MEDIA ANALYSIS FROM NORMAL VERSUS CONTAMINATED PERFUSION BIOREACTOR

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Normal Versus Contaminated Perfusion Bioreactor (P026)

Customer: [redacted]

Date: December 12, 2025

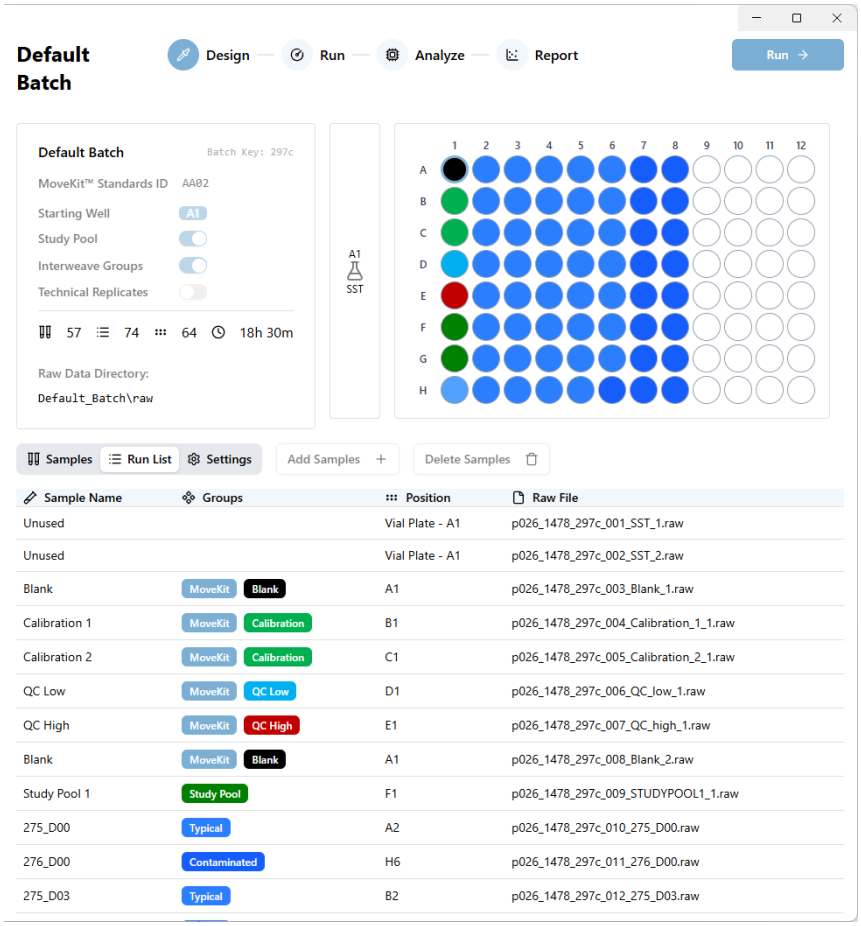
Platform: MoveKit CE **Sample Number:** 64

Executive Summary:

- 64 perfusion samples from two CHO perfusion bioreactors were analyzed by MoveKit™ CE using ZipChip and Exploris 240 Orbitrap
- Concentrations are reported for 111 metabolites which passed quality filters
- Results for most major amino acids are the same for the early timepoints between contaminated and typical bioreactors; however many other metabolites are highly differentiated at Day 6 (betaine, dipeptides, pyridoxal, nicotinic acid, others)
- A variety of metabolites were observed to spike at Day 20-21 in the typical bioreactor (argininosuccinic acid, dimethylarginine)

Study Design and Sample Preparation

Samples submitted for analysis are contained in **Table S1_Samples**. 64 cell culture media samples were analyzed after 10x dilution with phosphate buffered saline (PBS, Gibco). For the dilution, 20 µL of media sample was added to a 0.5 mL well plate, followed by 180 µL of PBS. The well plate was shaken briefly to mix the samples. A study pool quality control (SPQC) sample was created by mixing 20 µL of each of the diluted samples.



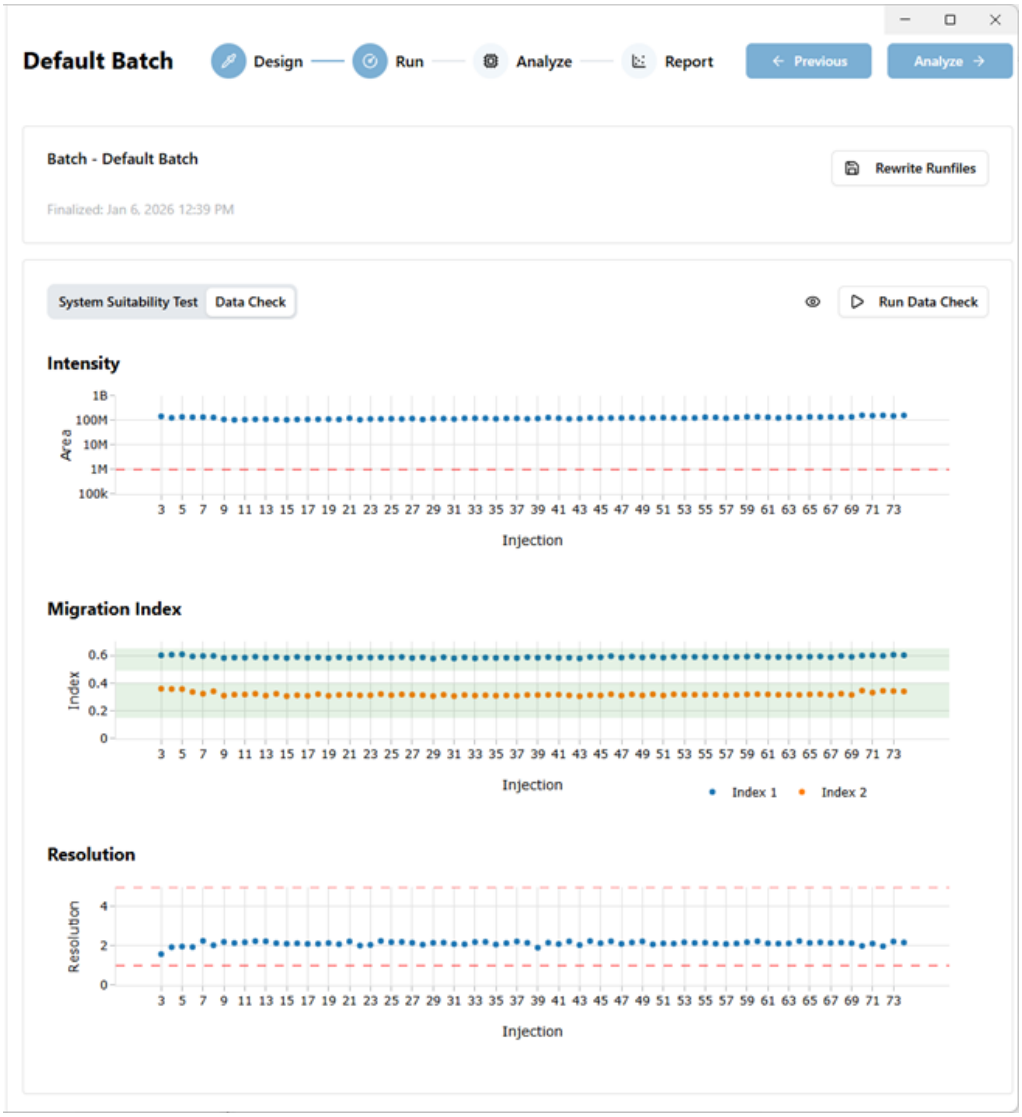
Metabolite extraction: Briefly, 140 µL of extraction reagent (methanol containing 45 stable isotope internal standards, MoveKit CE AA02) was added to a 1-µm hydrophobic filter plate, followed by 20 µL of sample, QC, calibrator or SPQC. The plate layout is shown in the batch design page (at right). The plate was

shaken for 15 minutes at room temperature. 40 µL of focusing reagent (ammonium acetate) was then added and the plate was shaken for another 5 minutes. Extracts were filtered through the filter using vacuum into a 96-well plate for analysis. The analysis sequence was created in MoveApp and is provided as **Table S2_Sequence**.

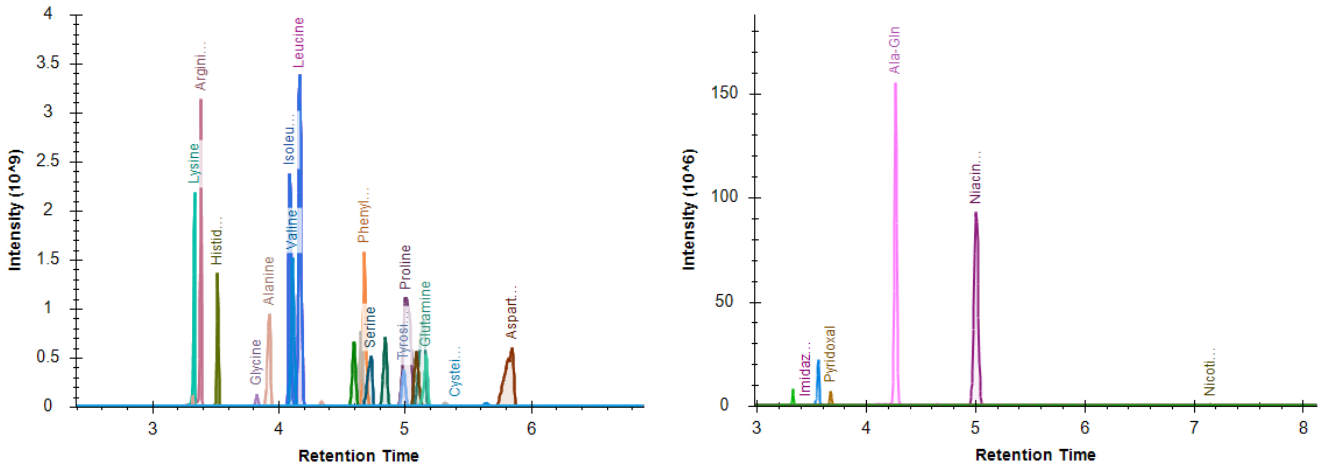
Data were collected on an Exploris 240 Biopharma and ZipChip CE-MS. An HR chip and peptides BGE were used for the ZipChip separation. The MS method utilized data-dependent acquisition to collect full-scan MS1 information from 70-800 m/z which was used for quantitative analysis against a Skyline template containing targeted compound identities, precursor m/z values, and indexed migration times. Migration time indexing was performed using a subset of 17 internal standards.

SST and Data Check

Initial system suitability testing (SST), and real-time data checks, were performed using MoveApp automation to ensure data quality. The SST passed all criteria including mass accuracy, BGE quality, Lys/Asp migration time, and Ile/Leu critical pair resolution. The data check passed overall intensity, multi-point migration index, and Ile/Leu critical pair resolution for all injections. A screenshot is shown at right from the data check. This preliminary data quality check runs in real-time and confirms no repeat injections are required. For more information on this step, please view a detailed description here.



ZipChip-HRMS separations utilized to perform metabolite quantification demonstrate high peak capacity and low flow rate, resulting in low matrix effects. An extracted ion chromatogram for amino acids (left) and a selection of vitamins and dipeptides (right) from the Study Pool QC from this sample set is shown below. Measured concentrations for these analytes range from ~14 mM (aspartic acid, Ala-Gln) to ~130 nM (citrulline).



Data Analysis / Quality Assessment

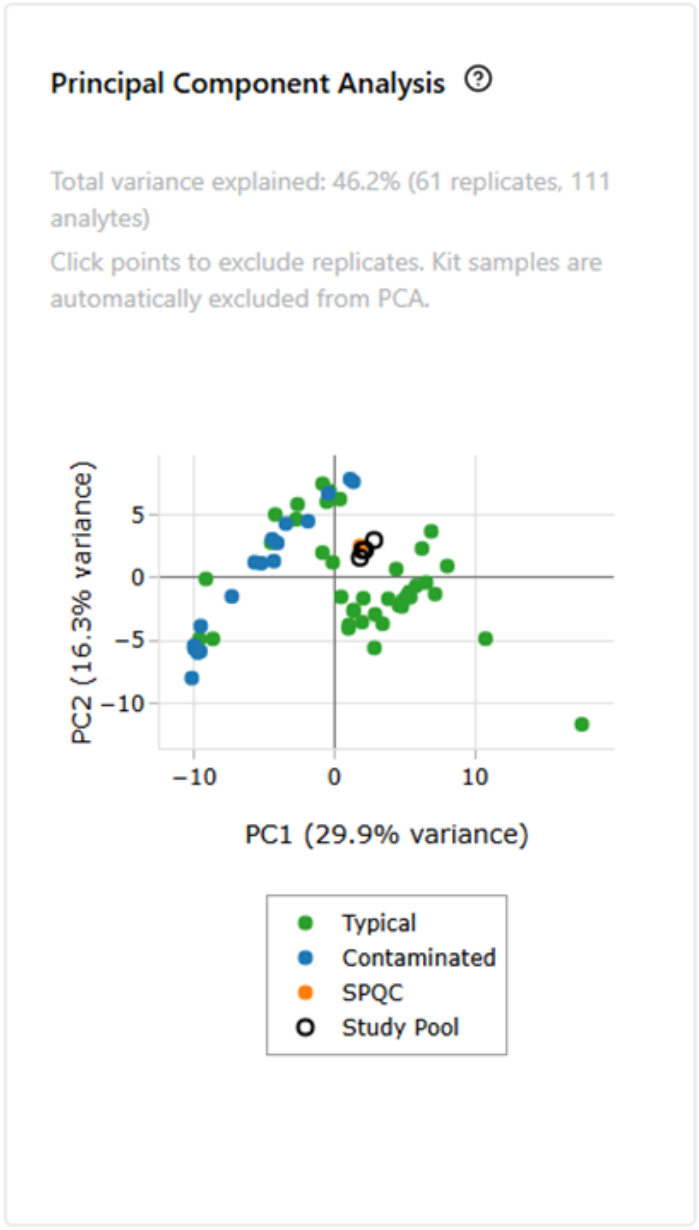
After quantitative data processing, concentration data were filtered to contain only those metabolites which were deemed to be reliably detected and had the following quality metrics:

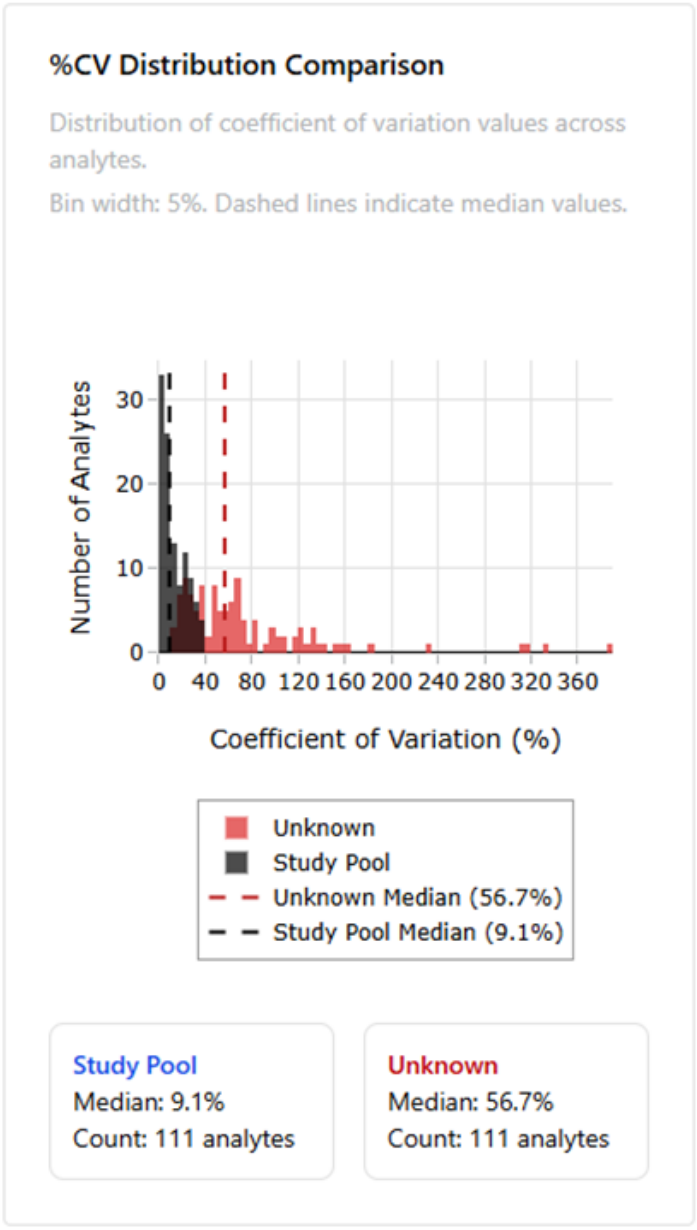
- <45%CV across the four study pool QC injections, and
- Up to 80% missing data across all perfusion bioreactor samples, and
- Values <LLOD (3x blank) replaced as missing.

A permissive missing value threshold (up to 80%) was used in order to allow the possibility for highly dynamic metabolites to show up in only a subset of the samples; they still were required to be detectable and reproducible in the SPQC.

Metabolite concentrations are reported in **Table S3_Concentrations**. 111 metabolites across the 64 perfusion bioreactor or 5 SPQC analyses remained after filtration (an extra SPQC was placed on the plate in addition to the two dedicated wells). PCA was performed using Z-score normalization and is shown below. In this plot there is one point for each sample (or Study Pool), with the purpose being to perform a dimension reduction of the large number of metabolites measured. In doing so we gain a simple, visual comparison of biological versus technical variance (i.e. scatter of green/blue versus black points). The PCA (below, left) shows much higher biological variance than technical variance, giving confidence to underlying biological comparisons when later performing statistical hypothesis tests. The outlier green point (bottom right) is the Day 6 datapoint in the 'contaminated' culture.

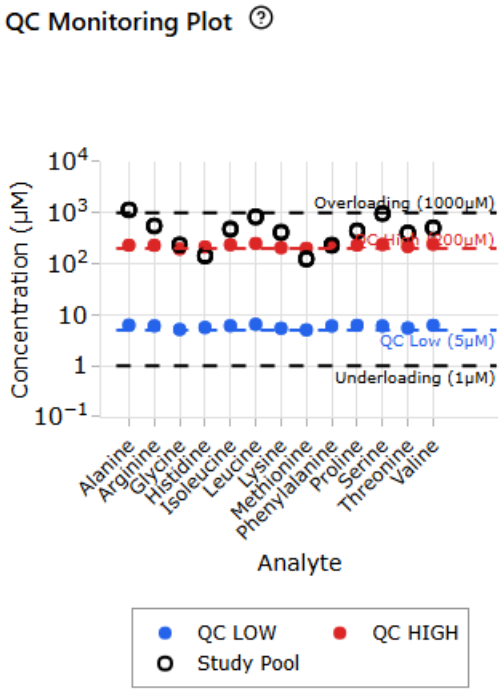
A variance histogram plot (below, right) plots %CV for all analytes, with each metabolite placed in its appropriate variance bin for SPQC or biological variance. Observing the distinct variance distributions allows us to have confidence that biological changes measured during statistical evaluation are not due to technical noise in the data. The median %CV for the study pool was 9.1 %, while variance for the biological samples was 56.7%. This gives confidence in the ability for the metabolites measured to be able to give high quality biological interpretations.





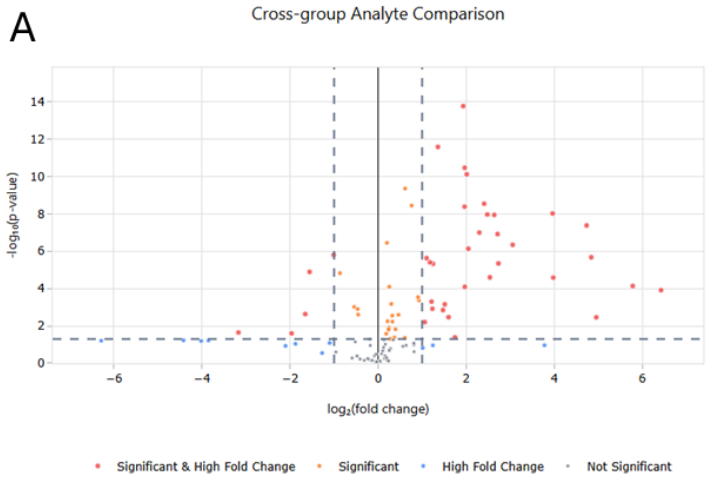
QC Monitoring Plot Interpretation. Overall quantitative QC monitoring of the study was performed by analyzing a mixture of amino acids at two concentrations, QC Low (5 uM) and QC High (200 uM), at the beginning and end of the study. These samples were prepared on the plate with the study samples and treated as unknowns. The QC monitoring plot (below) shows two important results:

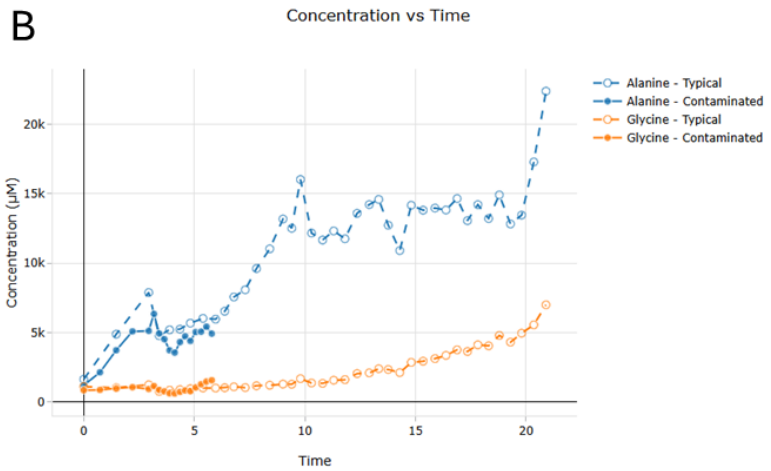
1. The reported concentration for QC Low and QC High values match their expected results
2. The reported values for the SPQC (after 10x dilution) fall within the expected linear range of MoveKit CE.



Preliminary Interpretation

The MoveApp was used to perform a simple statistical comparison (t-test) between the conditions, which admittedly has limited utility due to the overall difference in the experimental timecourse between the conditions (only through Day 6 for the contaminated condition). A volcano plot (Panel A at right) was used within the MoveApp to visualize those molecules most differentiated between conditions. Sometimes, as shown in Panel B, the statistical differences between conditions were a result of increasing concentration over time, with a shorter time period being collected for the contaminated bioreactor. Panel B shows an increase from ~2 mM to 20 mM for alanine, and from ~1 mM to ~6 mM to glycine, during the course of the typical perfusion bioreactor. Good correlation is seen for glycine and alanine.





Additionally, a number of interesting trends emerged, a few of which are captured in the visualizations below.

- **Panel A** shows the quantitative trend for pyridoxal (vitamin B6) and nicotinic acid (vitamin B3); a drastic decrease in pyridoxal and increase in nicotinic acid is observed in the contaminated bioreactor at Day 6. This is consistent with previous reports of bacterial contamination of bioreactors (Huang et al *Mol Ther Methods Clin Dev* **2022** Apr 13).
- **Panel B** shows utilization of Ala-Gln (Glutamax) by the cells during the early stages of both cultures. Apparently Glutamax is included in the initial media but is not replenished with perfusion.
- **Panel C** shows a characteristic spike in two other alanine-containing dipeptides (Ala-Ala and Ala-Glu), both of which spike in the contaminated bioreactor at Day 6.
- **Panel D** shows two example metabolites which increase drastically near the end of the typical perfusion bioreactor; argininosuccinic acid (ASA) and dimethylarginine; these metabolites are related to NOS pathway and oxidative stress.