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Process Optimization using High Throughput Automated Micro-bioreactors in CHO cultivation

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Dear Nandita,

Please find enclosed the manuscript entitled “Process Optimization using High Throughput Automated Micro-bioreactors in CHO cultivation” for the consideration at JoVE.
We confirm that the work has neither been published, nor is it under consideration for publication elsewhere.
The main objective of the paper is to introduce the software control of the automated micro-bioreactor.
These systems have a major advantage over the conventional bioreactors for process optimization. Using Design of Experiments, different process factors can be evaluated to reach the end results, for example, high product concentration. The designing of the protocol to run a Design of Experiment in an automated micro-bioreactor, with a maximum working volume of 15 mL, has been described in detail to benefit the users to conduct the desired cultivations for process optimization. Further, the analytics of the response is demonstrated in the script. In this case, Protein A chromatography is used to quantify the product concentration.
There is no conflict of interest to disclose.
Please address all correspondence regarding this manuscript to me as corresponding author (solle@iftc.uni-hannover.de).
Thank you for your consideration of this manuscript.

Yours sincerely,

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TITLE:

Process Optimization using High Throughput Automated Micro-Bioreactors in Chinese Hamster Ovary Cell Cultivation

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KEYWORDS:

Chinese Hamster ovary cells, Micro-bioreactor, Design of Experiment, Monoclonal antibody, Protein A chromatography, Process optimization

SUMMARY:

Here, we present a detailed procedure to run a Design of Experiment in an automated micro-bioreactor followed by cell harvest and protein quantification using a Protein A column.

ABSTRACT:

Optimization of bioprocesses to increase the yield of desired products is of importance in the biopharmaceutical industry. This can be achieved by strain selection and by developing bioprocess parameters. Shake flasks have been used for this purpose. They, however, lack the capability to control the process parameters such as pH and dissolved oxygen (DO). This limitation can be overcome with the help of an automated micro-bioreactor. These bioreactors mimic cultivation at a larger scale. One of the major advantages of this system is the integration of the Design of Experiment (DOE) in the software. This integration enables establishing a design where multiple process parameters can be varied simultaneously. The critical process parameters and optimum bioprocess conditions can be analyzed within the software. The focus of the work presented here is to introduce the user to the steps involved in process design in the software and incorporation of the DOE within the cultivation run.

INTRODUCTION:

The global biopharmaceutical market was worth more than US $250 billion in 2018 and has been continuously expanding[1]. Pharmaceutical companies are moving away from producing

small molecular drugs to biotechnologically produced therapeutics such as recombinant proteins. These alone are responsible for a revenue of more than $150 billion[1]. Mammalian cells are now extensively used for the production of these pharmaceutical recombinant proteins. In the current period, among the 68 approved products produced by mammalian cells, 57 are produced by Chinese Hamster ovary cells (CHO)[2]. CHO cells are specifically used for the production of recombinant proteins that require post-translational modifications. These cells are preferred as they grow in a suspension and thereby enable reproducible results in a serum free chemically defined medium[3,4]. The other advantage of using CHO cells is that the glycan structure of the product resembles that of the human monoclonal antibody (mAb) and results in higher recombinant protein yield and specific productivity due to gene amplification[5].

The yield of recombinant CHO (rCHO) cell culture has increased by a hundred-fold in the past two decades. This improvement is attributed to the optimization of the process parameters, feeding strategy and development of serum free chemically defined medium[6]. With the increase in requirements of the pharmaceutical products, the pressure increases on cost and time efficiency for the development of the production process[7]. To reduce the pressure while assuring product quality has redirected the focus of the pharmaceutical industry on Quality by Design (QbD). QbD is used to understand the product production as well as the process. A vital tool used in the ObD is the Design of Experiment (DOE). It helps increase understanding of the process by revealing the relationship between various input variables and resulting output data. Applying the DOE approach to optimize the bioprocess is beneficial during the early stages of the project in assimilating the process conditions and increasing the titer quantity and quality. This approach is beneficial when compared to the old-fashioned strategy: one-factor-at-a-time (OFAT). The statistical approaches to DOE using Classical, Shainin or Taguchi are far superior to the OFAT[8].

The process and media optimization can be performed in shake flasks. The flasks are relatively inexpensive. However, it is not possible to control parameters such as temperature, pH and dissolved oxygen (DO). To overcome these drawbacks, multiuse bench-top bioreactors ranging from working volume of 0.5 L to 5 L can be used. The reactors provide an extensive on-line monitoring and process control. However, the use of the multiuse bioreactor is time and labor intensive. In order to overcome these disadvantages, a novel single-use bioreactor that combines the comprehensive process of monitoring the bench-top bioreactor and easy handling of the shake flask is used. The high throughput screening system and single-use technology have contributed to enhance the efficiency of process performance and development[9].

In this article, the guidelines to load the recipe in the automated micro-bioreactor (AMBR) software are listed. The influence of different stirrer speeds and pH on the viable cell concentration (VCC) and titer is studied during the course of this experiment. The experimental result and analysis are carried out with design of experiment software MODDE 12. The product analytics are carried out in a high pressure liquid chromatography (HPLC) system with a Protein A column. It is based on the principle that the Fc region of the mAb binds to protein A with high

affinity[10,11]. With this method, it is possible to identify and quantify the mAb. The quantification is carried out over the measured elution peak areas at 280 nm.

PROTOCOL:

1. Preculture procedure

NOTE: Recombinant CHO DG44 cells with a viable cell concentration of 1 x 10^7 cells/mL are used for this protocol.

1.1. Thaw the vial containing 1.2 mL of cells to room temperature and immediately transfer the cell suspension to a 15 mL conical centrifuge tube containing 10 mL of cold seed medium.

1.2. Centrifuge the conical centrifuge tube for 5 minutes at 190 x *g* and room temperature and discard the supernatant.

1.3. Pre-heat 150 mL of the seed medium in a 500 mL shake flask to 36.8 °C.

1.4. Gently resuspend the cell pellet in 10 mL of pre-warmed seed medium and transfer the cells into the shake flask.

1.5. Use 1 mL of the sample from the flask to measure the initial VCC and viability using a cell counter.

NOTE: The viability should be above 70% after thawing for successful cultivation.

1.6. Incubate the shake flask in an orbital shaker (shaker diameter of 19 mm) at 36.8 °C and 7.5% CO_2 with a shaking rate of 120 rpm.

NOTE: These conditions vary depending on the cell strain and medium.

1.7. Three days after passaging the cells, remove the shake flask from the shaker and place it under the laminar flow cabinet. Take 1 mL of sample to measure the final cell concentration. Calculate the volume to be transferred to fresh pre-warmed seed medium such that the initial cell concentration in the new passage is 2 x 10^5 cells/mL.

1.8. Passage the cells 5 times in total before transferring to the bioreactor for the main cultivation.

2. Main cultivation

2.1. Measure the final cell concentration of the preculture. Calculate the volume to be transferred to the bioreactor such that the initial cell concentration in the reactor is 3 x 10^5 cells/mL.

2.2. Fill the reactor with production medium a day before the inoculation to equilibrate the reactor and set the process parameters such as temperature, pH and DO.

NOTE: The cultivation conditions are 36.8 °C and 60% dissolved oxygen concentration (DO). We tested stirrer speeds of 1050 rpm and 1300 rpm along with pHs of 6.9, 7.1, and 7.3. The total duration of the cultivation is 12 days until the cells are harvested. The batch process runs for 72 hours after which the feed medium is added every 24 hours. The protocol to be used for the cultivation is listed in detail in the next segment.

3. Writing the recipe in the automated micro-bioreactor software

NOTE: There are two ways of writing a recipe in the AMBR cell culture software: it is created either by using a wizard or by adding each step manually. For the purpose of this protocol, steps using the wizard are shown.

3.1. Creating a new experiment

3.1.1. Open the AMBR cell culture software and in the **Introduction** tab click on **Create New Experiment**.

3.2. Loading the recipe

3.2.1. In the **New Experiment** tab, enter the name of the experiment along with the date on which it is to be conducted.

3.2.1.1. Activate the check point for the culture station and the vessels to be used during the cultivation. The **Auto Add DOE Tags** will also be activated for an easy transition during the programming of the DOE experiment. Click on **Next** to switch to the next tab.

3.2.2. Set information about addition of media into the vessel along with antifoam, inoculum, feed and glucose.

3.2.2.1. Activate the **Add Media Plate** check point. Define the Plate type, name and location of the plate containing the medium.

CAUTION: Depending on the type of plate and if the plate contains a lid, activate the check on **Is Lidded** to ensure smooth functioning of the liquid handler

3.2.2.2. Click on **Add Media To Vessels.** Enter the volume of the media to be added into the vessels. Define the mapping of the transfer of the media from the plate to the vessels. Click on **Next** to switch to the next tab.

3.2.3. Set the cultivation conditions in the reactor.

3.2.3.1. After the media information has been fed into the software, assign the cultivation conditions. Click on **Condition Media** and fill in the temperature, target DO, upper pH limit and stirring RPM (Up stirring or Down stirring).

3.2.4. Set addition of inoculums into the vessels.

3.2.4.1. Activate **Add Cell Plate**. Define the plate type, name and location of the plate containing the medium.

3.2.4.2. Click on **Add Cells To Vessels**. Enter the time of inoculation and the volume of the media to be added to the vessels.

3.2.4.3. Define the path travelled by the liquid handler to the transfer of the cell from the plate to the vessels. Click on **Next** to switch to the next tab.

NOTE: Ensure **Reuse Pipette Tips** is deactivated to avoid cross-contamination and incorrect initial viable cell concentration.

3.2.5. Set addition of feed, glucose and antifoam.

NOTE: The procedure for addition of feed, glucose and antifoam is similar to each other. For the sake of this protocol the procedure is listed for “Feed”. This can be replicated for glucose and antifoam.

3.2.5.1. Activate the **Add Feed Plate** and define the plate type, name and location. Click on **Add Feed To Vessels** and enter the volume of the feed to be added to the vessels. Define the mapping of the transfer of the feed from the plate to the vessels.

3.2.5.2. Depending on the cultivation, add the number of feed addition. For this cultivation, the reactor is fed after 72 hours for every 24 hours.

3.2.5.3. Manually add the time delay between the feeding by entering the data into **Delay from cells added**. The first day of feeding is after 72 hours of inoculation and the next one is after 96 hours and so on.

NOTE: Antifoam addition is programmed to be added every day to avoid foaming during the cultivation.

3.2.6. Set sampling during the cultivation.

3.2.6.1. Activate the **Add Sample Plate** and define the plate type, name and location.

3.2.6.2. Check on **Take Sample from Vessels** and enter the volume of the sample to be removed from the vessels. Define the mapping of the transfer of the sample from the vessels to the plate. Ensure that the volume does not decrease below 10 mL during the entire course of cultivation.

3.2.6.3. Add the number of samples to be taken during the cultivation. Similar to feeding, add the time of the sample being removed from the vessel for each input sample point.

3.2.7. Save the process. It is now ready for execution.

NOTE: To ensure the smooth running of the protocol, switch to the **Process Steps** tab in the AMBR cell culture software and select **Process Step view** to visualize the flow of the recipe.

3.3. Design of experiment in the automated micro bioreactor

3.3.1. In order to run the DOE software of the bioreactor, ensure that the recipe in the main software is saved and ready to use.

3.3.2. Open the **AMBR 15 DOE** software and click on **Investigation** and select **New**.

3.3.2.1. Enter the name of the new DOE investigation in the **Create Investigation** dialog box.

3.3.2.2. In order to assign an experiment to the DOE investigation, open the recipe created to study the different parameters. Click on **Browse** and select the respective experiment.

3.3.3. Define the DOE factor.

3.3.3.1. The vessel tags are already enlisted in the column. To define the desired DOE factor, select the parameter and click on the column labeled **DOE factor**. Select **New** and add the units, abbreviation, lower and upper limit of the factors (e.g., temperature, DO, pH).

3.3.4. Define the response factor.

3.3.4.1. Once the DOE factors have been defined, define the response based on which the experimental analysis would be structured.

3.3.4.2. In the **Responses** tab, define the values to be considered for the analysis of the data.

3.3.4.3. Click on **Edit DOE Responses** and define the name of the response, abbreviation, units, minimum and maximum limits (e.g., titer, viable cell concentration).

3.3.4.4. Once the responses are defined, select the AMBR variable for each response and define the variable. A response can be automatically associated with a micro-bioreactor variable, Choose the required variable from the drop-down list.

3.3.4.5. Change the equation for each of the response depending on the requirement. The choice is between the minimum, maximum, first, last and average data.

3.3.5. Create a design.

3.3.5.1. Use the **Start Design Wizard** in order to select the type of experimental design, to add or remove the number of replicates and center points.

3.3.5.2. Select the objective, which determines the choice of designs and models:
Screening: Uses linear and interaction models to find the important factors
Optimization (RSM), Uses quadratic and cubic models for detailed modeling and optimization
Split objective: Models for formulation and process factors can be chosen separately

3.3.5.3. Once the objective is decided upon, select the model and the design along with the number of center points and replicates.

3.3.5.4. Click on **Finish** and switch to the next tab.

3.3.6. Define the experiment.

NOTE: The DOE factors are listed in the right column of the software. On selecting the desired factors, the vessels running that experiment with the desired parameter would be highlighted. The vessels within the culture station can be moved around by right clicking on the vessel and moving it to the desired location.

3.3.6.1. Create work packets that can be imported in the AMBR cell culture software. Depending on the number of experiments the different work packets are created and stored for further implementation

3.4. Execution of the experiment in the work packets created on the AMBR control laptop

3.4.1. In the **Experiment** tab, click on **Create DOE Experiment** and browse for the work packet created using the DOE software.

3.4.2. Initialize the process by clicking **Start**.

3.5. Analysis of experimental results

3.5.1. Once the experiment has been executed, export the data using **Export DOE Results**. The **Export DOE Results** window opens and the rows indicating the culture vessel and station are listed in the table.

3.5.2. Select the desired rows and click on **Exported Selected Rows** or **Export Experimental data** to store all the results and save the file for further analysis.

3.5.3. Import the data into the AMBR DOE module by switching to the **Results** tab and selecting **Import Results**.

3.5.4. Browse for the desired data file and click the **Analysis Results**.

3.5.5. Analyze the results further in MODDE.

4. Execution of cultivation in the automated micro-bioreactor

NOTE: The following steps are executed by the user with the help of the protocol written in the aforementioned software. The steps are carried out by the user unless mentioned otherwise.

4.1. Loading the vessels

4.1.1. Open the gamma sterilized culture vessels under a laminar flow cabinet and orient in the culture station as depicted in **Figure 2**.

4.1.2. Clean the clamp plate with 70% ethanol and double distilled water. Then, autoclave the plate and place on top of the vessel.

4.1.3. Mount the clamp plate with a stirrer plate, ensuring each pin is fixed firmly.

4.1.4. Tighten both the stirrer plate and clamp plate onto the stirring assembly.

4.2. Running the micro-bioreactor software

4.2.1. Use the program written in Section 3 to run the cultivation.

4.2.2. Visualize the process steps scheduled or completed in the **Process** tab. Alter the cultivation steps during the process run as needed by first pausing the liquid handler and then editing the process recipe.

4.2.3. Add antifoam to the vessels before the stirrer is started to ensure there is no excessive foaming during the cultivation. The antifoam will be added regularly, and the foam detected visually.

4.3. Addition of media into the vessel

4.3.1. Fill the 24 well plate provided with the micro-bioreactors manually with sterile media and place in the designated deck of the system. Ensure the plate is placed in the deck designated by

the written program (section 3). The filling of the vessel will take place as designed in section 3.2.2.

NOTE: The temperature and stirrer start immediately after the addition of the media and the antifoam. The sensor reader is activated 1 hour after the vessel is filled (Start Monitor step). Gassing to each vessel commences once the reader has been activated. Media is left to equilibrate for a minimum of 6 hours before pH recalibration and inoculation. The process parameters can be altered in the software as mentioned in section 3.2.3.

4.4. Inoculation

4.4.1. Measure the viable cell concentration after the 5^{th} passage. Calculate the number of cells to be transferred to the vessels to ensure that the initial cell concentration in all the vessels is 3 x 10^5 cells/mL.

4.4.2. Transfer the cells to a 24 deep well plate such that the volume of the suspension is at least 1.6 times the required volume. For a required volume of 2 mL of the inoculum, transfer 3.2 mL of cell suspension into each well in the plate.

4.4.3. Place the 24 well plate in the designated deck. The vessels will be inoculated as in section 3.2.4.

4.5. Daily sampling and analytics

4.5.1. Remove a 460 µL sample from the vessels every day using the liquid handler. Dilute 200 µL of the sample with 800 µL of filtered 1x PBS buffer (5x dilution), and then place in the cell counter.

4.5.2. Centrifuge the remaining sample for 5 min at 190 x *g* and room temperature and store the supernatant for further analysis (glucose, lactate, glutamine and glutamate).

4.5.3. Freeze 100 µL of the supernatant at -20 °C until the end of the cultivation for protein quantification.

4.6. End of cultivation

4.6.1. When the process parameter control (i.e., temperature, agitation, pH and DO) has terminated, stop the monitoring of the process.

4.6.2. Unscrew the stirrer plate and clamp plate.

4.6.3. Remove the culture vessels and clean the culture stations. Place the drying plates on the culture stations and screw them in.

4.6.4. Meanwhile, clean the clamp plates thoroughly with 70% ethanol and double distilled water.

4.6.5. Click on **Stop** in the bioreactor software once the drying cycle is completed.

4.7. Cell culture harvest

4.7.1. Harvest cells on Day 12 of the cultivation by manually removing the content of the vessels into 50 mL centrifuge tubes. Centrifuge the cell broth at 190 x *g* for 30 min.

4.7.2. Discard the cell pellet and store the supernatant at -20 °C.

5. Measuring mAb concentration

5.1. Use a 1.7 mL Protein A column for the quantification of the protein during the cultivation run.

5.2. Prepare the equilibration and elution buffer before thawing the samples.

5.2.1. Use a solution of 0.5 M Na_2HPO_4 containing 0.5 M NaCl with a pH of 7.9 as the equilibration buffer and a solution of 100 mM glycine containing 0.5 M NaCl with a pH of 2 as the elution buffer.

5.3. Filter both buffers through a 0.2 µm membrane and degas before being placed for the analysis.

5.4. Purge the high-performance liquid chromatography system (HPLC) with freshly prepared equilibration buffer.

5.5. Load the protein A column on to the HPLC system.

5.6. Carry out chromatography with a flow rate of 1 mL/min. Set the column oven temperature at 30 °C and autosampler temperature at 10 °C

5.7. Thaw the frozen samples at room temperature and filter 225 µL of each sample through a 0.22 µm PVDF membrane. Dilute the samples with higher concentration of the desired protein are in a 1:20 ratio with equilibration buffer and filter through the membrane before placing in the autosampler.

5.8. Place the samples in the autosampler. Load the method and sequence in the software and start the sequence.

NOTE: The method is comprised of three phases (see **Figure 1**): injection of the sample into the column for the first two minutes; followed by elution buffer for 8 min and column regeneration with equilibration buffer for 10 min.

[Place **Figure 1** here]

REPRESENTATIVE RESULTS:

An overview of the cultivation performed in this study is presented in **Figure 2**.

[Place **Figure 2** here]

The cell growth in the automated micro-bioreactors is comparable to the multi-use bioreactors. This is depicted in **Figure 3.** The cell concentration from the three different scales is compared and it is observed that the 15 mL automated micro-bioreactor mimics the 2 L glass bioreactor. The results from the shake flask are also compared to exhibit the benefit of the AMBR.

[Place **Figure 3** here]

The influence of different stirrer speed and pH is studied in the automated micro-bioreactors. Viable cell concentration (VCC) is one of the vital parameters to compare the cultivation. **Figure 4** represents the comparison of the VCC and monoclonal antibody concentration in the different micro-bioreactors**. Figure 5** represents the response contour plot of the two responses considered for the comparison, namely, VCC and mAb concentration. The values are comparable in the vessels with the same pH and different stirrer speed, indicating that the stirrer speed selected for this process has no significant influence on the process output. For future cultivations, the stirrer speed of 1050 rpm would be used in order to avoid foaming.

The pH, however, has a conspicuous impact on the process output data. The negative influence of pH 6.9 on the VCC can be observed in the **Figure 4A**. The growth of the cells improved significantly under the culture at pH 7.3 compared to pH 7.1. In **Figure 5B**, the monoclonal antibody concentration of the different cultivations is compared. The production of the mAb is slower in the vessels maintained at pH 7.3; however, the final product concentration is comparable to the vessel maintained at pH 7.1.

[Place **Figure 4** here]

[Place **Figure 5** here]

One of the advantages of using the AMBR is the continuous monitoring and control of the cultivation. The monitoring of the pH can be observed in **Figure 6**. The pH is controlled at the set point using CO_2. The bolus feeding from Day 3 is responsible for the spike in the values.

[Place **Figure 6** here]

The process parameters used for the future tests was narrowed down to a stirrer speed of 1050 rpm and pH of 7.1.

FIGURE LEGENDS:

Figure 1: **Protein A chromatogram, representing the different phases during a single run.**

Figure 2: Schematic representation of the experimental conditions to test pH and stirrer speed profiles in the culture stations. The figure also represents the correct layout to place the vessels.

Figure 3: Comparison of the viable cell concentration at different scales. 15 mL micro-bioreactor, 150 mL shake flask, and 2 L multi-use glass bioreactor.

Figure 4: **Result of the DOE experiment.** (**A**) Viable cell concentration profile of the cultivation run to study the influence of pH and stirrer speed (**B**) Monoclonal antibody concentration profile over the fed-batch process in the respective cultivation conditions.

Figure 5: Response contour plot indicating the influence of pH and stirrer speed on the maximum viable cell concentration and monoclonal antibody, respectively.

Figure 6: pH monitoring in the automated micro bioreactors for the cultivation, with set points of pH 6.9, 7.1 and 7.3 and a stirrer speed of 1050 rpm.

DISCUSSION:

Optimization of the process to increase the yield is of crucial importance in the biopharmaceutical industry. Shake flasks could potentially be used for the screening of the strain; however, the monitoring of the process parameters such as pH and DO are unavailable in the flasks. The micro-bioreactors have an advantage as they allow continuous monitoring and control of the process. These control loops in the micro-bioreactor also provide a condition similar to those at larger scale and thus, deliver results that are comparable to the larger scale bioreactors. Another advantage of the micro-bioreactor is the wide range of conditions that can be tested within the same time frame and at a lower cost when compared to bench top bioreactors in terms of time and labor[12,13] .The smaller size is also advantageous in terms of lower running costs due to the reduced amount of substrate and reduced space requirement for parallel operations; however, the cost of the vessels must be considered as it may be more expensive to run a the cultivation in the single use micro-bioreactors when compared to the bench top multiuse bioreactor.

While there are several advantages to the system, there are a few considerable disadvantages to using the micro-bioreactors. Changing the pH and the DO for each of the vessel within one culture station is possible; however, the stirrer speed and the temperature cannot be changed for an individual vessel. This increases the number of experiments carried out to establish the optimum stirrer speed and temperature. The online measurements are restricted to pH and DO. Real time process monitoring of the critical process parameters (CPP), such as temperature,

pH, DO, would be beneficial to avoid the delay between sampling and analysis. It has the potential to be a useful tool for optimizing productivity[14]. Development in the field of spectroscopic measurements in micro-bioreactors may prove to be beneficial in the future models.

The advantages outweigh the disadvantages of using the automated micro-bioreactor. However, there are a few criteria to be considered when operating these systems. First and foremost is the designing of the script to successfully run the cultivation. It is of vital importance that the script written is inclusive of all the crucial steps such as definition of the plates along with the correct mapping of the plate and vessels. All the parameters must be checked before starting the experiment. Ensure no process steps are scheduled to occur at the same time. This will result in the workstation choosing one step over the other.

Another important criterion to be focused upon is the use of the clamp plates. The plates are autoclaved before every cultivation; this could lead to damage of the O-rings. Visually check the O-rings before autoclaving. Faulty O-rings could result in unexpected variation in the DO and pH control. The other factor for faulty readings could be a loose connection between the clamp plate and the vessels. Ensure the clamp plate and the stirrer plates are secured tightly to the stirring assembly. Use the tightening tool provided with the system.

During the cultivation, it is crucial to visually monitor the foam. A minimum of 20 µL of antifoam is required at the start of the cultivation. Foaming will result in liquid being present in the trough of the clamp plate. Excess of the foam in the clamp plate will lead to overflow, leading to the foam being collected at the bottom of the culture station, obscuring the pH and DO spots to be read by the sensor.

Another important factor to focus upon is the use of the DOE software. The integrated DOE software enables quick designing of the process incorporating the relevant conditions. The visualization of the vessels being used ensures that no factor has been overlooked. One of the advantages of DOE analysis is that the experiments can be configured via work packets, configuring each bioreactor with defined bioprocessing parameters. All the data generated is analyzed using MODDE. The software manages the large amount of data generated during the course of the experiment. A generalized subset design setup generates a sequence of reduced design set, thereby, solving the problem with multivariate calibration.

A detailed procedure to run a Design of Experiment in an automated micro-bioreactor is demonstrated in this article. The protocol was designed to focus on the feed batch process. The DOE software aided in establishing the optimum process parameters to increase the biomass and the titer concentration. The cultivation data were also compared to an experiment conducted in a shake flask and a 2 L bench-top bioreactor. The results demonstrate the reproducibility and scalability of the process. The goal of the protocol was to demonstrate the use of automated micro-bioreactors in a feed batch process and to analyze the bioprocessing parameters using the DOE software. It can be concluded that the automated micro-bioreactors

are useful for the process development and these can be extended to a semiperfusion system[15].

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The authors have nothing to disclose.

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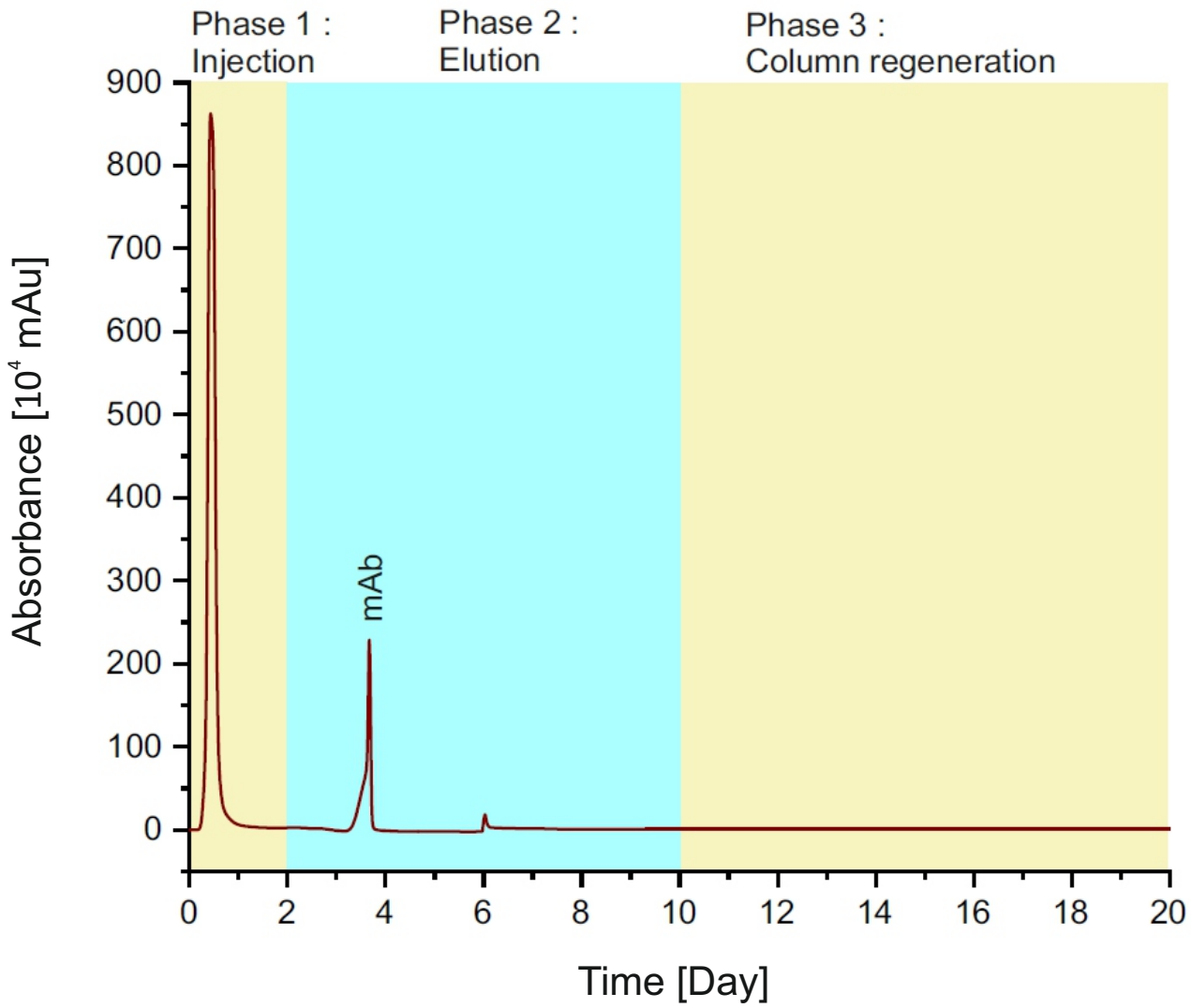
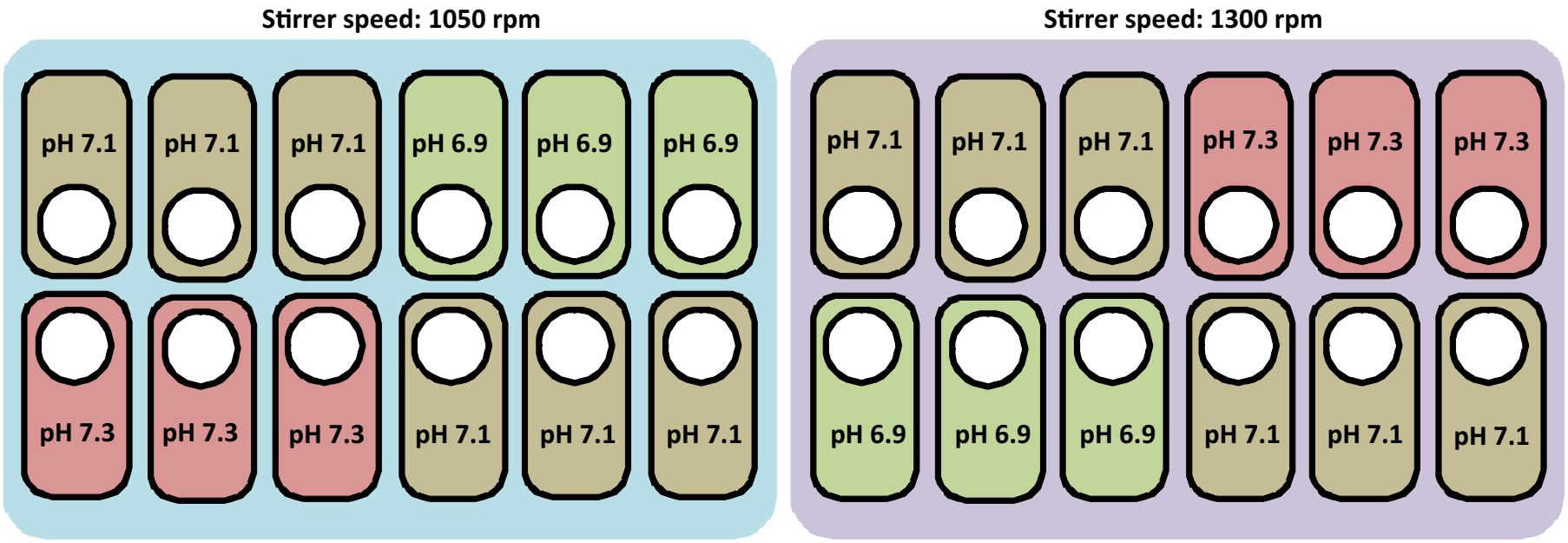
Figure



Figure



Figure

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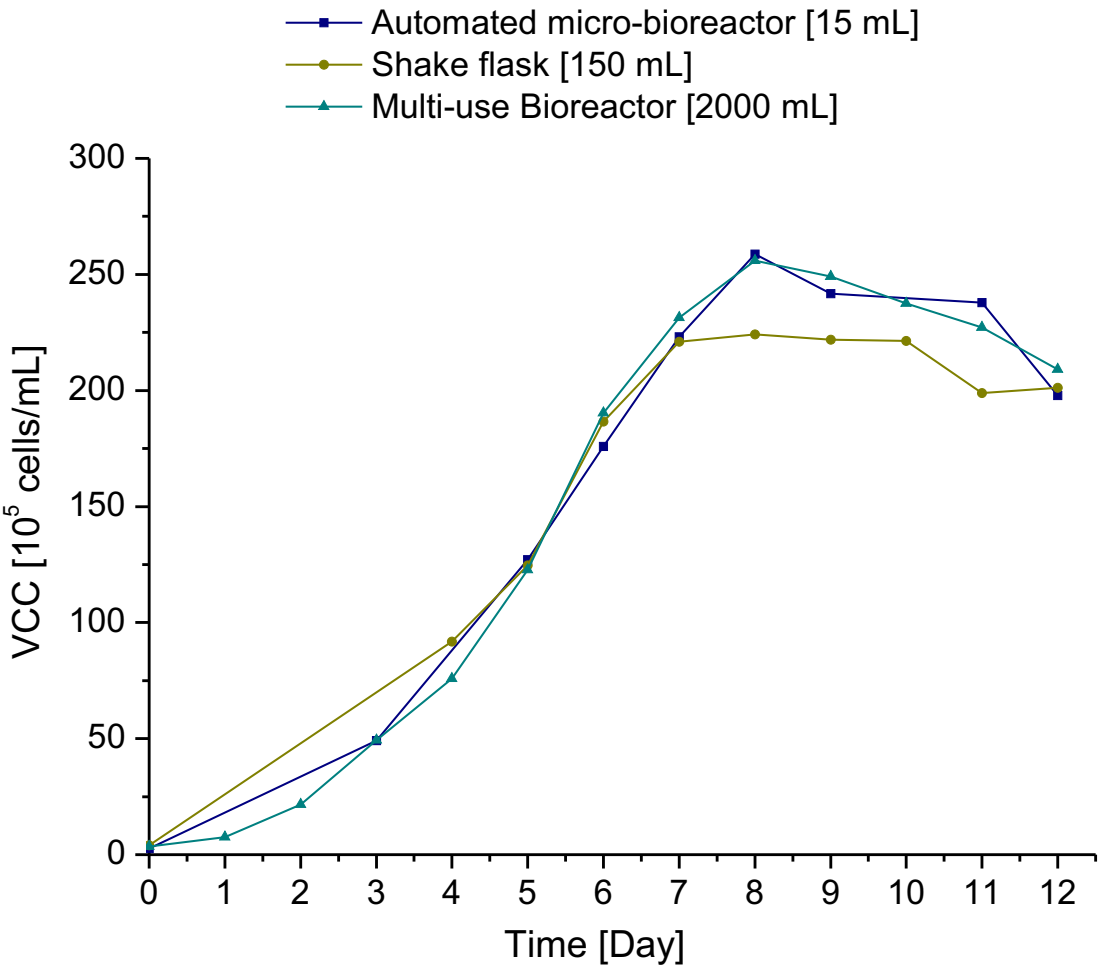

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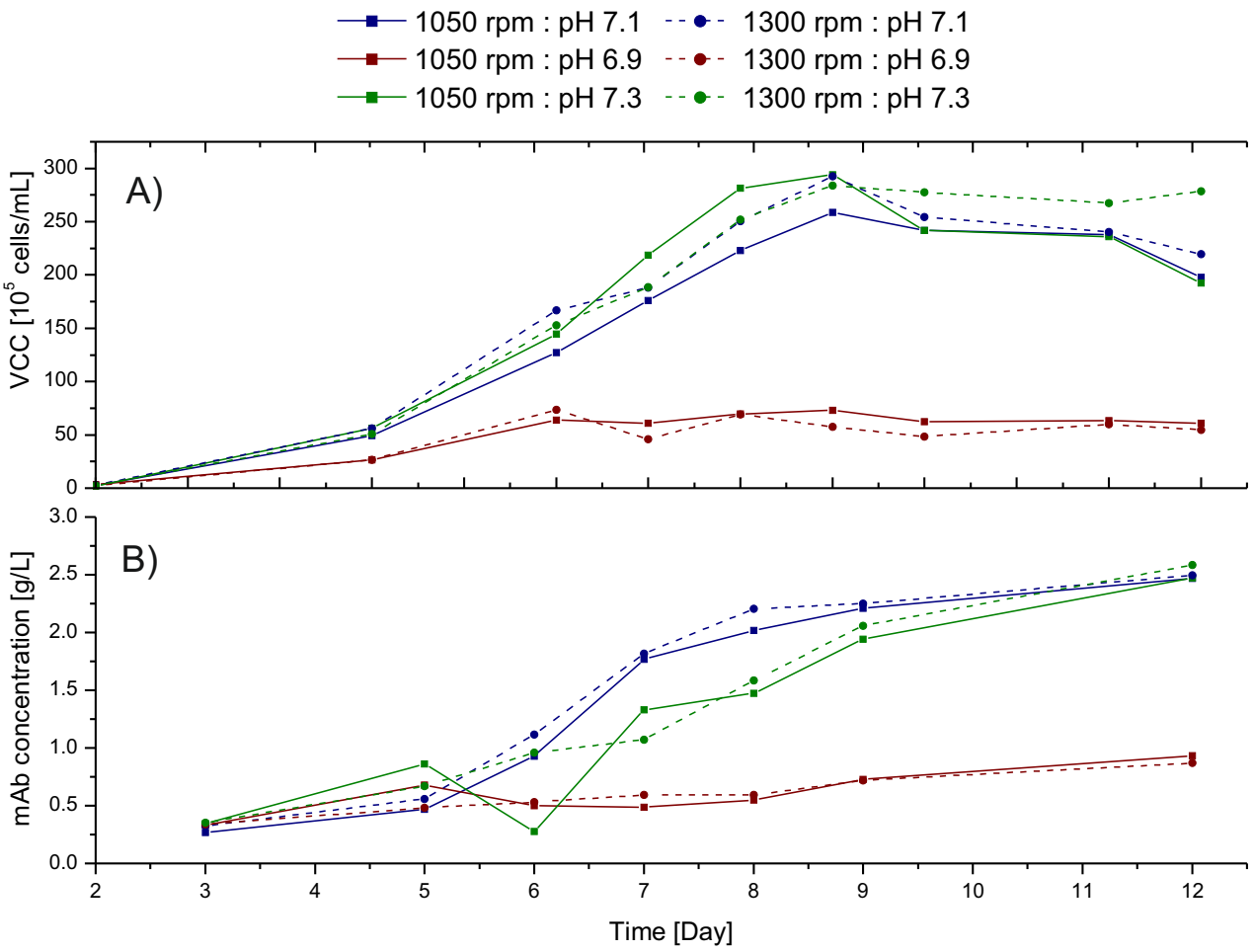

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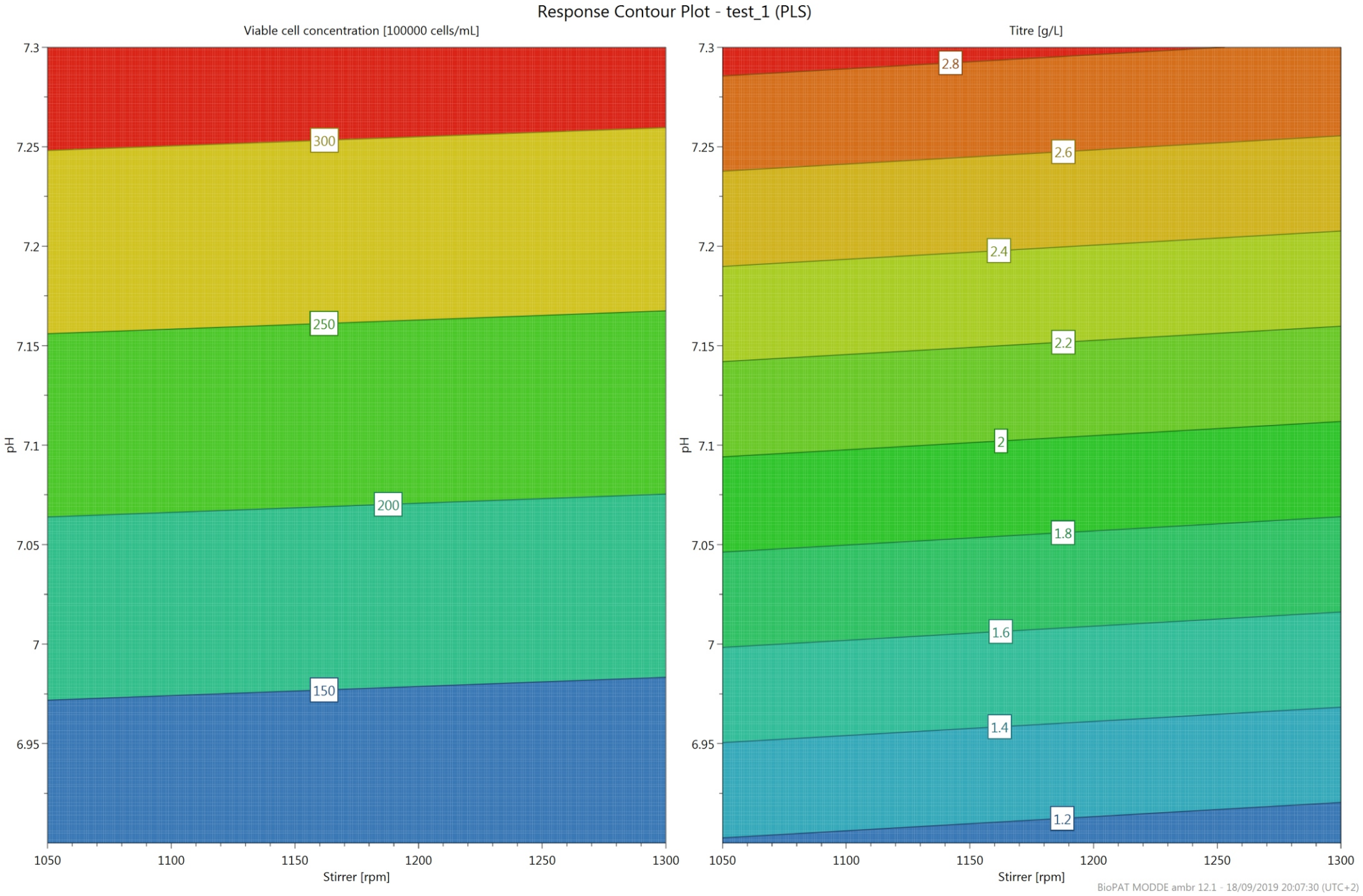

Figure

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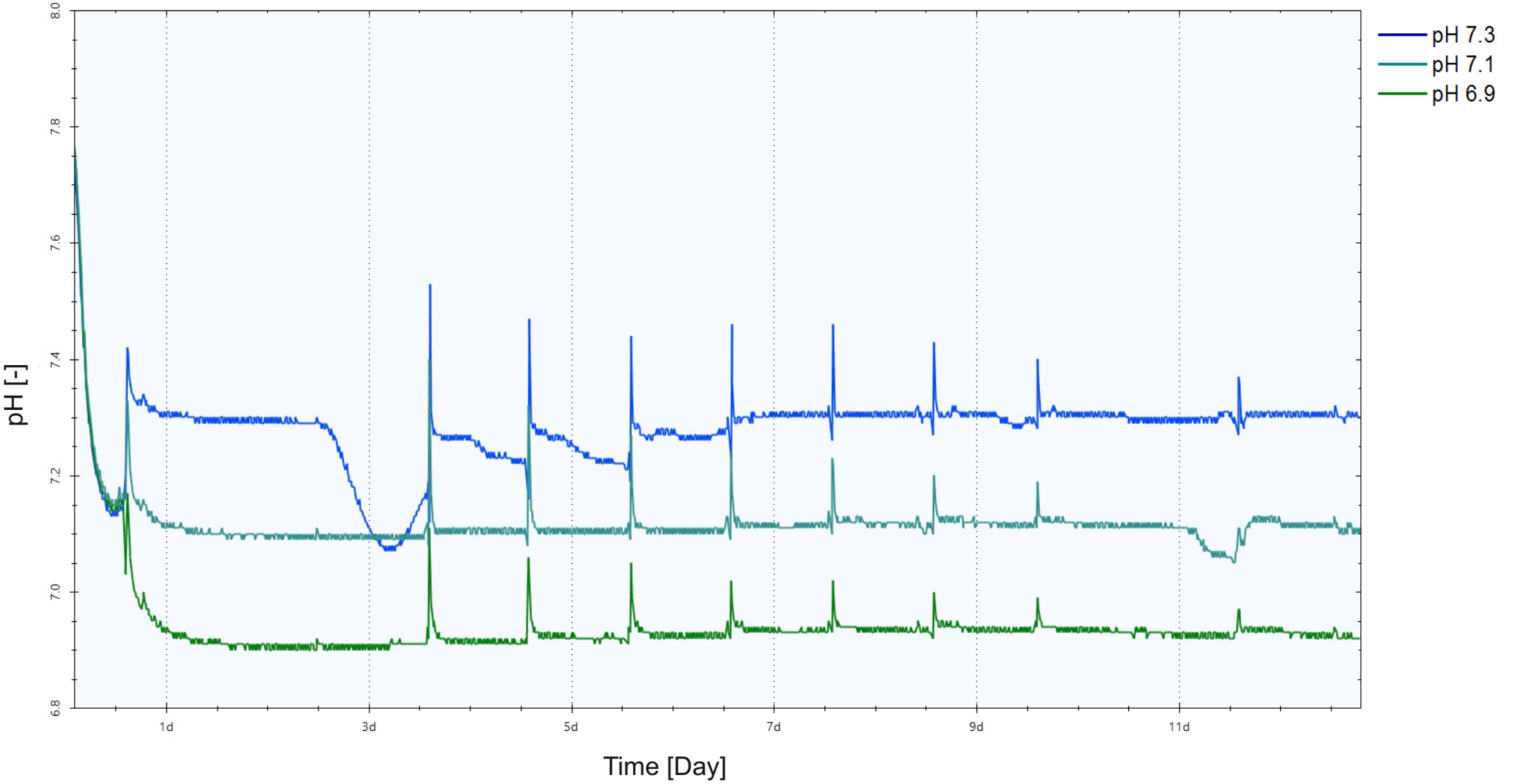

Table of Materials

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
1 mL disposable pipette tips, sterilized	Sartorius Stedim Biotech GmbH	A-0040	
200 mM L-glutamine	Corning, Merck	25-005-CV	
24 Well deep well plates	Sartorius Stedim Biotech GmbH	A-0038	
5 mL disposable pipette tips, sterilized	Sartorius Stedim Biotech GmbH	A-0039	
ambr 15 automated microbioreactor system	Sartorius Stedim Biotech GmbH	001-2804	
ambr 15 Cell Culture 24 Disposable Bioreactors - Sparged	Sartorius Stedim Biotech GmbH	001-1B86	
Antifoam C Emulsion	Sigma-Aldrich, Merck	A8011	
Bottle Top Sterile filter	Corning, Merck	CLS431474	0.1 µm pore size
CEDEX Detergent (3% Mucosol)	Roche Innovatis AG	05-650-658-001	
Cell counter	Roche Innovatis AG	05-650-216-001	CEDEX HiRes
CHO DG44 cell line	Cellca, Sartorius Stedim Biotech GmbH		
CHOKO Feed Media A (FMA)	Sigma-Aldrich, Merck	CR80025	
CHOKO Feed Media B (FMB)	Sigma-Aldrich, Merck	CR80026	
CHOKO Production Medium	Sigma-Aldrich, Merck	CR80027	
CHOKO Stock Culture Meium	Sigma-Aldrich, Merck	CR80028	
Chromaster high pressure liquid chromatography system	VWR International		
Conical Centrifuge tube	Corning, Merck	SIAL0790	
Ethanol	Merck	1070179026	
Glycine	Carl Roth	56-40-6	
HPLC Vials	VWR International	SUPLSU860181	
PBS	Sigma-Aldrich,Merck	P4417	
Protein A Column	Thermo Fisher Scientific	1502226	POROS™ A 1.7 mL
Sodium chloride	Sigma-Aldrich,Merck	7647-14-5	

Sodium phosphate dibasic anhydrous	Sigma-Aldrich,Merck	7558-79-4	
Trypan Blue	VWR International	VWRVK940	
YSI	YSI Inc	2900D	YSI 2900 Select

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Title of Article: PROCESS OPTIMIZATION USING HIGH THROUGHPUT AUTOMATED MICRO-BIOREACTORS IN CHO CULTIVATION

Author(s): TAMANNA NAGRAIK, ALINA GONZALEZ SALCEDO, DÖRTE SOLLE, THOMAS SCHEPER

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Editorial comments:

General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Reply:

Thank you the editorial comment. The script has been proof read.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Reply:

The guidelines are followed while writing the manuscript. The page margin, font and the size are as per the protocol.

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For example: Falcon, ambr 15

Reply:

Thank you for the comment. The names such as ambr 15 and falcon have been replaced by ambr(automated micro-bioreactor) and conical centrifuge tubes.

Protocol:
1. Please ensure that all numbered steps/substeps are written in the imperative.
2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Disclosures:
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Reply:

The statement acknowledging the project partners has been mentioned in the segment.

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1. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.
2. Please do not abbreviate journal titles.
Reply:

Thank you for helping with the direction to list the authors. The necessary changes have been made.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Reply:

The table of material has been updated with the list of the chemicals used.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The authors describe a detailed protocol on how to setup and run parallel CHO cultivation with daily bolus feeding using the ambr 15 micro-bioreactor system. Although having no hands-on experience for the ambr 15 system, this reviewer feels that one could follow the protocol to run parallel cultivations, especially for being familiar with cell culture handling in general. The authors state that they applied the system for design-of-experiments, however, this reviewer is missing this aspect. Shown data is limited to the analyzed samples, more specifically any plot describing the results of a DOE is missing. Also, it is not clear whether the ambr system comes with an own DOE software, or if it comes with a MODDE license, or how these are inter-connected. Anyhow, a few major comments arise in the view of this reviewer. After these have been addressed, the protocol should be suitable for publication in JoVE.

Major Concerns:

L60 - 63: Please add one or two sentences on the shortcomings of "classical" DOE, e.g. not different fractional factorial designs. A reference for the interested reader wanting to dive deeper into DOE would be nice.

Reply:

Thank you for your feedback. As mentioned by you I have added a reference giving an insight into the different DoE approaches.

L 66, 67: To the knowledge of this reviewer, there are shake flask systems with online monitoring of pH and DO available from, e.g., Presens or Sartorius.

Reply:

The reviewer is right in the statement that there are many shake flasks with the possibility to monitor the pH and DO; however, the controlling of these parameters is vital for cultivation. It is possible with the micro-bioreactors.

L 410: When talking about the production rate, please provide a number and how this rate was determined.

Reply:

The sentence is rephrased as the focus of the method article was the programming of the software to run the cultivation

.
L 433: The authors often state the advantage of online monitoring in the system. It would be nice to actually see these online data.

Reply:

Thank you for your comment. I have attached the figure indicating the continuous measurement of the pH and DO

L 440, 441: Please provide a comparison of rough cost.

Reply:

The statement about the cost is with reference to the time and the labor. With single-use reactors, the user would not have to spend time in cleaning and sterilisation of the bioreactor before and after the cultivation.

L 473, 474: This manuscript is lacking any description/ discussion on a DOE plan as well as on the outcome of a DOE plan, which is in contrast what the authors state in their manuscript.

Reply:

Thank you for the comment. This method article was to introduce the new users to the method of script writing in the software.

L482, 483: Where is the data on shake flask and bioreactor cultivation? What was compared? What are the conclusions from this comparison?

Reply:

Thank you for the point overlooked. The comparison of the results are showcased in the representative results.

L 484, 486: In contrast to the stated goal of this protocol, this reviewer misses the aspects of DOE (plans, evaluation, interpretation).

Reply:

The interpretation of the DOE is depicted in the results. The focus was to introduce new users to the software.

Minor Concerns:

L 39: Please provide a reference for the 250 Bn statement.

Reply:

The reference is added but was also mentioned in line 42.

L 77: replace titre concentration by titre

Reply:

Thank you. The word has been replaced as suggested by the reviewer.

L 89: What is freeze viable cell concentration?

Reply:

The word freeze was deleted from the line.

L 96: More thorough description of "Gently"

Reply:

The word gently was deleted from the line.

L 98: Clarify the difference between VCC and viability? How are both values calculated / measured?

Reply:

Thank you for review. The VCC is defined as viable cell concentration in line 98. These were measured using a cell counter.

L 102: When using a shaken cultivation system, always provide the shaking diameter.

Reply:

The error has been rectified in the mentioned line.

L 104: Is cells meant instead of cell?

Reply:

Thank you. The word has been replaced as suggested by the reviewer.

L 204 - 207: Is there any software protection against a too low volume?

Reply:

The predicted volume is indicated in the software. It is up to the user to ensure it doesn't drop below 10mL

L 241 - 243: Can only one response be defined per experiment?

Reply:

Thank you for the review. In the section 3.3.4.3, it has been mentioned that the user can edit the number of responses. This isn't restricted to one response per experiment.

L 259: Extrapolation from model is rather risky. Is there any measure of credibility or confidence that can be described in this section?

Reply:

The reviewer was correct in the statement that the word extrapolate was inappropriate. It has been replaced accordingly.

L 318: The antifoam is detected visually?

Reply:

The sentence is corrected to "the foam is detected visually"

L 355: The process parameters are not terminated, but their control.

Reply:

Thank you for helping make this more comprehensible. The necessary change has been made to the statement

L 404: This sentence sounds weird. Please rephrase.

Reply:

The sentence has been rephrased

L 447: Provide a few examples of CPPs

Reply:

A few examples of the parameters are mentioned in the line.

L 452: replace machineries with systems.

Reply:

Thank you. The word has been replaced as suggested by the reviewer.

L 453 - 456: This sounds rather trivial to this reviewer. Correct, specific handling of a certain piece of technology is almost vital to producing reasonable data from such device.

Reply:

The automated micro-bioreactors are designed to be robust, however, if the script is incorrect, the system run would be hindered and this could eventually lead to termination of the cultivation process.

L 464: If it is crucial to monitor foaming, is this also done during the nights?

Reply:

The antifoam is added everyday to combat the formation of foam.

L 477: What is SIMCA?

Reply:

It is multivariate data analysis software named Soft Independent Modeling of Class Analogy (SIMCA). The sentence was deleted due to irrelevance to the method article.

Reviewer #2:

Manuscript Summary:
In this manuscript, the authors illustrate a protocol for performing a DOE experiment in microbioreactors (specifically the ambr15 system). The focus of the video (based on the highlighting) will be on the use of the software rather than the manipulations of the bioreactor.

Major Concerns:

I question whether a demonstration of the software is really the best thing for a video. In addition, the hard part of doing a DOE experiment is deciding what the experimental variables and their ranges should be.

Reply:

Thank you for the comment. The method article focuses on how the script to run a DOE can be written. This is to give new users an introduction into the software

In a number of places, the authors cite flow rates and other bioprocess parameters without noting that these choices will depend on the system under study. The flow rate through a column depends on the column size and packing material. The choice of CO_2 level in the incubator depends on the medium, etc. This should be clarified in the text.

Reply:

Kindly refer to the “Table of material” for the exact column used for the quantification of the protein of interest. The information about the column volume has been added to the script.

Minor Concerns:

On page 3, the authors write "To reduce the pressure while assuring product quality has redirected the focus of the pharmaceutical industry on Quality by Design (QbD)". This is not really true. The FDA is driving QbD much more than the industry.

Reply:

The reviewer is right as the FDA does focus on QbD but the pharmaceutical industry is also focussing on it to be able to fulfil the FDA requirements.

On page 4, point 1.2 in the procedure, the size of the vial of frozen cells and the Falcon tube need to be specified.

Reply:

Thank you for the point. The volume of the vial and the conical centrifuge tube has been added to the script.

On page 11, the authors write "The influence of pH 7.3 on the VCC is comparatively higher

than the influence of pH 7.1." This is poorly written. I believe what they mean is that the VCC is improved under culture at pH 7.3 compared to 7.1.

Reply:

Thank you for finding the irregularity in the sentence. The necessary changes have been made in the abovementioned comment.