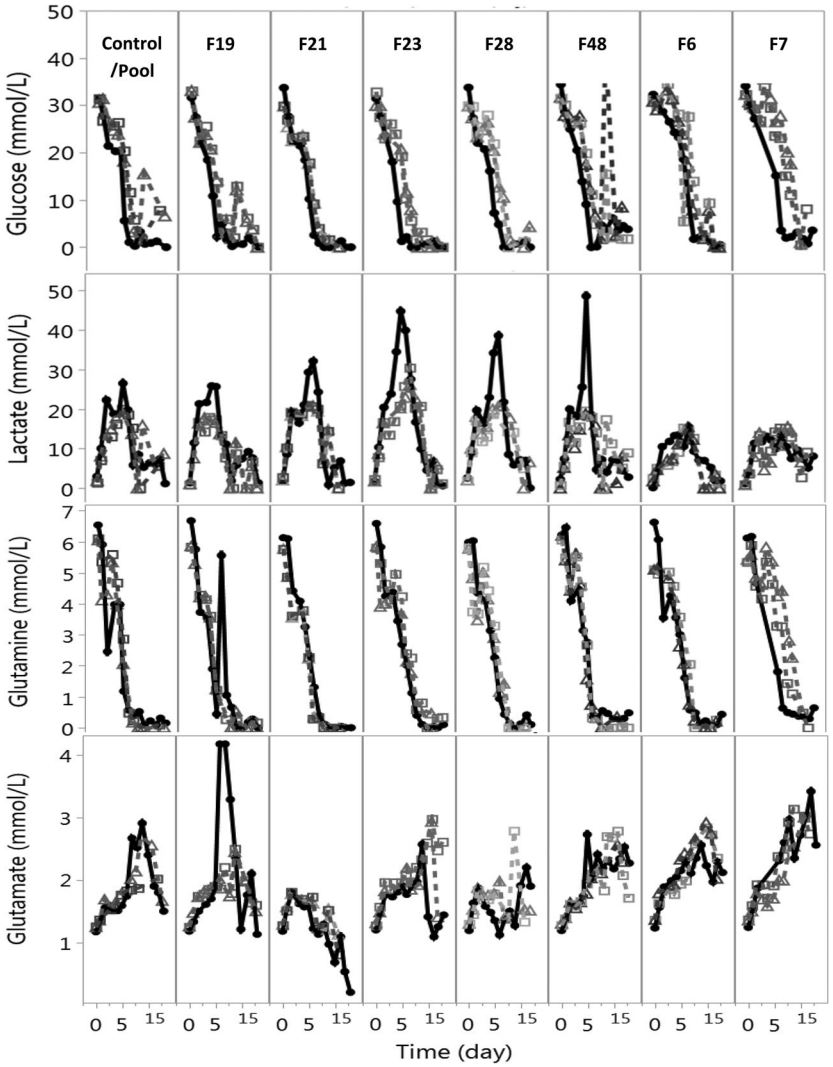

FIGURE 7 Metabolite profiles from ambr15 duplicates and 10 L reactor satellites. (●) Bioreactor, (-□-) ambr15 duplicate A, (-Δ-) ambr15 duplicate B. An overlay of metabolite profiles shows good correlation between ambr15 scale and benchtop bioreactors. Glucose feed strategy differences accounted for some discrepancies in glucose and lactate profiles. Additionally, growth delays in clone F7 that were not attributed directly to the perfusion mimic accounted for the consumption shifts in the F7 trends

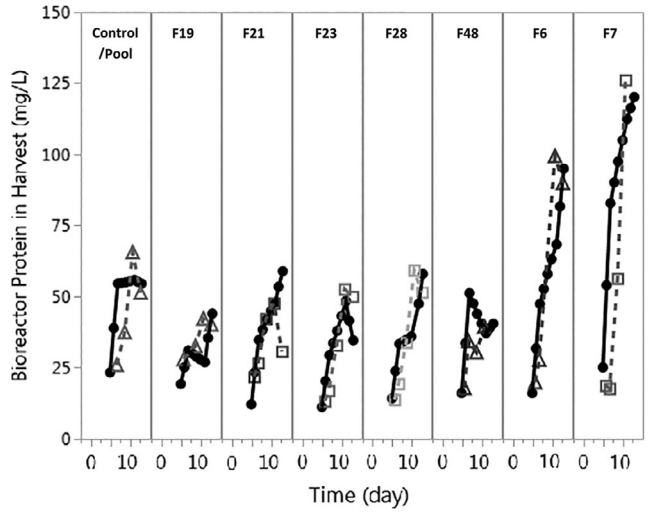

FIGURE 8 Titer profiles from ambr15 duplicates and 10 L reactor satellites. (●) Bioreactor, (-□-) ambr15 duplicate A, (-Δ-) ambr15 duplicate B. Only one, randomly selected duplicate was analyzed for titer from each clone, due to resource constraints. Profiles overlapped across scales, with a growth delay in clone F7 resulting in a similar delay in titer accumulation

performed similarly in ambr15, with three of the top four clones run at both scales being the same.

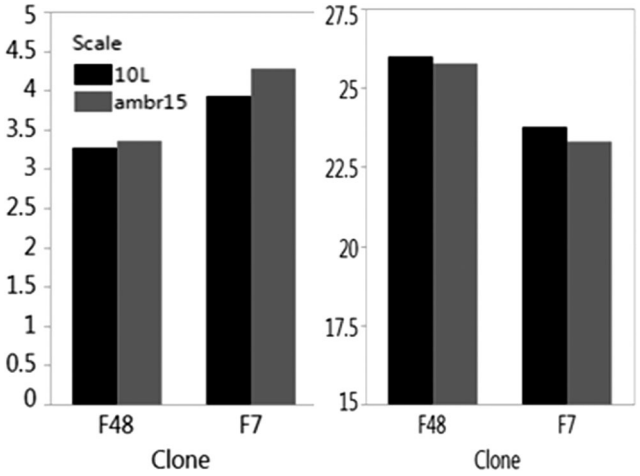

FIGURE 9 Product quality: two clones were analyzed for product quality (PQ). Bench scale clone-to-clone PQ differences were replicated in the ambr15 perfusion mimic, with scale differences less than 5%

4 | CONCLUSIONS

By adapting the ambr15 to operate as an optimized perfusion mimic, a high throughput screening platform was established that was capable of handling perfusion cell culture processes with the associated high densities that are currently being targeted. The process was applied in two different case studies (media development and clone screening) and showed excellent fidelity between ambr15 and the bench scale bioreactor system. The use of the ambr15 gives a significant improvement over tubespin, shake flask and deep-well plate perfusion mimics, due to in-line pH and DO control[18] and a better kLa to shear rate ratio.[27–29]

This is especially crucial for high density perfusion cultures that will have higher oxygen demands and may be damaged by the RPM required to supply sufficient kLa in a standard orbital shaking format.[30,31] With consistent geometry from high throughput to production scale, transfer of process parameters across all scales is significantly streamlined.[20,31]

Prior to macro writing and when utilizing manual media exchanges, operation of the 48 reactor system took comparable effort to a tubespin experiment run with about half as many conditions, but with improved probability of repeatable performance, post-scale up. When using VB scripts and automated media exchanges through use of the ambr15 liquid handler arm, required FTEs could be reduced to about half. A liquid handler was configured to process the ambr15 reactors, conducting the bolus media exchange and performing the cell count dilutions. This resulted in a further improvement in efficiency, reducing FTEs to approximately 0.25 FTEs per day to operate 48 reactors in parallel.

The result of utilizing standard bioreactor geometry with high throughput bandwidth allows for perfusion experimenters to execute higher powered DOEs, definitive screening designs, and response surface methodologies which were previously performed in bench scale bioreactor systems. Additionally, the control of pH allows for experiments and screening studies not possible with previous high throughput perfusion mimic methods.[32] The introduction of the Ambr250 perfusion system by Sartorius Stedim gives many of the abilities stated above, but with the promise of true perfusion. However, the system must still demonstrate that it can handle the high densities seen in current perfusion processes, with significant reduction in FTE needs, to justify its steep price tag (approximately $1 million) and high cost of consumables. Additionally, neither this perfusion mimic, nor the ambr250 perfusion system contain microspargers, and this is a potential source of differences for long duration, high density bioreactor runs, where the shear from microbubbles can have an impact on cell culture performance.[33] Addressing these concerns with the perfusion mimic is ongoing, but it has already demonstrated cell culture performance fidelity to larger scales, and the flexibility of experimental application, confirms the value of this system as a perfusion process development tool.

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