Upstream Process Development with a k_La Criterion Within the BIOne Single-Use Bioreactor

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BIOPROCESSING

Strategically Engineering Process Parameters for Upstream Bioprocesses

Modern upstream bioprocess development is evolving. The Quality by Design (QbD) directive by the United States Food and Drug Administration (FDA) expressed how a simple demonstration of bioprocess functionality should no longer be considered a sufficient capstone for process development.[1] Rather, a higher level of process understanding should be obtained. Such understanding should be established through the execution of robust characterization studies and demonstrated through the engineering of strategic process parameters. Such strategic process parameters are essential for the consistent manufacturing of biological drug substances. Therefore, strategic process parameters are necessary to meet quality standards and support final drug product safety and efficacy.

Upstream process parameters can be characterized as either volume dependent or volume independent.[2] Volume independent parameters are those which are held constant for a process across different bioreactor systems and scales. These parameters include temperature, media composition, and system pH. In contrast, volume dependent parameters are not necessarily held constant during process transfers. Definitions for these parameters will instead be dependent on vessel-specific geometric specifications. Therefore, these parameters must be strategically defined during cross-system process transfers. Volume dependent parameters include bioreactor working volume, agitation rate, and bottom air sparge rate.

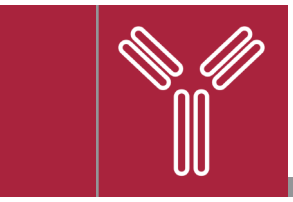
Volume dependent parameters are typically transferred across different bioreactor systems through linear scaling based upon conserving a central criterion. The volume dependent parameters for a new process scale will be engineered to ensure that the resulting criterion value is consistent with that which was observed at the original process scale.[3] Such a strategy can ultimately produce upstream processes across multiple scales which share a consistent foundational element.

Central criteria selected for process transfers and development have historically been based upon traditional engineering inputs. Examples of such inputs include power input per unit vessel volume (P/V), sparged gas flow rate per vessel volume (vvm), agitator impeller tip speed (ts), or superficial sparged gas velocity (U_S).[4,5] The specific criterion selected as central for process scaling will likely vary across different process development laboratories and facilities.

Challenges and Limitations of Upstream Bioprocess Transfer when Utilizing Traditional Engineering Inputs as Central Criteria

The suitability of utilizing traditional engineering inputs as central criteria may be limited for some upstream bioprocess development and transfer applications. For instance, utilizing such criteria may necessitate geometric consistency across bioreactor systems. For example, upstream system characteristics such as vessel aspect ratio or sparger type may have to be consistent across scales to support the utilization of certain central criteria. This limitation might be of particular concern to biopharmaceutical manufacturers who wish to explore the integration of unique disposable manufacturing trains into their upstream bioprocesses. A lack of geometric commonalities between nontraditional and traditional bioreactor systems may increase challenges during future process transfers when using such scaling criteria.

Defining parameters by utilizing a traditional engineering input as a central criterion may result in an upstream bioprocess with an undesired extracellular environment. For instance, maintaining a constant vvm criterion across benchtop bioreactors and production bioreactors systems can potentially result in increased bioreactor foaming and discrepancies in dissolved carbon dioxide (dCO_2) concentrations across scales.[6] Such dis-



crepancies can result in inconsistencies in cell culture growth and productivity across systems.

Ultimately, establishing a traditional engineering input as a central criterion for process transfer may misdirect emphasis to maintaining consistent system inputs, rather than maintaining consistent extracellular environments. A central criterion which aptly describes the extracellular environment could be selected rather than one that simply describes an engineering input. Shifting emphasis from matching engineering inputs to harmonizing extracellular environments has the potential to drive greater system understanding during process development and transfer work. Therefore, it can be argued that such a shift in focus would align strongly with the QbD directive of the FDA.

Bioreactor oxygen transfer rate and dCO_2 concentrations have been identified as potential central criterion candidates which describe relevant aspects of extracellular environments. This developmental work was focused towards engineering volume dependent parameter definitions for the BIOne Single-Use Bioreactor (SUB) which would produce a suitable oxygen transfer rate within the system. This strategy was performed by utilizing the volumetric oxygen mass transfer coefficient (k_La) as a central criterion for process development. Using a robust mass-transfer characterization model, volume-depended parameters were engineered to produce an extracellular environment with a suitable oxygen transfer rate. The parameters were then optimized to both minimize cell stress and maintain optimal dissolved carbon dioxide concentrations. This strategy proved highly effective in the successful scaling of a process from a shaker flask to a stir-tank reactor (STR) system.

A k_La Criterion can be Highly Suitable for Cell Culture Process Development

An essential component of a suitable cell culture bioreactor environment is the dissolved oxygen (DO) percentage within the medium. Across bioreactor systems and models, this parameter is maintained primarily through two basic processes. Molecular oxygen is introduced into the culture medium through both the sparging of gases directly into the liquid, and from the agitation-assisted dissolution of oxygen across the interface boundary between the medium surface and headspace. The cumulative rate by which oxygen is transferred from the gaseous to the aqueous phase within the overall bioreactor system is defined by the Oxygen Transfer Rate (OTR).

Characterization of the OTR within an upstream bioprocess is essential for process development and optimization. Molecular oxygen is required for the oxidative phosphorylation metabolic pathways (OXPHOS) within aerobic cultures. This pathway becomes upregulated as cells transition from exponential growth to stationary phase.[7,8] It is during stationary phase when peak titer production occurs.[9] Therefore, maintaining a sufficient dissolved oxygen concentration can be necessary for optimal cell culture productivity.

When volume dependent parameters are not correctly defined, dissolved oxygen can become a limiting metabolic substrate within bioreactor systems. If the OTR is not sufficient to meet the demands of highly aerobic cultures, OXPHOS metabolic potential could be limited.[10] Localized hypoxic conditions within the bioreactor environment also have the potential to induce additional undesired process effects, such as increased aggregation of mammalian cells.[11] Ultimately, if OTR is not sufficient within a system, both cell culture health and productivity will likely be sub-optimal. Therefore, a thorough understanding of the OTR for a bioreactor system can be beneficial to mitigate the risk of an undesired productivity limit on a culture.

The OTR within a bioreactor can be defined as the product of the volumetric oxygen mass transfer coefficient (k_La) and the oxygen concentration gradient ($C^* - C$) within the system. This relationship is shown in **Equation 1**. The oxygen concentration gradient will be dependent on the definition of the dissolved oxygen process parameter. As this parameter is volume independent, it will be likely be held constant across process scales during development. Thus, if k_La is also kept constant, the OTR will be consistent across all scales during development. Such significant process relevance makes k_La a suitable candidate to serve as a process development criterion.

$$OTR = k_La \times (C^* - C)$$

Equation 1: Functional Derivation of Oxygen Transfer Rate

k_La = Volumetric Mass Transfer Coefficient of Oxygen
C^* = Dissolved Oxygen Saturation Threshold
C = Dissolved Oxygen Percentage

Many factors can influence the rate of oxygen transfer within a bioreactor system. This coefficient can be influenced by physical, chemical, and biological aspects of the overall bioreactor system. Such factors include system volume, agitation rate, sparged air flow rate, media salt and surfactant concentrations, extracellular protein concentrations, and overall system biomass accumulation.[12,13,14,15] The variety of factors that can significantly influence the k_La of a bioprocess, makes characterization of the mass transfer coefficient within a bioreactor system a considerable challenge. This challenge is further increased as many of these factors fluctuate throughout the duration of the process. However, through robust and thorough analysis methods, the mass transfer of the bioreactor system can successfully be characterized and understood.

Characterizing k_La within the BIOne Single-Use Bioreactor through Design of Experiment (DoE) Multivariate Analysis facilitates Robust Modeling and Improved System Understanding

The BIOne Single-Use Bioreactor (SUB) system is a novel liner-based benchtop disposable bioreactor system, designed and manufactured by Distek, Inc. It has been recognized that improved oxygen transfer rate characterization of the BIOne system has the potential to expand the process application utility of the bioreactor system. Increased understanding regarding the effects of volume dependent process inputs on the k_La output within the system would make the bioreactor highly suitable for process development with k_La as the established central criterion.

Robust mass transfer characterization can be completed through the execution of a Response Surface Design of Experiment (DoE) mass transfer evaluation. A DoE-based design offers the potential for increased systems characterization knowledge and understanding; thus, the methodology aligns well with the FDA directive of QbD.[16] As compared to more traditional one factor at a time (OFAT) analysis, a DoE facilitates multivariate analysis which allows for a detailed examination of the overall impact of both the main effects and effects interactions on the response output.[17] These attributes make a DoE evaluation highly appropriate for mass-transfer characterization within a bioreactor system.

The main effects selected as input factors for the characterization of the Distek BIOne SUB bioreactor system were volume-dependent parameters: agitation rate (rpm), sparged bottom air flow rate (sccm), and vessel working volume (mL). To increase the utility of the evaluation, a model medium was used during the characterization. This model medium was utilized to replicate the bicarbonate concentration, surfactant concentration, and osmolality typically found within various cell culture media types. The model medium was prepared as described in Matsunaga et al., 2009.[18]

Biological process outputs with the potential to influence mass transfer, such as extracellular protein and overall biomass, were not considered during the BIOne SUB k_La evaluation. The substantial variability of these outputs across individual bioprocesses makes attempting to control these inputs unsuitable for an overall characterization study. Such controls would ultimately restrict the overall utility of the analysis. Therefore, all mass transfer characterization work for the Distek BIOne SUB was performed *in vitro* with a cell-free bioreactor environment.

For characterization of the Distek BIOne SUB a specific type of Response Surface DoE evaluation, the central composite design (CCD), was been identified as suitable for use.[19] The design space for this CCD, was based upon the both the operational and practical limits of the 2-L BIOne system. The bounds of the design space are described in **Figure 1**. The k_La response output for each given set of conditions would be determined through the static gassing out method, described in **Figure 2**. Upon completion of all evaluation points within the design space, multivariate analysis on the system was performed using JMP15 (SAS) software.[20]

A.

Pattern	Vessel Volume (mL)	Agitation Rate (rpm)	Sparged Air Flow (sccm)
+++	2000	450	150
−−+	900	50	150
+−+	2000	50	150
−−−	900	50	15
A00	2000	250	82.5
000	1450	250	82.5
+−−	2000	450	15
−++	900	450	150
0A0	1450	450	82.5
−+−	900	450	15
+−−	2000	50	15
0a0	1450	50	82.5
00A	1450	250	150
a00	900	250	82.5
00a	1450	250	15
000	1450	250	82.5

B.

Figure 1. Materials and methods for experimental determination of oxygen mass transfer coefficient (k_La) within bioreactor. A. BIOne Single-Use Bioreactor (SUB) 2-L, drilled hole (flute) sparger, with single impeller used for evaluation. **B.** Design space for Central Composite Design DoE used during characterization.

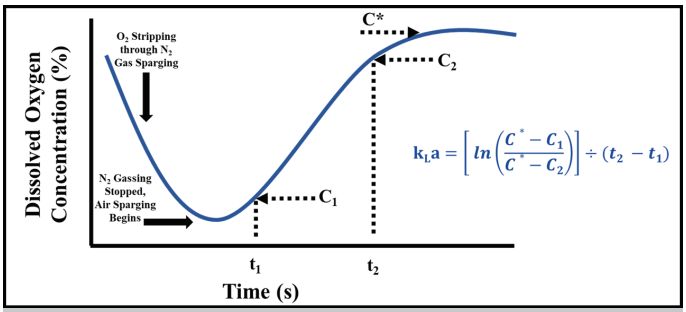


Figure 2. Experimental determination of oxygen mass transfer coefficient (k_La) through static gassing-out method. Oxygen is removed from the system through nitrogen gas sparging. Upon completing of oxygen stripping, the nitrogen sparging is stopped. Air is then sparged into the system. Using the formula shown above, the oxygen mass transfer coefficient may be calculated for each evaluation point.

Agitation and Working Volume Demonstrate Largest Effects on k_La Response within DoE Design Space

Upon completion of DoE multivariate analysis, the significance of the mass transfer model was evaluated. The results of this analysis demonstrated that the model was highly significant. A summary of these statistical results is shown in **Figure 3**.

To evaluate the impact of the main effects on the system k_La, effect ratios were calculated based on the individual sum of squares calculated for each input. These individual values were analyzed in relation to the total sum of squares for the entire model. Through this approach, a main effects summary for the system could be visualized. This effects summary, also shown in **Figure 3**, demonstrates that both working volume and agitation rate have the greatest impact on the overall k_La within the evaluated design space. The three-dimensional curvature in the corresponding response surface models support these results.

The effect summary for the model supports the suitability of low-aeration upstream bioprocess strategies within the BIOne SUB. Such strategies may be advantageous for the bioreactor process development of more fragile, shear sensitive cell cultures. It has been demonstrated that hydrodynamic shear forces caused by sparged aeration may be more detrimental to cell culture health than shear forces generated through mechanical agitation.[21] Research has suggested that this phenomenon may be due to the interfacial energy dissipation resulting from bubbles bursting at the surface of the bioreactor medium.[22] Therefore, the BIOne SUB may be suitable for upstream process development for shear sensitive cell types as agitation, rather than aeration, drives oxygen transfer within the system.

To demonstrate the predictive accuracy of the model, four sets of random k_La testing parameters were evaluated. Experimental k_La values were determined for each of these conditions. These values were then compared to the model predicted k_La values for each set of parameters. For the model to be considered accurate, the mean experimental output had to be within the 95% confidence interval of the model prediction. As shown in **Figure 4**, the accuracy of the model was successfully demonstrated.

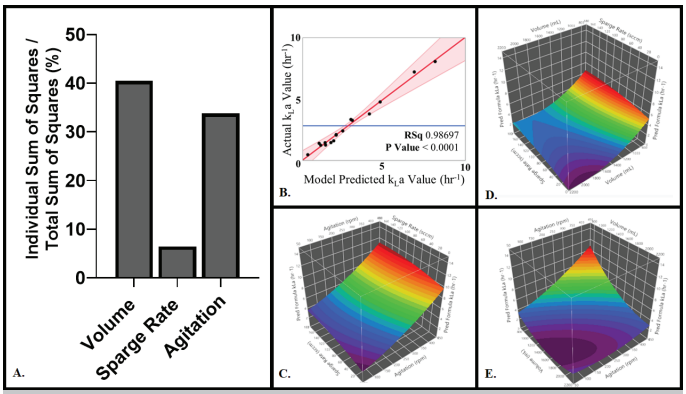


Figure 3. Mass Transfer Effects Summary in BIOne SUB. A. Main effects summary. Results demonstrate that system working volume and agitation are primary input drivers of k_La within system. **B.** Model summary demonstrates significance of mass transfer model. **C.** Interaction effects of sparged air rate and agitation on k_La. **D.** Interaction effects of sparged air rate and volume on k_La. **E.** Interaction effects of agitation and volume on k_La.

Mass Transfer Characterization Model Provides Roadmap to Defining Volume-Dependent Parameters for Scale-Up of a Shear-Sensitive Cell Line from Orbital Shake Flask to Stir-Tank Bioreactor

The HEK-293 cell line has proven itself as a versatile biological platform for multiple biopharmaceutical production processes.[23,24,25] However, one of the challenges working with this cell model is its sensitivity to hydrodynamic shear forces.[26] This attribute made this cell model a suitable choice to evaluate the low-aeration process potential within the BIOne SUB.

Within a gentle, orbital shaker flask system, the HEK-293 cell line has been demonstrated to obtain peak viable cell densities of approximately 3.0×10^6 cells per mL when cultivated during a batch process within FreeStyle™ 293 Expression Medium (Gibco, A1435-01).[27] It was determined that such a metric could

serve as a viable standard for expression of the same cells in the same basal medium within the BIOne SUB. Therefore, using k_La as a central criterion, a novel HEK-293 upstream bioprocess was engineered.

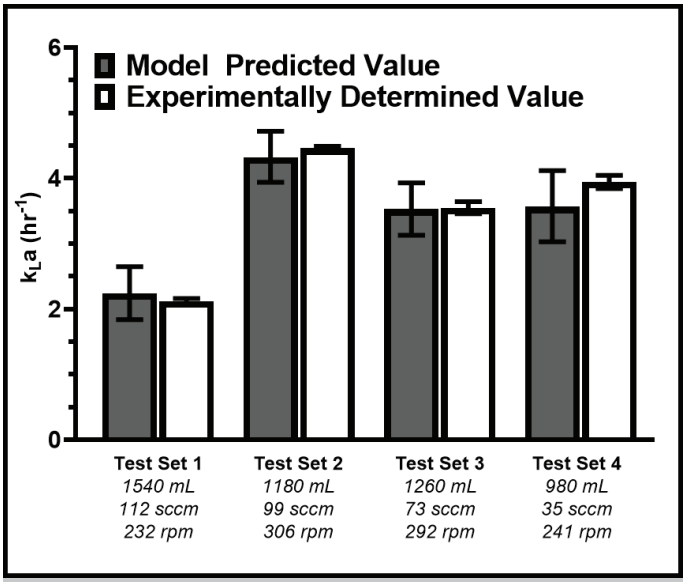


Figure 4. Evaluation of accuracy of BIOne SUB mass transfer model. Model was evaluated for accuracy using four sets of randomly generated Test Set parameters. For model to be successfully demonstrated accurate, mean experimental values needed to be within 95% confidence interval bounds of model prediction. All test set parameters were successfully demonstrated to be within passing standards.

Experimental data are mean values from n=3 trials. Bars shown on precited value columns represent 95% confidence interval for model predictions. Bars shown on experimental values represent standard error.

Foundational literature described how a system k_La value of 0.25 to 5 hr^{-1} is likely sufficient to support the aerobic requirements of most mammalian cell lines during simple batch processes. The midpoint of this range was targeted as the criterion value for defining volume dependent parameters for the BIOne SUB HEK-293 batch process.[28] Using the previously generated mass transfer characterization model, the following operational definitions were created: 220 rpm agitation rate, 1000 mL working volume, and with no minimum air sparge rate.

The HEK-293 line was cultured for 10 days in a batch process. The FreeStyle™ 293 Expression Medium was supplemented with 0.1% Pluronic F-68 (Gibco, 24040-032) to provide additional protection against hydrodynamic shear forces. Additionally, the medium was supplemented with 30 ppm Antifoam C emulsion (Sigma, 28011). Previous work has demonstrated effective foaming mitigation at this concentration without adverse effects to cell health.[29]

It was recognized that dissolved carbon dioxide (dCO_2) concentrations in the medium could potentially become problematic during the process due to the low aeration operational strategy. Therefore, overlay air flow was supplemented and tuned during the batch process to facilitate carbon dioxide stripping from the medium. Adjustments for this tuning were made based upon analytical measurements obtained through integration of an online dCO_2 probe (Mettler Toledo, Pro-Analytics).

Results Demonstrate that the BIOne SUB and a k_La Criterion can Support the Successful Onboarding of Novel Upstream Bioprocesses

Using the operational definitions engineered with the BIOne SUB mass transfer model and the target k_La criterion, a shear sensitive HEK-293 cell line was successfully cultured in a batch process. As shown in **Figure 5**, the cell growth trend was demonstrated to be comparable to what was described during HEK-293 orbital shaker flask processes performed within the same basal medium.[28] These results support that the hydrodynamic shear forces and overall cell stress may be comparable between the two systems under certain operating parameters. This commonality demonstrates the BIOne SUB can be a suitable system for the cultivation of more shear-sensitive mammalian cell types.

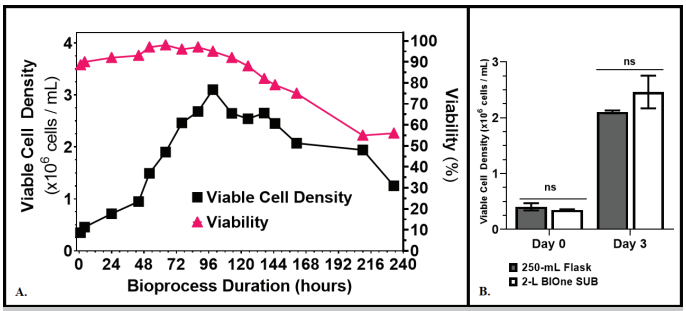


Figure 5. HEK-293 cell line successfully cultured within BIOne SUB in Batch Process. A. Viable cell density and viability trends for 10-day HEK-293 batch process within single-use bioreactor. **B.** Viable cell density comparison between bioreactor and orbital shaker flask controls suggest process comparability.

The culture pH trends shown in **Figure 6** supports that the OTR of the system was sufficient to sustain the aerobic requirement of the culture. The change in pH from the lower to upper bound of the deadband suggests that the culture underwent a metabolic shift as they transitioned from net lactate production

aerobic glycolysis to OXPHOS. The high viability of the culture during this time suggests that the OTR of the system was capable of supporting such a transition. The overlay air dCO_2 mitigation strategy, also summarized in **Figure 6**, was demonstrated to be successful.

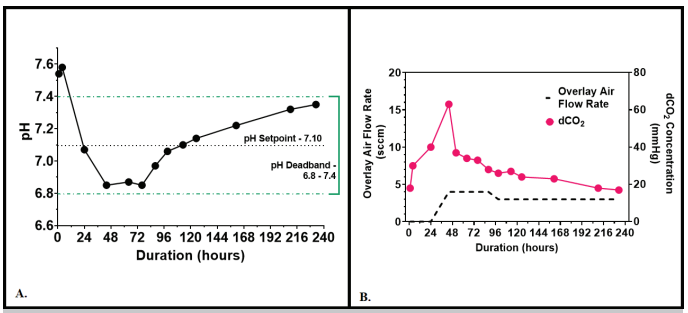


Figure 6. pH and dCO_2 process trends demonstrate sufficient OTR and effective dCO_2 stripping.. A. pH trend demonstrates lactate shift metabolic change in culture, suggesting OTR was sufficient to support flux from aerobic glycolysis to OXPHOS. **B.** Overlay air tuning strategy successfully mitigated risk of high dCO_2 concentrations in medium.

Conclusion

The QbD directive from the FDA states that extensive bioprocess characterization and understanding should be essential elements of future bioprocess developmental work. It can be suggested that process development utilizing a central criterion which describes the extracellular environment supports this philosophy. As such, we performed novel HEK-293 batch process development within the BIOne SUB using a k_La central criterion to define operations definitions for our volume dependent parameters.

To complete this work, robust DoE mass transfer characterization was first performed on the BIOne SUB using the static gassing-out k_La determination method. The resulting model provided comprehensive system insights regarding how fluctuations in process inputs would affect the oxygen transfer rate within the system. This increased system understanding facilitated the successful process development using a k_La central criterion. A shear sensitive HEK-293 batch cell culture process was effectively transferred from an orbital shaker flask into an STR bioreactor system.

The success of this study supports the suitability of k_La as central criterion for bioprocess development. Additionally, these results demonstrated that the BIOne SUB can be highly suitable for novel upstream process developmental work. Upstream bioprocess scientists and engineers may wish to consider utilizing the BIOne SUB and a k_La central criterion for their own process development and characterization studies.

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