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Approaches to Establishing a Perfusion Mimic Process in Ambr® 15 Cell Culture Generation 2

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Introduction

Perfusion processes have gained popularity due to the possibility to reach higher cell densities and extend the protein production phase. Increasingly, these processes are adopted at the early stages of process development to ensure that clones, media, and feeds that are taken forward are selected under the most representative conditions. To date, this method of processing has been a challenge at the microbioreactor scale due to the infeasibility of implementing a hollow fiber filter at this scale. Therefore, alternative methods of cell separation and media exchange are needed.

The Ambr® 15 Cell Culture system has been shown to provide improved results when compared to shake flask or shaking plate cultures from the high level of automation combined with reliable and independent process control for pH and DO. With low working volumes, the small scale stirred bioreactor vessels offer considerable advantages for screening of cell lines and media when running intensified processes, particularly in terms of costs.

Find out more: www.sartorius.com/en/products/fermentation-bioreactors/ambr-multi-parallel-bioreactors/ambr-15-cell-culture

Materials and Methods

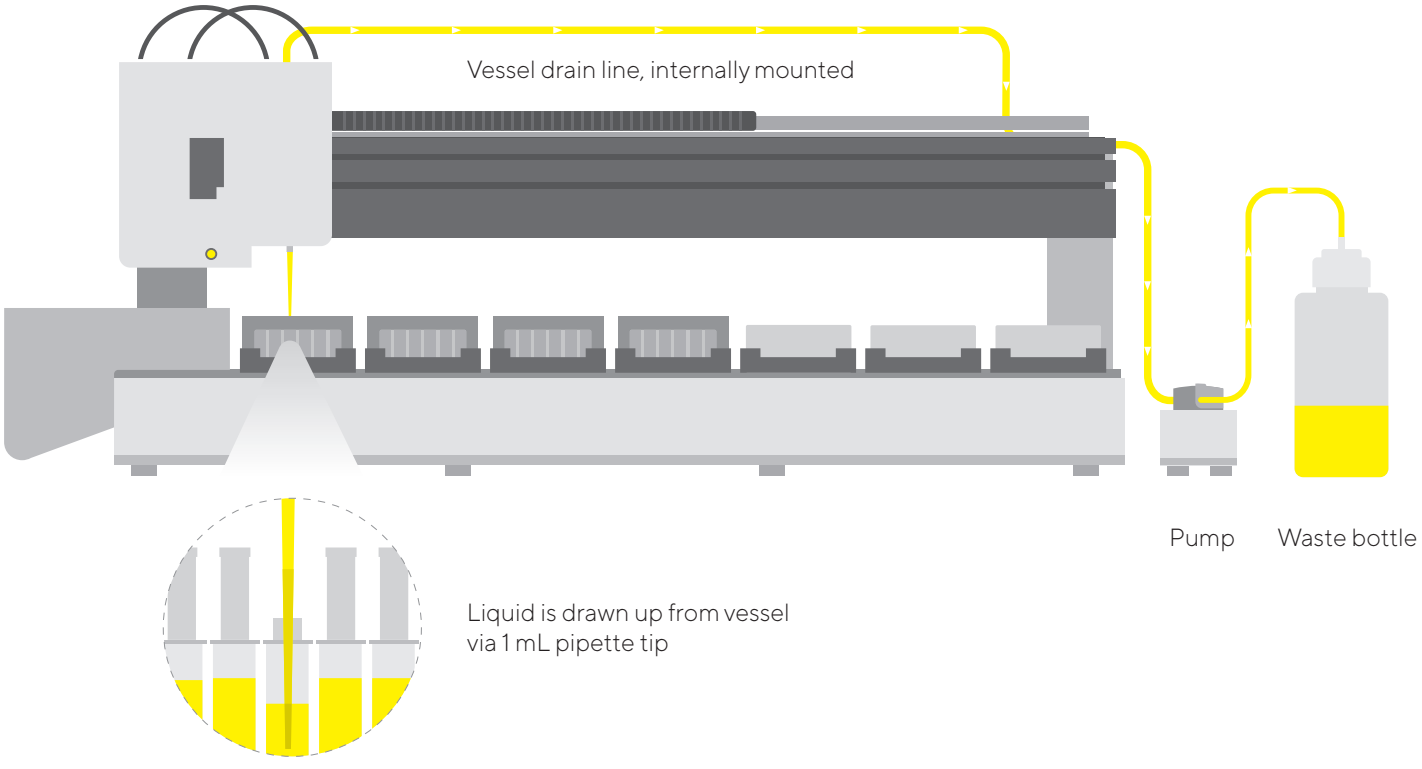
Here, two perfusion mimic protocols have been established in the Ambr® 15 Cell Culture system to show how different approaches can be applied to facilitate understanding of intensified processes at the microscale. Both methods follow traditional perfusion theory of exchanging spent media for fresh media at a semi-continuous rate, while retaining cells in the bioreactor. However, without the use of a hollow fiber filter, each method separates cells and exchanges media in different ways, and can be utilized depending on the needs of the specific cell line and resources of the laboratory.

New capabilities have been introduced into the Ambr® 15 Cell Culture Generation 2 system that are better able to support perfusion mimic applications at the microscale. These include the new Rapid Vessel Drain functionality (see Figure 1), which allows removal of large volumes of culture or spent media directly from each bioreactor in a single step; and additional software steps to automate vessel draining and refill of cultures with fresh medium. Both of these are used in the implementation of the protocols outlined here.

In both the centrifugation method and the cell settling method, sparged Ambr® 15 Cell Culture microbioreactor vessels were inoculated at a seeding density of 2.5e6 cells/mL and at a working volume of 10 mL. A perfusion rate of 1 vvd (vessel volumes per day) was used, with a target cell density of 20e6 cells/mL. Cell bleeding was performed to maintain the target cell density using the Rapid Vessel Drain feature of the Generation 2 liquid handler, along with the Passage Culture Vessel step in the software that uses an equation to determine the volume of culture to be removed. The Passage Culture Vessel feature of the Generation 2 system is intended to more easily perform cell passages within the microbioreactors, and since the concept to remove culture and replace with fresh media is the same as in cell bleeding, this feature can be applied to perfusion mimic applications, as done here.

The experiments were run for approximately two weeks with samples for cell counts, metabolites, and titers taken daily. The Cedex HiRes was used for cell counts, and the Cedex Bio analyzer was used for titer quantification and metabolite analysis. A mAb-producing CHO DG44 cell line was grown in chemically defined, animal origin free (AOF) media, both developed by Sartorius Stedim Cellca.

Figure 1. Rapid vessel drain schematic for Ambr® 15 Cell Culture 48 vessel system



Daily additions of antifoam are needed to preemptively prevent foam from accumulating on the surface of the culture. Furthermore, maintaining positive pressure into the vessels is essential to prevent backflow of culture through the sparge tube into the clamp plate, and potentially damaging the system. A constant minimum flow of air as part of the gassing strategy should be implemented for this purpose.

See Figure 2 below for a summary of the setpoints used during set-up of the experiments.

Process Parameter	Setpoint
Working volume	10 mL
DO setpoint	60%
pH setpoint	7.00 +/- 0.10
Stir speed	1200 rpm (increased to maintain DO setpoint if needed)
Gassing strategy	O_2 on demand up to 0.91 mL/min Constant air at 0.10 mL/min CO_2 on demand up to 1.24 mL/min
Foam control	Daily 20µL additions of 2% Antifoam C

Figure 2. Process parameters for both perfusion mimic methods evaluated.

Centrifugation Method

Centrifugation is one method of separating cells from the supernatant, whereby vessels are centrifuged to create a cell pellet, and the remaining supernatant is exchanged with fresh media at a whole vessel volume once per day (1 vvd). The daily cycle of steps for this method began with taking samples from the vessels and analyzing for pH, O_2 and CO_2 concentrations, cell count, cell viability, and metabolites. Process controls for pH, DO, stirring, and temperature were then paused on the Ambr® 15 software to allow for the removal of vessels from the system, which were placed into specially made Sartorius adapters that fit into Centrisart® G-16 and G-16C centrifuge buckets. These centrifuge adapters have the capacity to fit three Ambr® 15 vessels each, allowing twelve vessels (an entire culture station) to be centrifuged at the same time.

Vessels were centrifuged for five minutes, then returned to the culture station, and the clamp plate replaced. Using the liquid handler, samples were taken for titer analysis from the supernatant. Since the angled design of the centrifuge adapter ensured that the cells aggregated in the corner of the vessel furthest away from the pH and DO spots, and also away from the sampling port, the sampling of the supernatant was easily performed with minimal disturbance to the cell pellet. As the cell pellet grew larger due to the increased total amount of cells over the duration of the experiment, the relative centrifugal force (RCF) used to spin down the vessels was also increased from 200 g up to 400 g

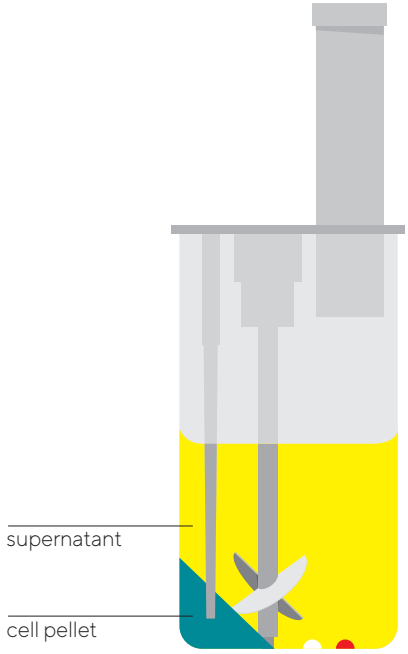


Figure 3. Cell pellet position in Ambr® 15 microbioreactor following centrifugation

to ensure that the cells were packed tightly in the formed pellet, and able to be effectively retained in the vessel.

Following sampling for titer, the remaining supernatant was removed using the Rapid Vessel Drain step in the software, which allowed for a quick (~5 seconds) aspiration from the vessel, leaving only the cell pellet. Fresh media was added using the liquid handler to refill each vessel to 10.125 mL. The target resuspension volume was 10.125 mL to allow for a 100 µL sample volume to be taken for cell count, post-media exchange and post-bleeding, along with the 25 µL excess taken by the liquid handler when sampling. Process controls for pH, DO, stirring (ramped in increments of 100 rpm every 30 seconds), and temperature were then resumed. The steps in which the vessels were out of process control were performed in rapid succession to minimize the duration of process disruption.

After media exchange, the culture was stirred for 15 minutes to ensure that the cell pellet had sufficiently been resuspended into the media. Once a homogenous culture has been achieved, cell bleeding was carried out if the viable cell count for the day exceeded 20e6 cells/mL. The Passage Culture Vessel step in the software was utilized to perform cell bleeding, which removed a volume of the culture from the vessel and replaced it with fresh media to reach a specified target VCD (viable cell density). Additional glucose was then added to the vessels if required, as well as a 20 µL addition of antifoam. An antifoam addition is crucial soon after media resuspension to re-supply the culture with a foam-controlling agent, as the media exchange completely strips the culture of any remaining antifoam.

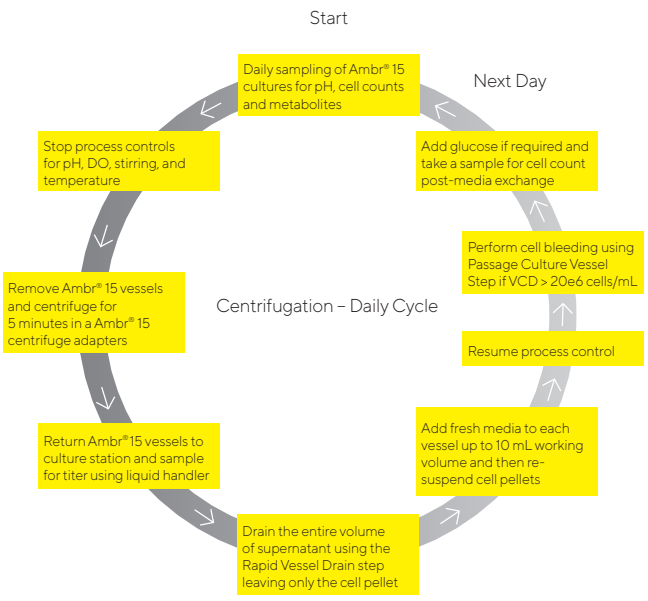


Figure 4. Daily cycle of steps performed in the centrifugation method

After the steps were completed for the day, a 0.1 mL sample was taken from each vessel to confirm the cell count post-media exchange and post-bleeding. The vessels were also checked periodically to ensure that the DO setpoint was maintained; if not, the stir speed was gradually increased in 100 rpm increments until the DO setpoint was achieved and remained steady.

It is important to note that in later experiments, an extra step was included in the daily cycle that improved the accuracy of the cell counts (data not shown). In these experiments, the weight of the cell pellet was determined in order to add a more precise media resuspension volume, resulting in a more controlled culture volume and subsequent cell count. To determine the volume of the cell pellet, process controls were paused again after the entire supernatant was drained from each bioreactor vessel, leaving only the cell pellet. Vessels were removed and weighed. The weight of each vessel minus the weight of an empty vessel resulted in the weight of the cell pellet:

Cell pellet (g) = vessel with cell pellet (g) - empty vessel (g)

Equation 1. Determination of cell pellet weight.

After weighing each vessel and placing back into the system, fresh media was added using the liquid handler to refill each vessel to 10.125 mL. This was done using the following equation, assuming 1 g/mL density of the cell pellet:

Fresh media for resuspension (mL) = 10.125 mL - weight of cell pellet (g)

Equation 2. Determination of volume of media needed for resuspension.

Following media exchange, the daily process flow continued where controls for pH, DO, stirring (ramped in increments of 100 rpm every 30 seconds), and temperature were resumed; and the culture was stirred for 15 minutes before cell bleeding was performed.

Cell Settling Method

For the cell settling method, stirring in the vessels was stopped to allow time for cells in the culture to settle to the bottom of the bioreactor before exchanging a portion of the supernatant with fresh media. Initial experiments, testing various settling times and different number of media exchanges performed per day, indicated that a settling time of 35 minutes and a media exchange three

Figure 5. Ambr® 15 centrifuge adapters, available as a set of 4

times per day provided the optimal conditions to minimize cell loss and maintain good cell viability. This meant exchanging 1/3rd of the working volume every 8 hours for a 1 vvd perfusion rate. To undergo this process, the following steps were performed at a frequency of three times per day.

Firstly, process controls for pH, DO, and stirring were paused, and a minimum gas flow of 0.0001 mL/min air was set in the software, to allow the cells to settle in the vessel. This minimum gas flow of 0.0001 mL/min air was maintained through the sparge tube to prevent liquid backflow into the clamp plate. This ensured that no culture was lost during the cell settling step, and also prevented damage to the system itself.

After a duration of 22 minutes from the start of settling, a sample was taken for titer from each bioreactor using the liquid handler, and transferred to a separate dedicated well for each vessel in a 24-well plate. To prevent the pipette tip from disturbing the cells settled at the bottom of the vessel, a liquid handling script was used to take the sample from a height of 8.5 mL, meaning a volume was removed in order for 8.5 mL to remain in the vessel. After 35 minutes from the start of settling, the Rapid Vessel Drain step in the software was utilized to remove the remaining top 1/3rd of the culture, leaving 6.5 mL of culture in the bioreactor. Fresh media was then added back to each vessel to take the working volume back to 10 mL, and process controls for pH, DO, and stirring (ramped in increments of 200 rpm every 5 seconds) were resumed. The total settling time from the start of cell settling to the media refill was 55 minutes for an entire culture station comprising of twelve vessels. The cultivation then continued for approximately eight hours before occurring again.

Steps for culture removal and media addition were written into the Ambr® 15 software and automated to be performed every eight hours, which included overnight activities with no manual interactions or supervision required. Daily samples for pH, O_2 and CO_2 concentrations, cell count, cell viability, and metabolites were scheduled to be taken between the cell settling events, as was the cell bleeding. Cell bleeding was performed if the viable cell density for the day exceeded 20e6 cells/mL using the Passage Culture Vessel step in the software, which exchanged a certain volume of culture with fresh media to reach a specified VCD, using the Rapid Vessel Drain. A sample was taken from each vessel to confirm the cell counts post-bleeding. Glucose levels were also checked to determine if extra additions were needed, and 20 µL antifoam was added to each bioreactor for foam control. The 24-well plate containing the aliquots for titer samples that were taken overnight was then removed to be analyzed, and the 24-well plate was replaced with a new one for the next night's sample aliquots.

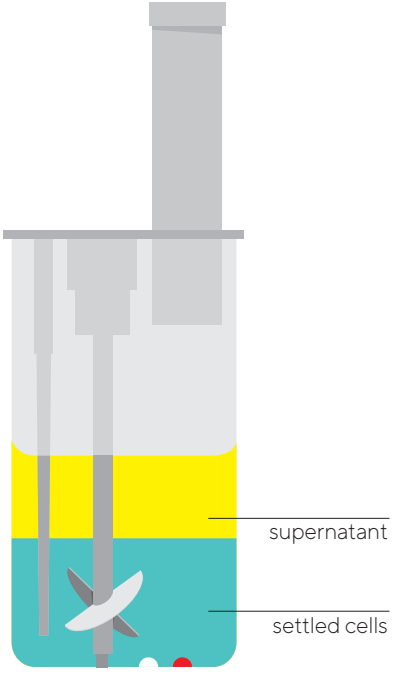


Figure 6. Ambr® 15 microbioreactor following cell settling

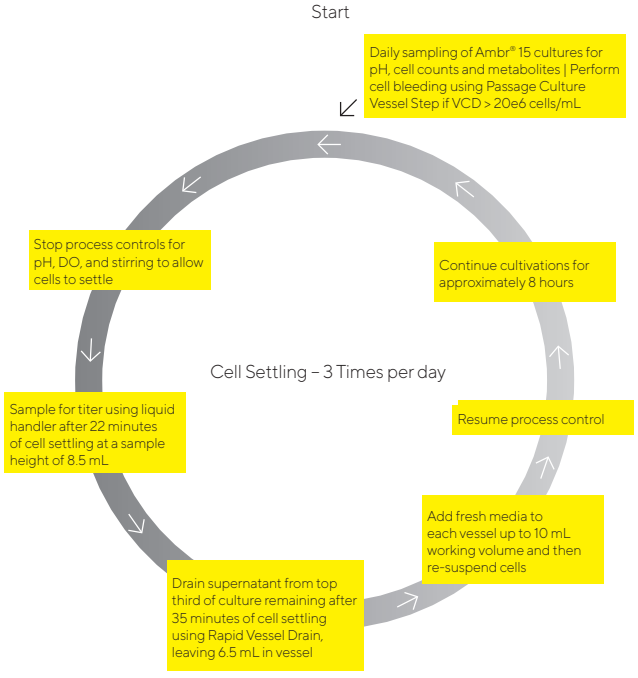


Figure 7. Daily cycle of steps performed in the cell settling method

Results

Centrifugation Method Results

Cell growth and viability profiles from cultures utilizing the centrifugation method are shown in Figure 8. After the initial growth phase, cultures were able to be maintained at the target cell density of 20e6 cells/mL. While some variability can be seen in the growth profiles between days 5 and 8, a higher level of consistency is achieved later in the process following changes made to improve performance during the experiment. One of these changes was to increase the relative centrifugal force (RCF) during the centrifugation of the cells as the total amount of cells increased, which helped to create a more tightly packed cell pellet, preventing cell loss at this step. Another change was to allow stirring of the culture for at least 15 minutes after media exchange was performed to ensure that the culture was well-mixed following resuspension of the cell pellet.

Both of these changes were implemented in response to observations seen during the course of running the experiment, and appeared to improve the reproducibility of the growth profiles. In addition, consideration of the weight of the cell pellet during the media exchange step was later included in the daily process flow that also improved the consistency of the cultures (data not shown here).

An average daily product titer of 1.0 g/L was achieved once the cultures were in their steady state of maintaining 20e6 cells/mL from day 5 onwards, and the cumulative product titer reached a maximum of 14 g/L by day 15 (see Figure 9). The slight difference in titer seen is due to those vessels growing to a higher VCD on days 6 and 7, resulting in higher titer; the trend trajectory aligns back with the other vessels once the cultures become more consistent after making the changes noted above.

As shown, cultures using the centrifugation method resulted in consistent data once specific steps were refined and optimized. While the process involved manual steps to remove vessels from the system to centrifuge cultures, this was only performed once per day to achieve 1 vvd perfusion rate and therefore could be scheduled to occur when operators were in the lab.

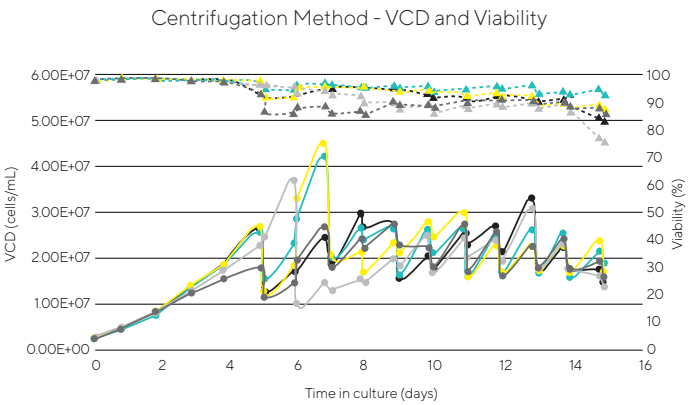


Figure 8. VCD and viability for cultures using the centrifugation method, n=5

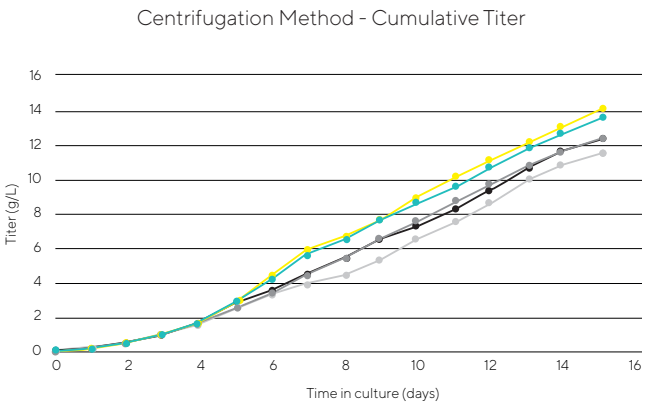


Figure 9. Cumulative titer for cultures using the centrifugation method, n=5

Cell Settling Method Results

Cell growth and viability profiles from cultures utilizing the cell settling method are shown in Figure 10. Highly consistent VCDs and high cell viabilities were obtained throughout the culture duration across the 11 replicates. There was a slight drop in growth on day 12 due to an error in the order of the steps performed, but the effect of this was consistent in all the vessels, and the cultures recovered on the following days. An average daily product titer of 0.8 g/L was achieved from day 5 onwards when steady state of the culture was maintained, and the cumulative product titer reached a maximum of 9 g/L by day 14 (see Figure 11).

As shown, cultures using the cell settling method resulted in very consistent data. Since the cell settling events were programmed to occur automatically (3x daily), these steps did not require an operator to be present, and could therefore occur overnight. Careful consideration of the software steps is needed to carry out this procedure successfully, but once achieved, the process is able to be run with little manual interaction needed. In order to fully automate all steps, including the cell counting, an integrated cell counter could also be used.

Conclusion

Results from these experiments show that high throughput perfusion mimic processes can be achieved in Ambr® 15 Cell Culture microbioreactors with good consistency amongst the replicates and with sustained cell viability and protein production. The Ambr® 15 Cell Culture Generation 2 system provides key features, like the Rapid Vessel Drain and the Passage Culture Vessel steps, that support perfusion mimic protocols and allows for more efficient and automated approaches to be applied when undertaking intensified process studies. The two methods presented provide different options for carrying out perfusion mimic studies depending on the feasibility of each in individual laboratories. Optimization of perfusion mimic protocols together with comparison of data generated across different scales would further support implementation of these methods for scale-down screening of clones under intensified conditions.

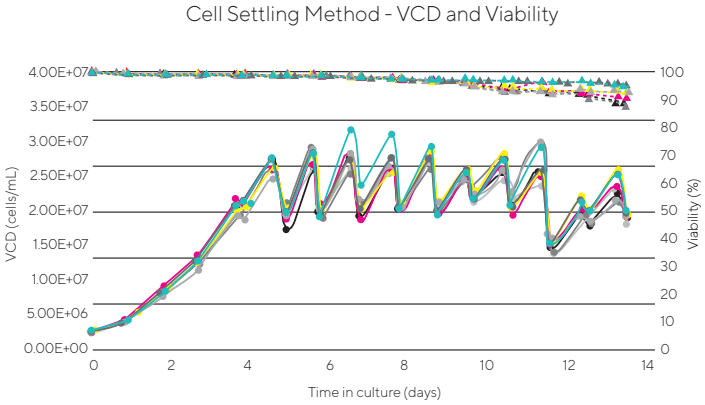


Figure 10. VCD and viability for cultures using the cell settling method, n=11

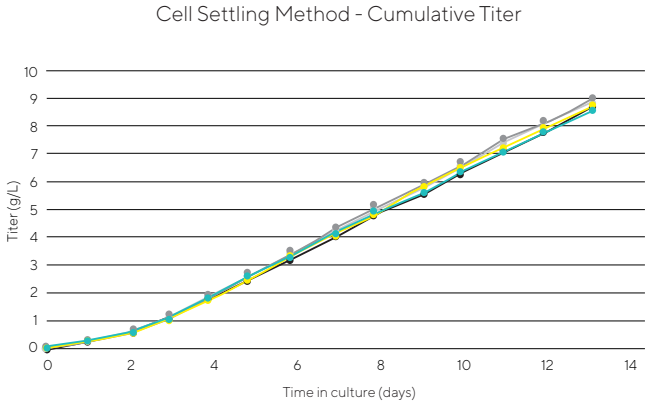


Figure 11. Cumulative titer for cultures using the cell settling method, n=11

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