PART II: Univ of Delaware, Papoutsakis

AMBIC

Industry/University Cooperative Research Center (I/UCRC)
Advanced Mammalian Biomanufacturing Innovation Center (AMBIC)

Dissecting Spent Media to Elucidate Components and Mechanisms that Affect Cell Culture Performance

Terry Papoutsakis (UD), Maciek Antoniewicz (UMich)

Mentor Meeting -- November 2, 2023

A National Science Foundation-facilitated
academic – industry – government consortia
to advance precompetitive knowledge in biomanufacturing

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Time did not permit to present the UD component

- Had only 5 mins available after Antoniewicz presentation
- Presented only a few slides
- This expanded set provides a better and more complete story line

Focus on generating perfusion media and the role of Extracellular Vesicles (EVs)

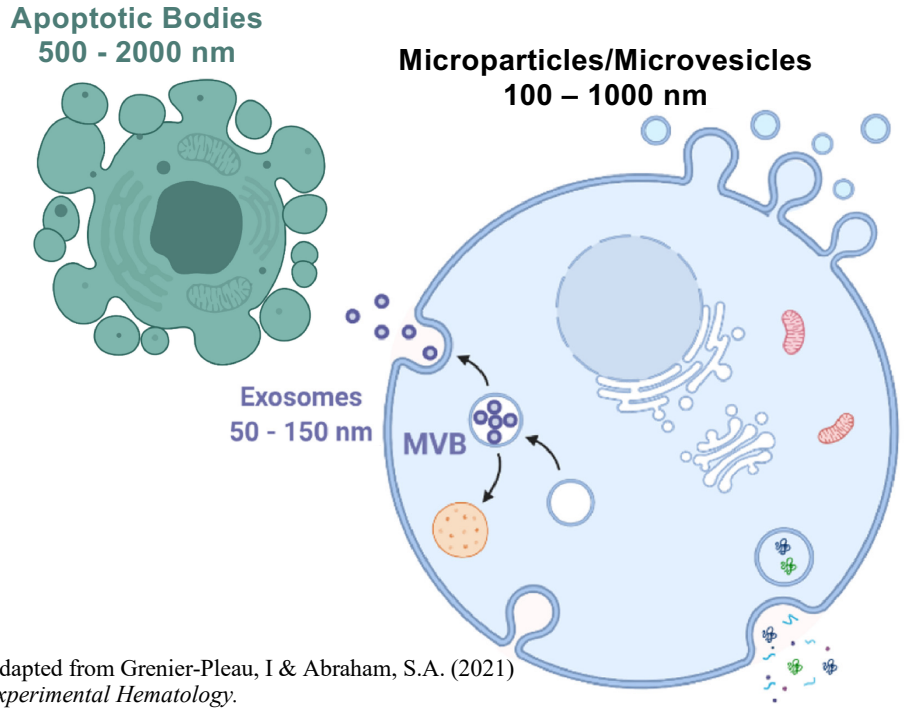
CHO-K1 VRC01 mAb producing cell line

UNIVERSITY OF DELAWARE

A quick primer first on EVs, CHO EVs and their microRNA (miR) cargo

Extracellular Vesicles (EVs) are native mediators of cell-to-cell communication

Nano-sized membrane particles formed from cell membranes that facilitate the transfer of biomolecules (e.g. proteins, RNAs, lipids, DNAs)



Adapted from Grenier-Pleau, I & Abraham, S.A. (2021) *Experimental Hematology.*

Types of EVs:

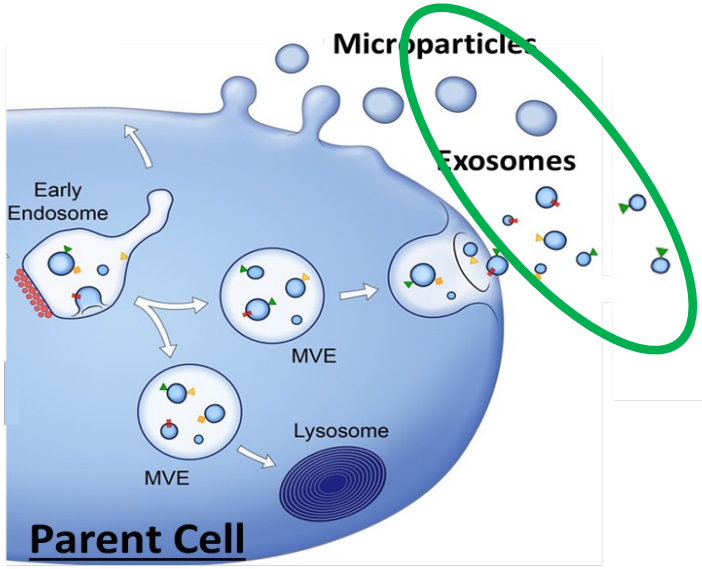
Exosomes – endosomal biogenesis; 50-150 nm

Microparticles – plasma membrane biogenesis; 100-1000 nm

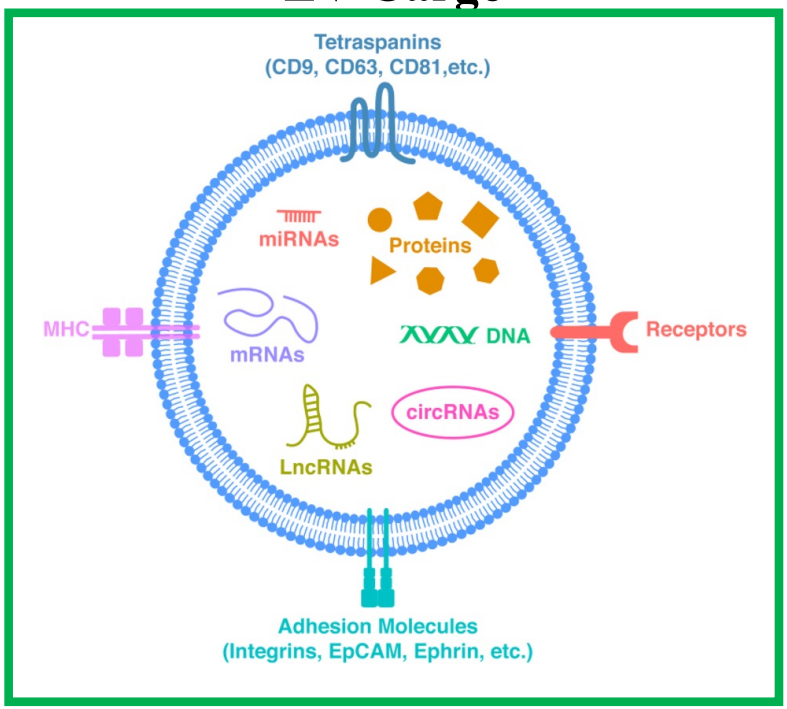
Apoptotic bodies – formed during apoptosis; >500 nm

EVs mediate cell-to-cell communication through cargo transfer and receptor binding

EV cargo from parent cells is exchanged with target cell after membrane fusion or endosomal escape



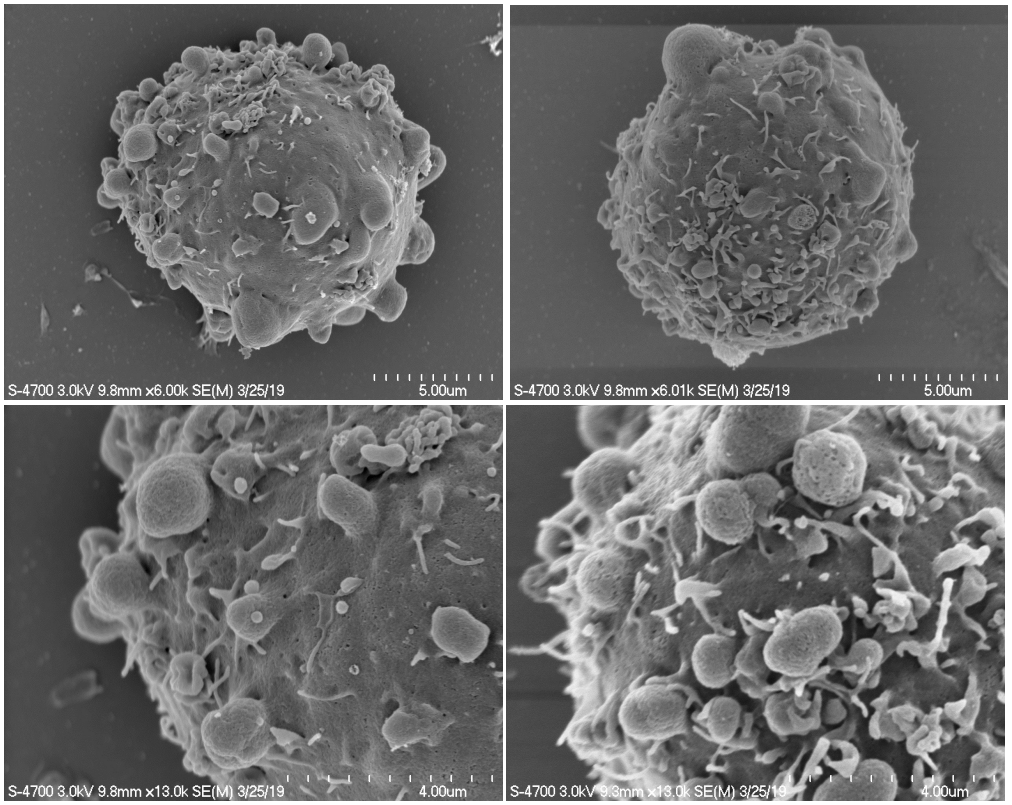
Adapted from Raposo, G., & Stoorvogel W. (2013) *J Cell Biol.*



Tao, S.C., & Guo, S.C. (2020) *Cell Commun Signal.*

Evaluating the exchange of EVs in culture and the potential for material sharing amongst cells

- Scanning electron microscopy (SEM) imaging of CHO cells shows many EV-like structures on cell membrane
    - Identify specific EV exchange events at the cell membrane
- EVs can be traced in culture via RNAs and proteins

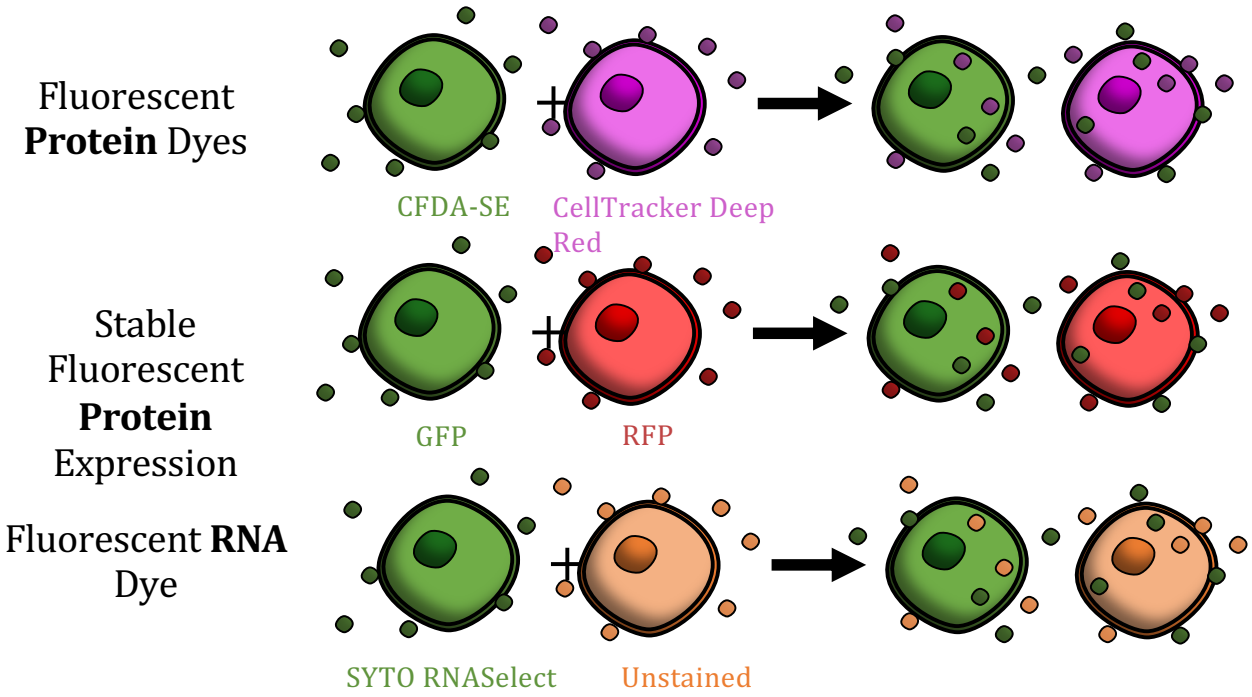


Belliveau, J., & Papoutsakis, E. T. (2022). *Biotechnol Bioeng.*

Tracking native exchange of CHO EVs in culture

- Co-culturing CHO cells expressing different fluorescent proteins or stained with different fluorescent dyes demonstrates the vast exchange of protein and RNAs between cells via EVs

- Microscopy and flow cytometry provides a visual basis for EV exchange and EV uptake at the surface

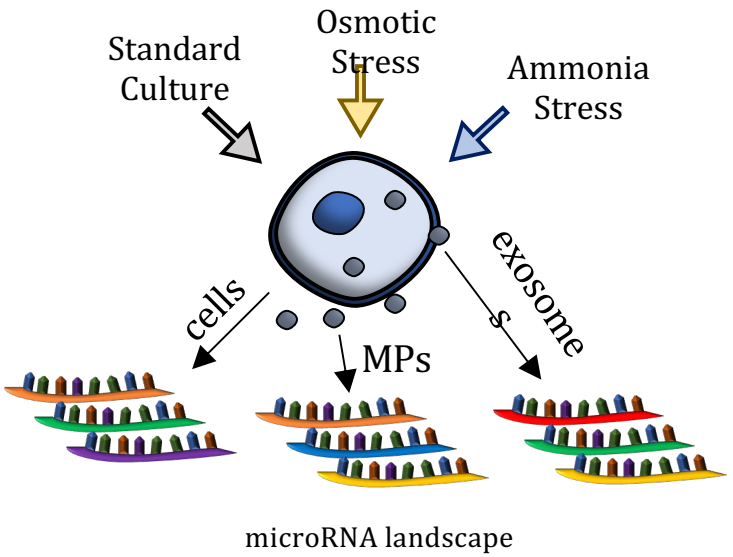


The microRNA landscape of CHO cells and EVs grown in standard and stress conditions

Standard Culture – batch culture grown in shake flasks

Ammonia Stress – accumulates as a waste product that inhibits cell growth and decreases titer and quality

Osmotic Stress – accumulates due to pH control and feedings; reduces cell growth rate



microRNA landscape

Key Questions:

1. Is the microRNA profile of the EV and its parent cell the same?

2. Does the microRNA content of CHO cells and EVs change with different culture conditions?

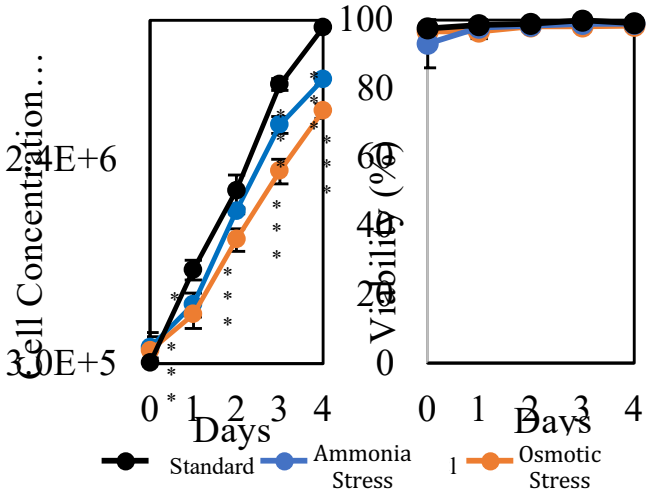
3. What are the potential roles of the microRNAs?

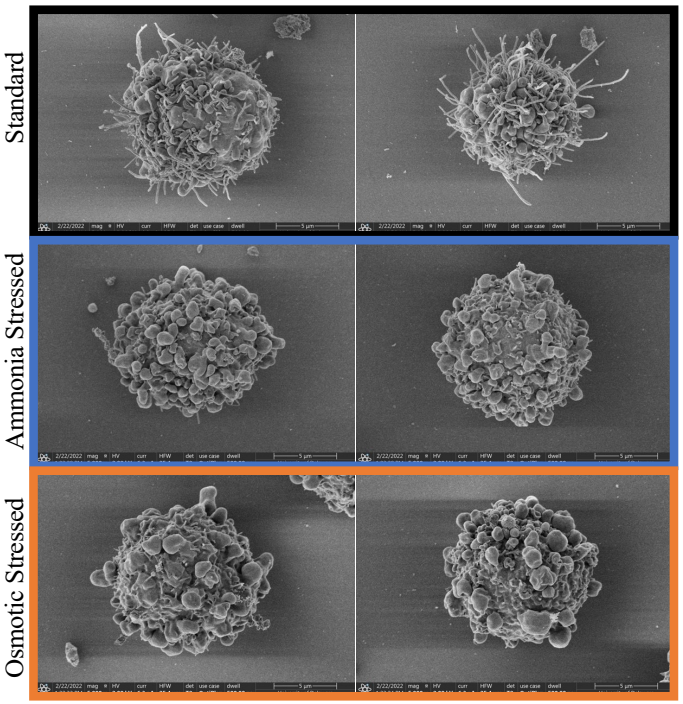
Aim 2

Appropriate stress conditions alter cell growth and morphology

- Select stress conditions that are industrially relevant and induce a stress response in cells that can be observed, without sacrificing overall cell growth and viability
  - Hypothesize a change in cell growth will likely have a change in RNA profile

- Bolus dose of 9 mM ammonium chloride (Ammonia stress) or 60 mM sodium chloride (Osmotic stress) at the start of culture





Belliveau, J., & Papoutsakis, E. T. (2023). *Biotechnol Bioeng.*

3 biological replicates, Student t-test with p values of *0.05, **0.01, and ***<0.001

Cells grown under standard conditions produce EVs with different miR profile

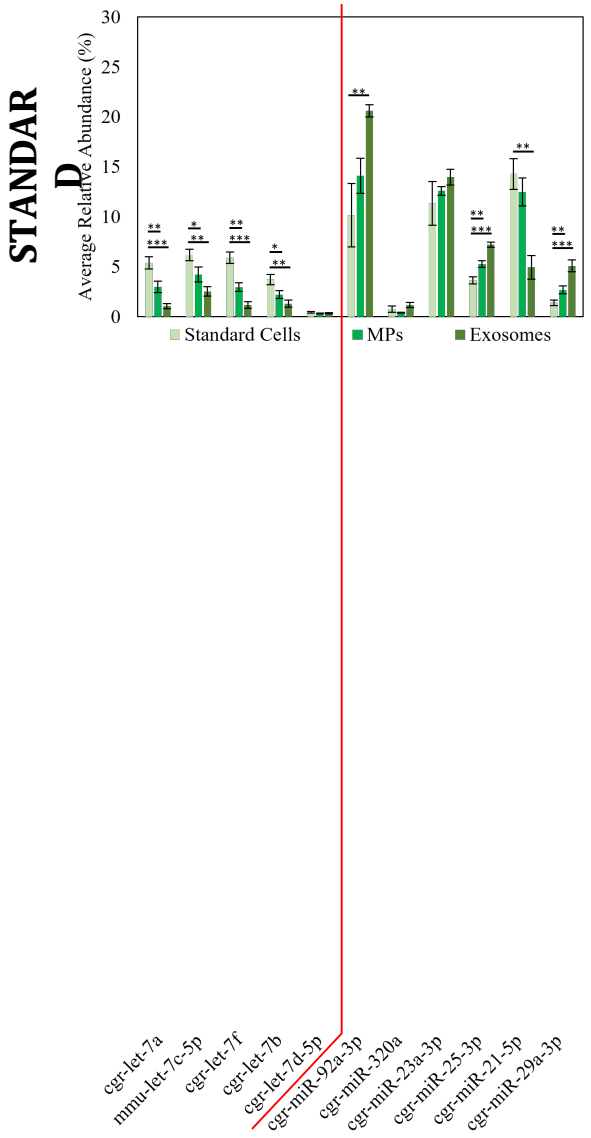
- Unique miR profiles identified in cells, MPs, and exosomes
- Approximately 5 miRs represent 50% of the total identified miRs
- The relative abundance of miRs differs in cells, MPs, and exosomes

- EVs are not a direct copy of the parent cell
- Suggests a miR sorting process that may be involved in cell-to-cell communication

Belliveau, J., & Papoutsakis, E. T. (2023). *Biotechnol Bioeng.*

miR landscape shifts with stress

- Application of culture stress (e.g. ammonia and osmotic) results in a change of the miR profile
  - Fewer total and unique miRs identified in stressed cultures
- Shift in specific miR abundances for stressed cells
- Stressed cultures have increased abundance in the let-7 family of miRs and decrease in abundance of miRs found in standard cultures



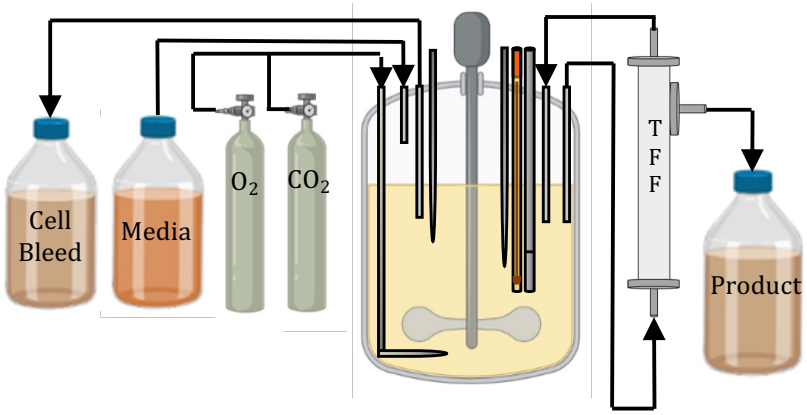
3 biological replicates, Student t-test with p values of *0.05, **0.01, and ***<0.001

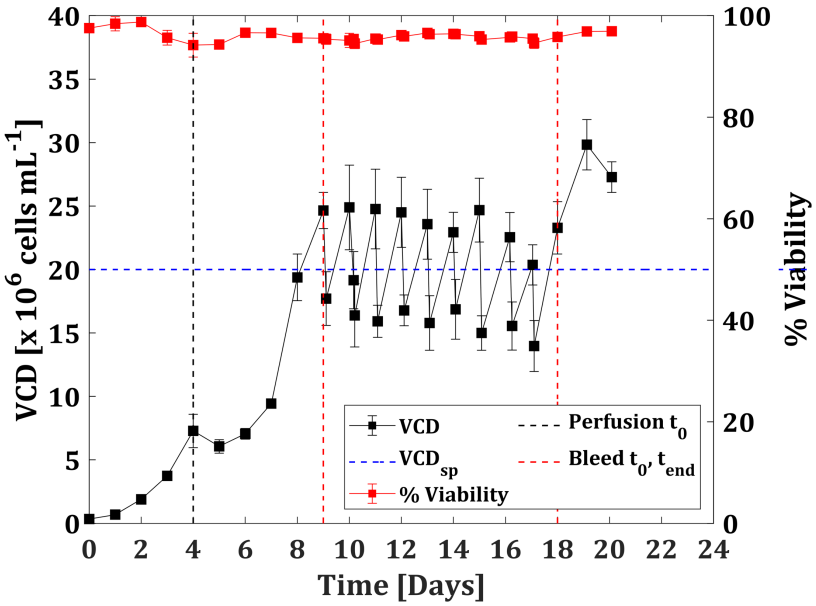
Belliveau, J., & Papoutsakis, E. T. (2023). *Biotechnol Bioeng.*

Two types of perfusion experiments to collect spent media

- Real perfusion
- Simulated perfusion operation

Real Perfusion: Operation of the cell retention module requires re-assessment of experimental configuration

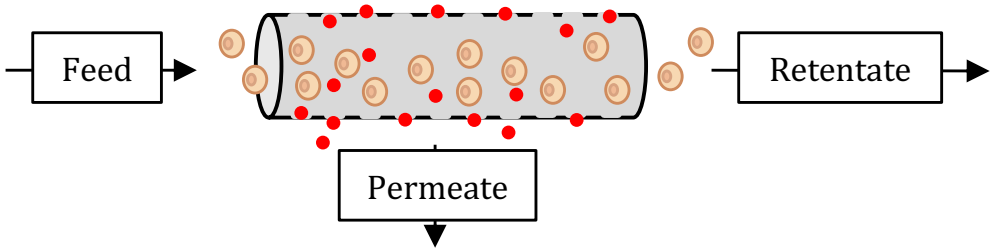




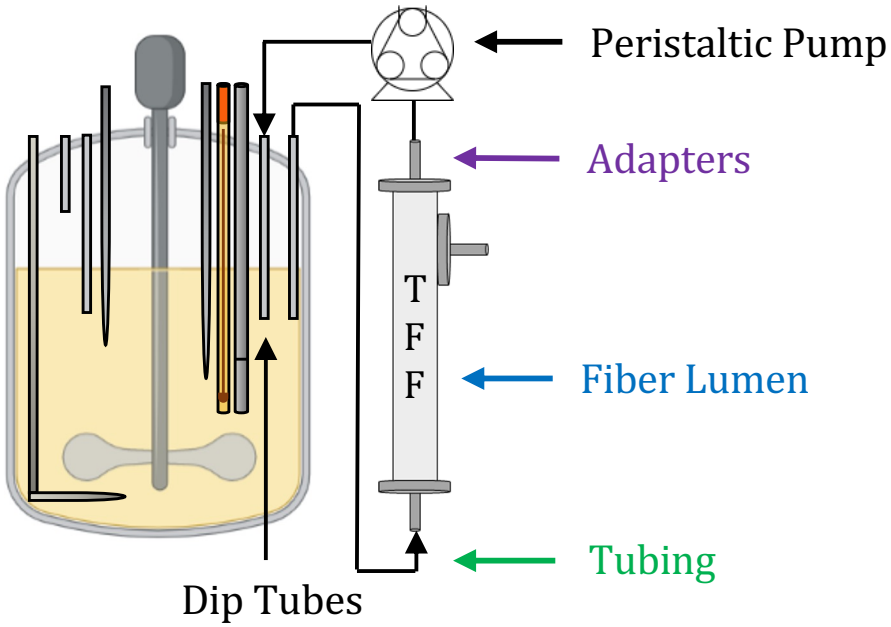
$VCD_{sp} = 20^{x}10^{6}$ cells mL^{-1}
Perfusion Rate = 1 vvd^{-1}

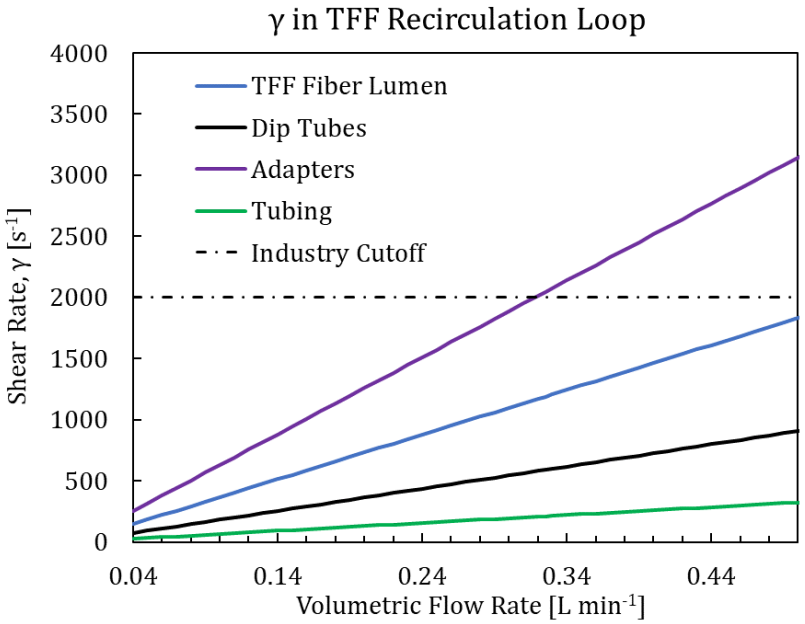
VCD decrease between Day 4-6 initially attributed to a process disturbance.

VCD decrease in the **final transient regime** promoted by **filter fouling.**

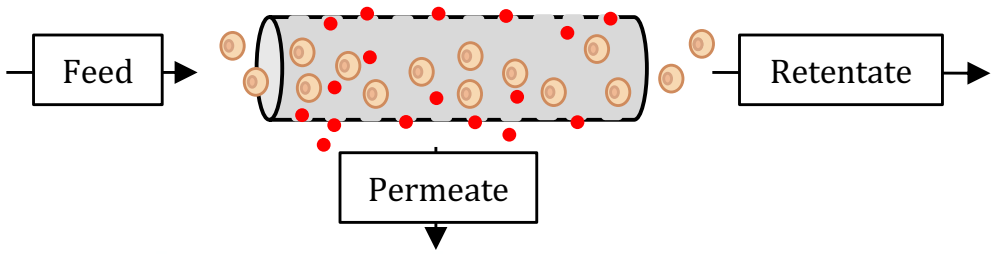


Volumetric flow rate is informed by the wall shear rate through the fiber lumen of the TFF





$$Shear\ Rate, \gamma = \frac{4Q_{TFF}}{\pi R^3}$$

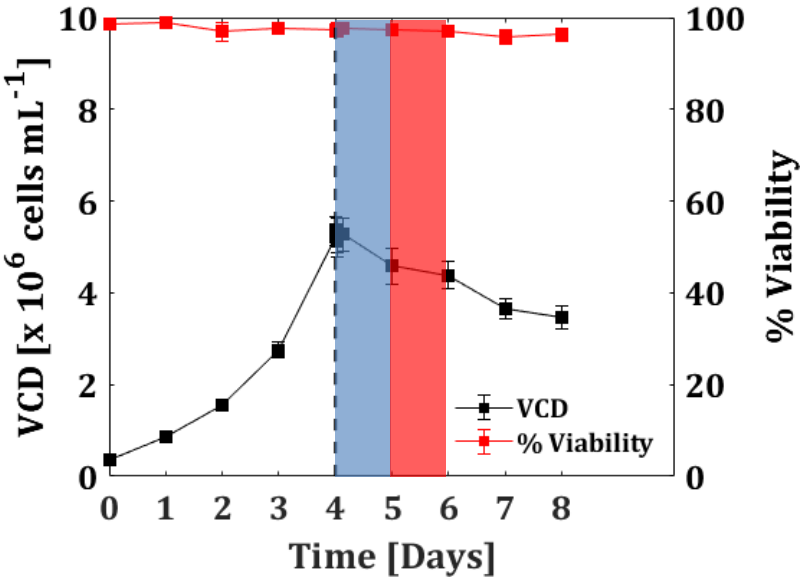


$\gamma = 1000\ s^{-1}$ within the hollow fiber lumen is a rule of thumb as informed by discussions with industry. (Shawn Barret from Sanofi)

γ at **the peristaltic pump** cannot be calculated directly.

[1] Karst, D. J. et al.(2016). *Biochemical Engineering Journal*, *110*, 17-26.
[2] Clincke, M. F. et al. (2013). *Biotechnology progress*, *29*(3), 754-767.
[3] Wang, S. et al. (2017). *Journal of Biotechnology*, *246*, 52-60.
[4] Stressmann, M. (2008). Membrane fouling in constant permeate flux cross-flow microfiltration of biological solutions.

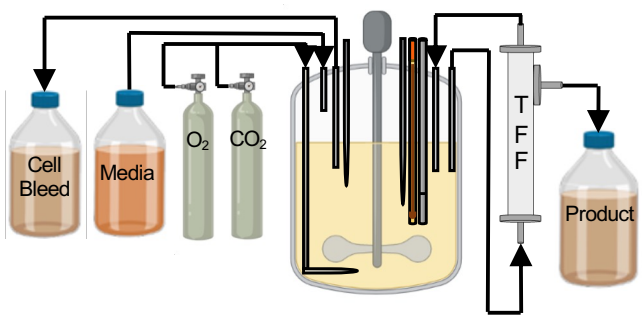
HCCF collection during transient regime from perfusion troubleshooting experiment 1



CHO-K1 VRC01 cell line

Base case perfusion media formulation:
HyClone ActiPro basal media
3% (v/v) HyClone Cell Boost 7a
0.3% (v/v) HyClone Cell Boost 7b\
Supplemental glucose targeting $C_{Glc} = 9\ g\ L^{-1}$

Perfusion Bioreactor



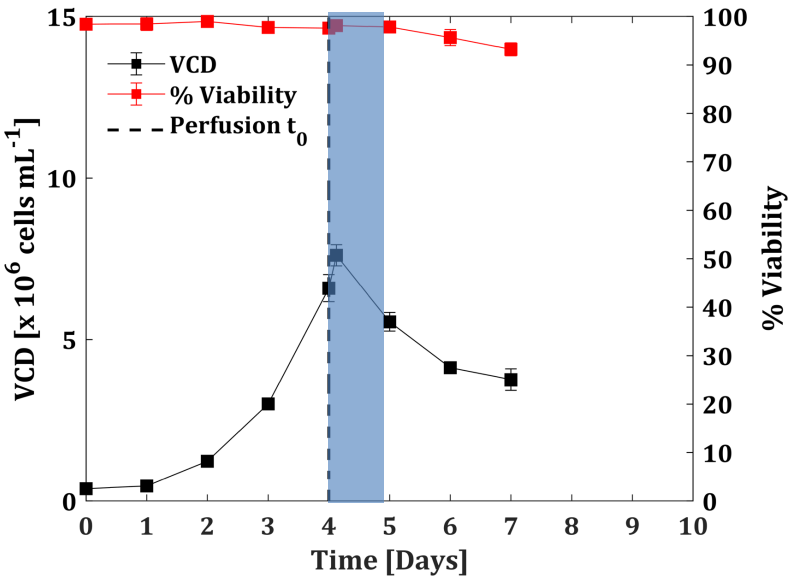
24-hour HCCF collection period at 4 °C over two intervals:

Day 4-5; representing culture fluid at the end of batch phase and beginning of perfusion

Day 5-6; representing culture fluid after one day of perfusion operation

Storage at -80 °C until analysis

HCCF collection during transient regime from perfusion troubleshooting experiment 2



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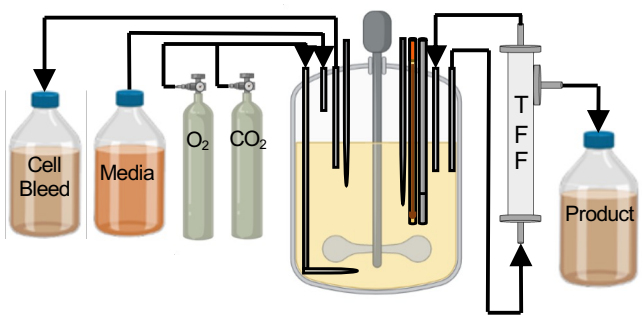
Perfusion Bioreactor



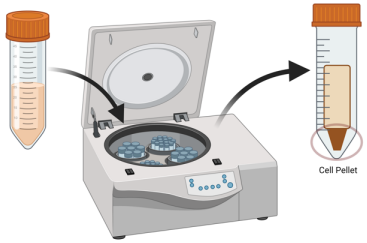
24-hour HCCF collection period at 4 °C over two intervals:

Day 4-5; representing culture fluid at the end of batch phase and beginning of perfusion

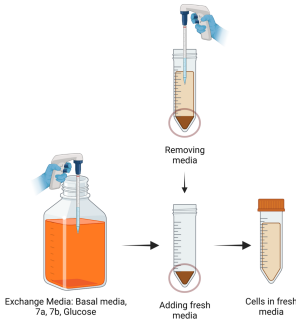
Storage at -80 °C until analysis

Semi-Perfusion in Spin Tubes
Complete Time-Series Metabolite Profiles

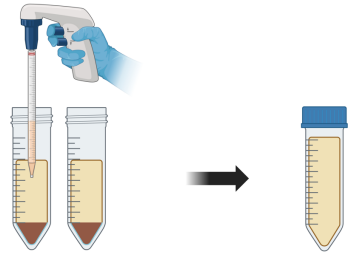
Scale-down model of perfusion in semi-continuous spin-tube and T-flask cultures to generate EVs



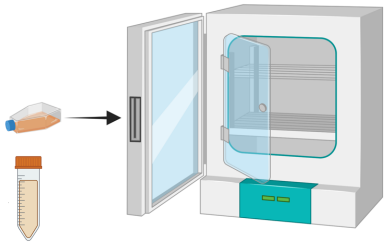
Step 1: Centrifuge cell culture at 180g for 5 minutes to pellet cells.



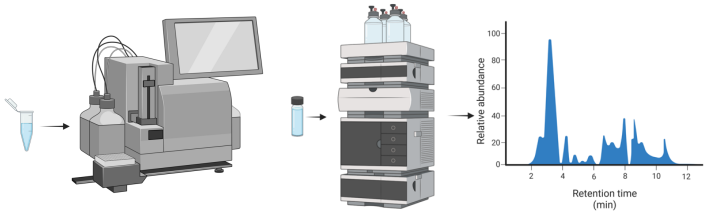
Step 2: Media exchange via supernatant removal and cell resuspension in fresh, pre-warmed media.



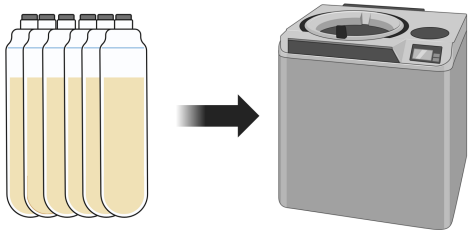
Step 3: Pool supernatant across biological duplicates to harvest EVs.



Step 4: 24-hour incubation with orbital agitation at 250 rpm for spin tubes and 150 rpm for T-flasks.

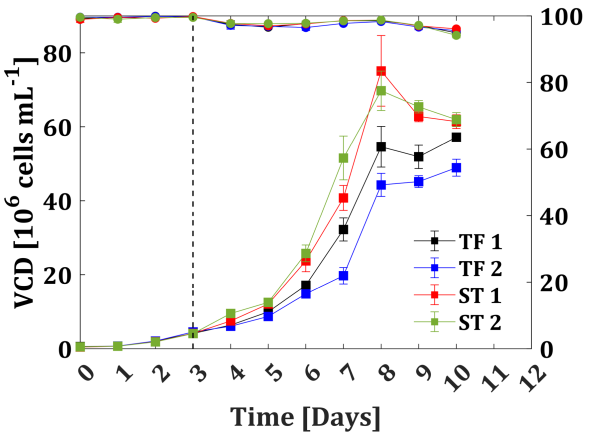


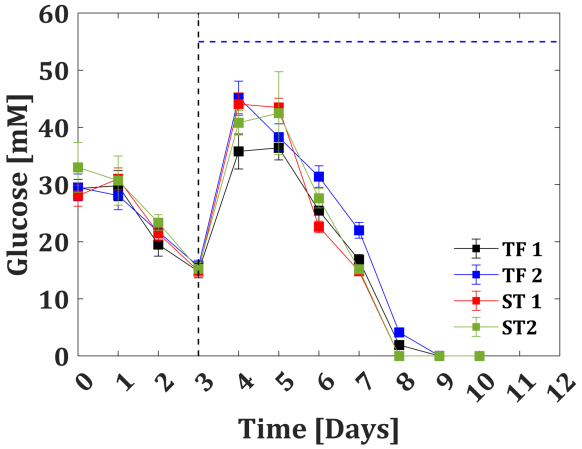
Step 5: Off-line metabolite measurements via HPLC (amino acids and titer) and YSI Biochemistry Analyzer (glucose, lactate, ammonia).

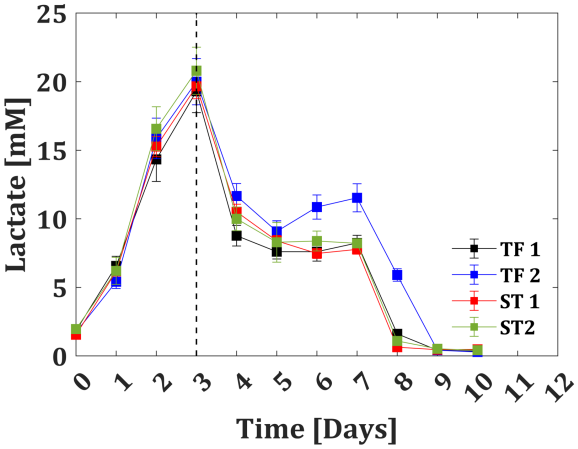


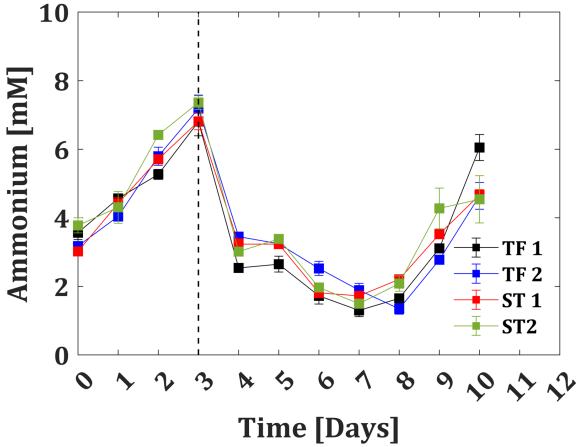
Step 6: Harvest EVs (microparticles and exosomes) via differential ultracentrifugation.

Base case media formulation with sufficient oxygenation enables a short PSS









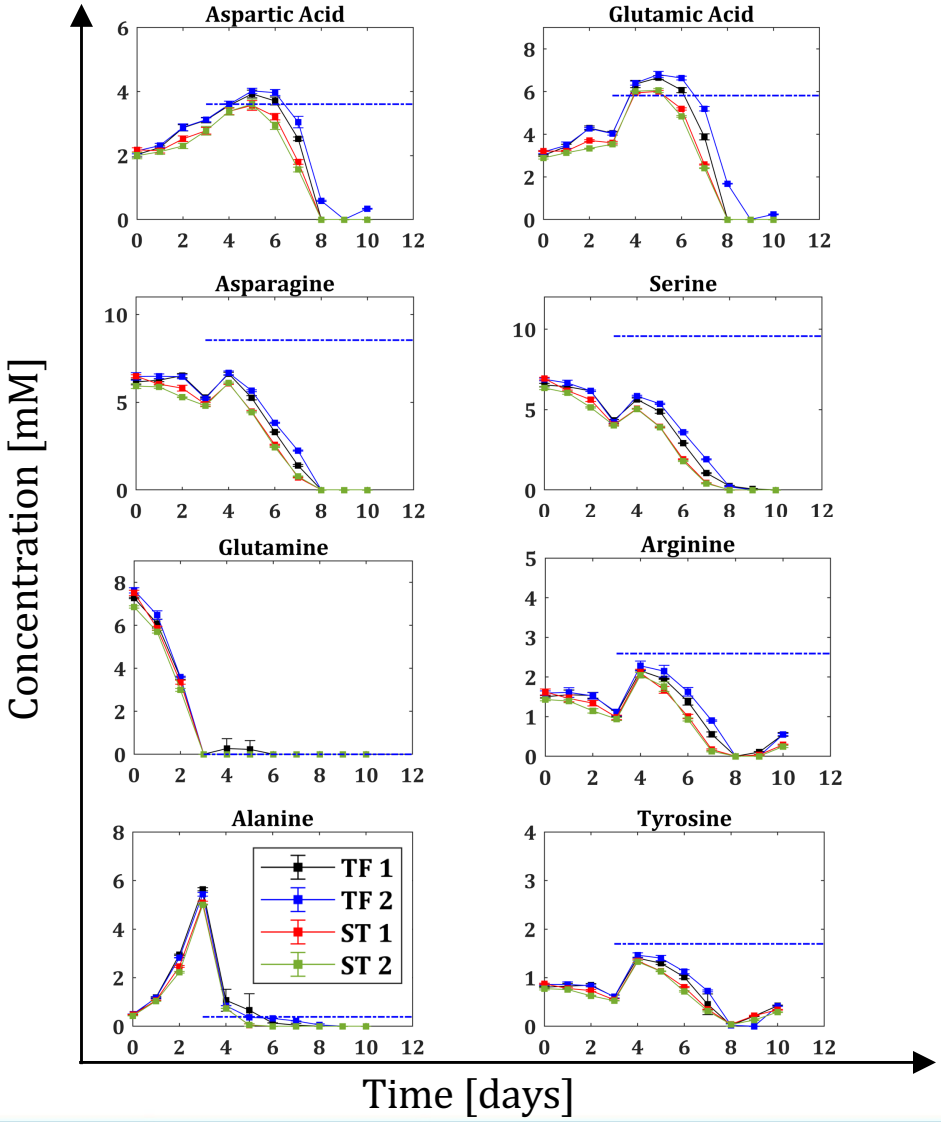
Glucose depletion and low lactate **evidence sufficient gas transfer.**

High $C_{Glucose}$ in media sustains the short PSS as the cultures approach fed-batch dynamics.

Ammonium accumulation reveals a non-holistic metabolic PSS.

Introducing a cell bleed in future experiments will promote a longer PSS.

The same amino acids are consistently depleted in both semi and continuous perfusion cultures



Asn, Ser, and Arg depleted ~24 hours prior to Asp and Glu in the Spin Tube cultures.

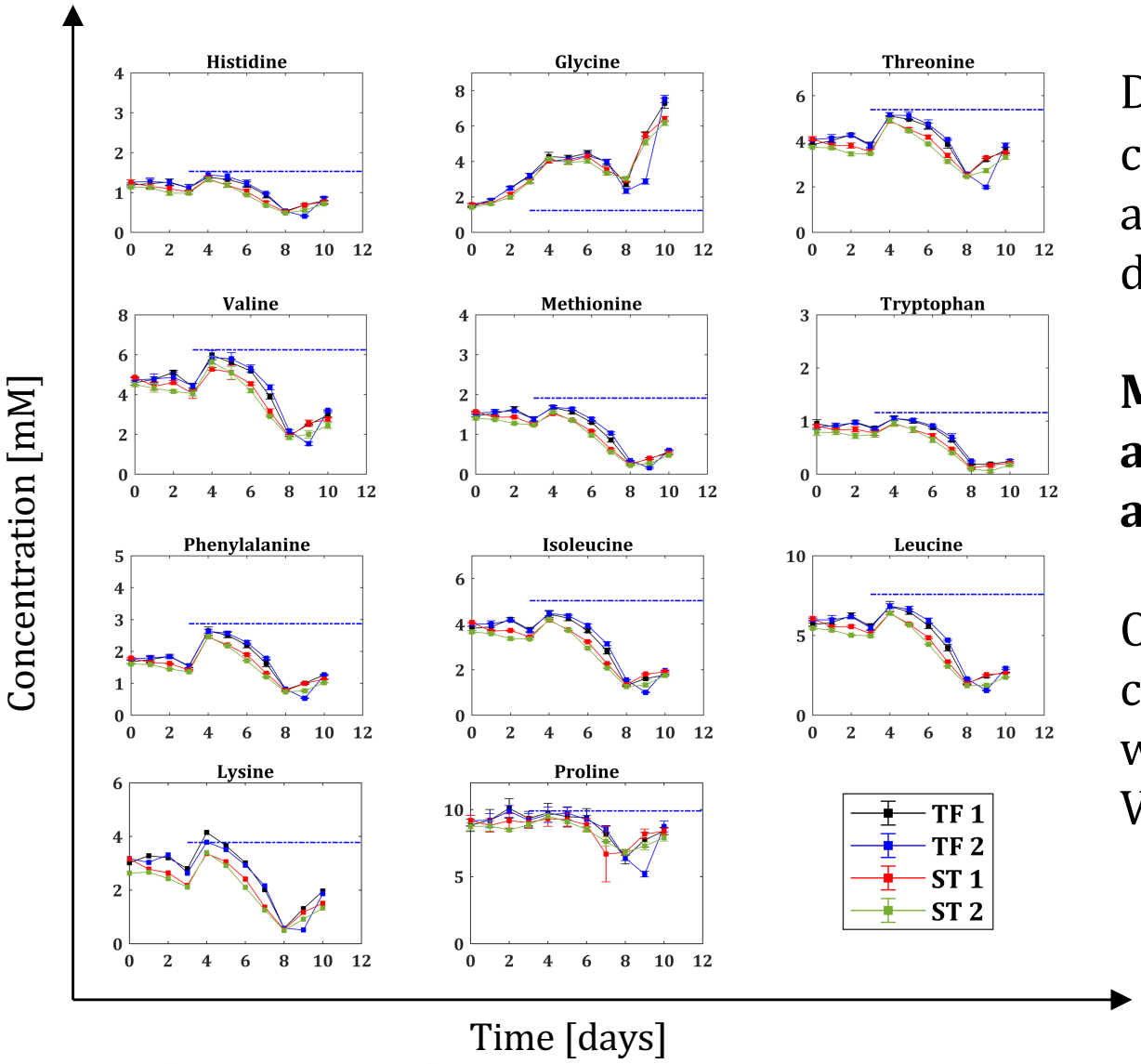
Asn and Ser are present in the highest concentrations in the base case formulation.

Arg and Tyr are consistently the only essential amino acids to face depletion at high VCDs.

Ala consumption suggests non-inhibitory Amm levels despite Amm accumulation.[4]

[4] Synoground, B. F., McGraw, C. E., Elliott, K. S., Leuze, C., Roth, J. R., Harcum, S. W., & Sandoval, N. R. (2021). Transient ammonia stress on Chinese hamster ovary (CHO) cells yield alterations to alanine metabolism and IgG glycosylation profiles. *Biotechnology Journal*, *16*(7), 2100098..

Most essential amino acids are not depleted

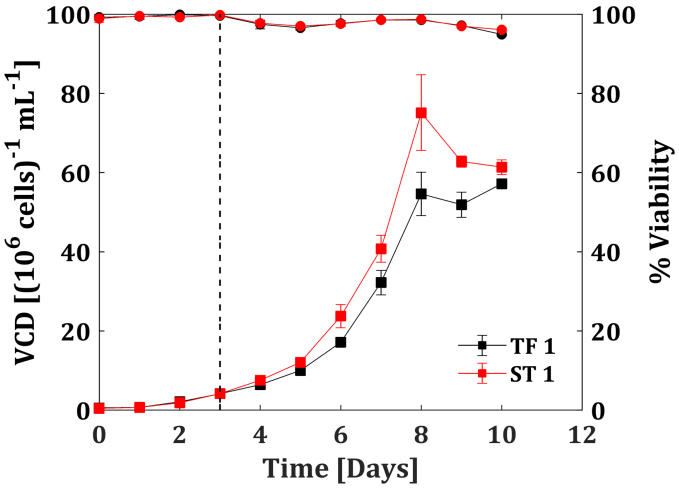


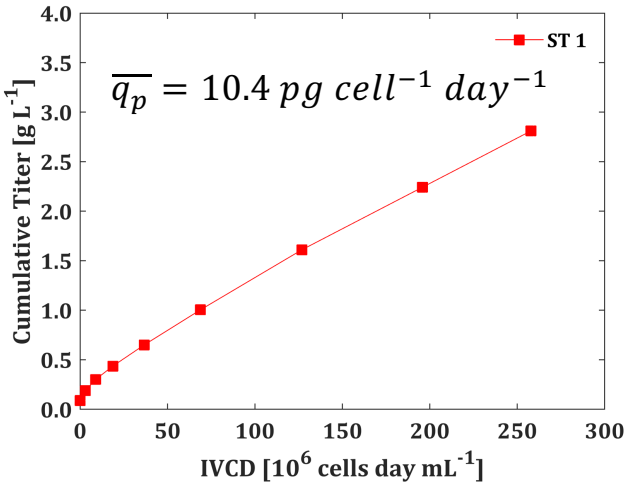
Despite relatively low concentrations of essential amino acids, only Met and Trp near depletion.

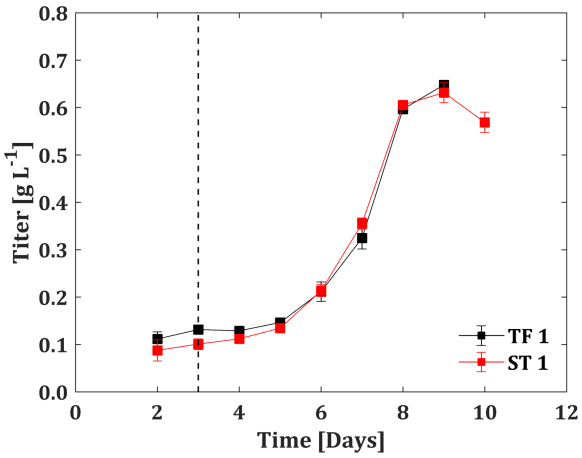
Metabolic demand on essential amino acids decreases as $\mu \rightarrow 0$ after Day 8.

Oscillatory Gly (nonessential) concentration may imply a re-wiring of Ser catabolism as VCD_{max} is approached.

Titer profiles under semi-perfusion without a cell bleed establish a productivity baseline



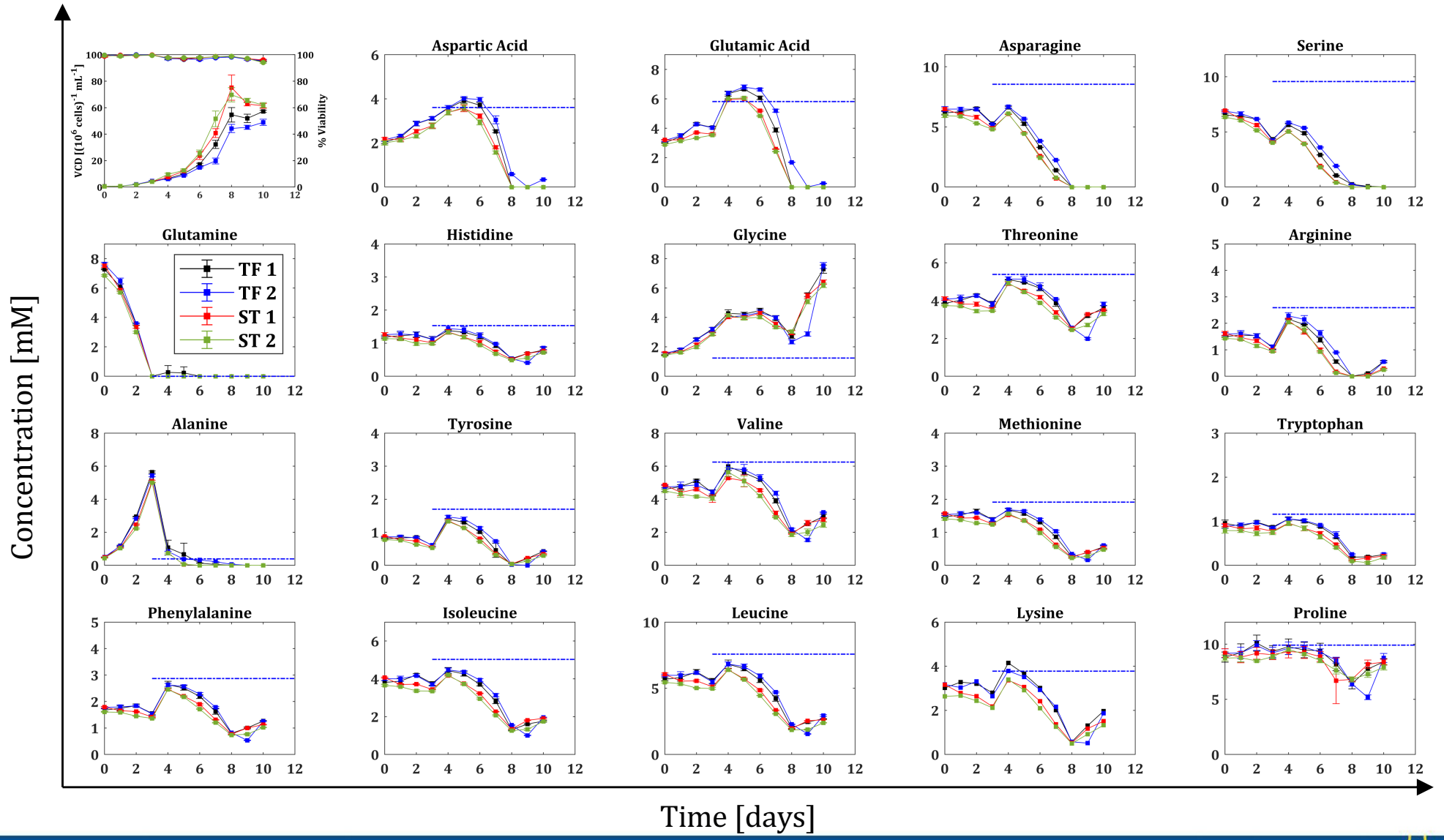




Previous semi-perfusion experiments at larger working volumes were faced with a drop in daily titer upon attaining $VCD_{max.}$

Continued high q_p at a $\mu \sim 0$; characteristic of the goal of metabolic control.

All measured amino acids



Supernatant pooled across biological duplicates and across consecutive days to harvest EVs

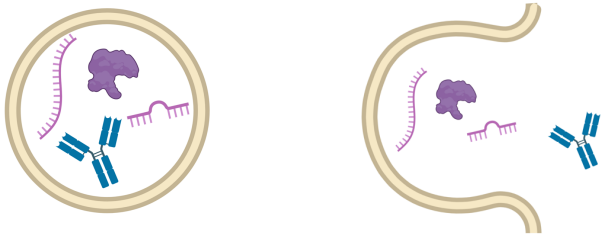
Sample ID	Pooled Supernatant	Volume [mL]	Experimental Condition
1	Days 5-6	30	High glucose T-flask duplicates
2	Days 7-8	30	High glucose T-flask duplicates
3	Days 9-10	30	High glucose T-flask duplicates
4	Days 6-7	30	Low glucose T-flask duplicates
5	Days 8-9	30	Low glucose T-flask duplicates
6	Days 5-10	30	High glucose spin tube duplicates

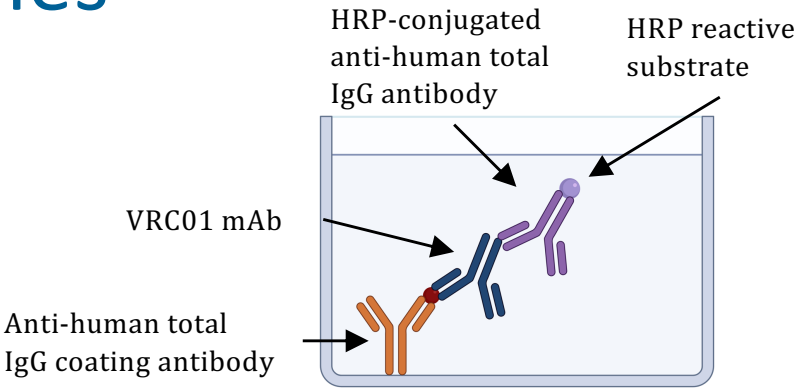
Only microparticles (MPs) were quantified via flow cytometry.

Sample ID	Microparticles mL^{-1}
1	1.05E+07
2	7.47E+06
3	1.30E+07
4	5.66E+06
5	1.55E+07
6	1.83E+07

Given high VCDs, MP counts are **underestimates.** MP and exosome pellets could not be resuspended after harvest and during sample preparation for flow cytometry.

ELISA assay to detect mAbs in concentrated lysate from isolated MPs and exosomes





Step 1: Harvested microparticles and exosomes lysed to release cargo.[1]

Step 2: Concentrate lysate with Vivaspin 500 centrifugal filters.

Step 3: Human IgG ELISA assay to quantify VRC01 mAb released from EVs.

Question: Does secreted mAb pellet with MPs and exosomes during ultracentrifugation?

MP and exosome pellets washed with PBS to remove co-pelleted mAb prior to lysis.[2]

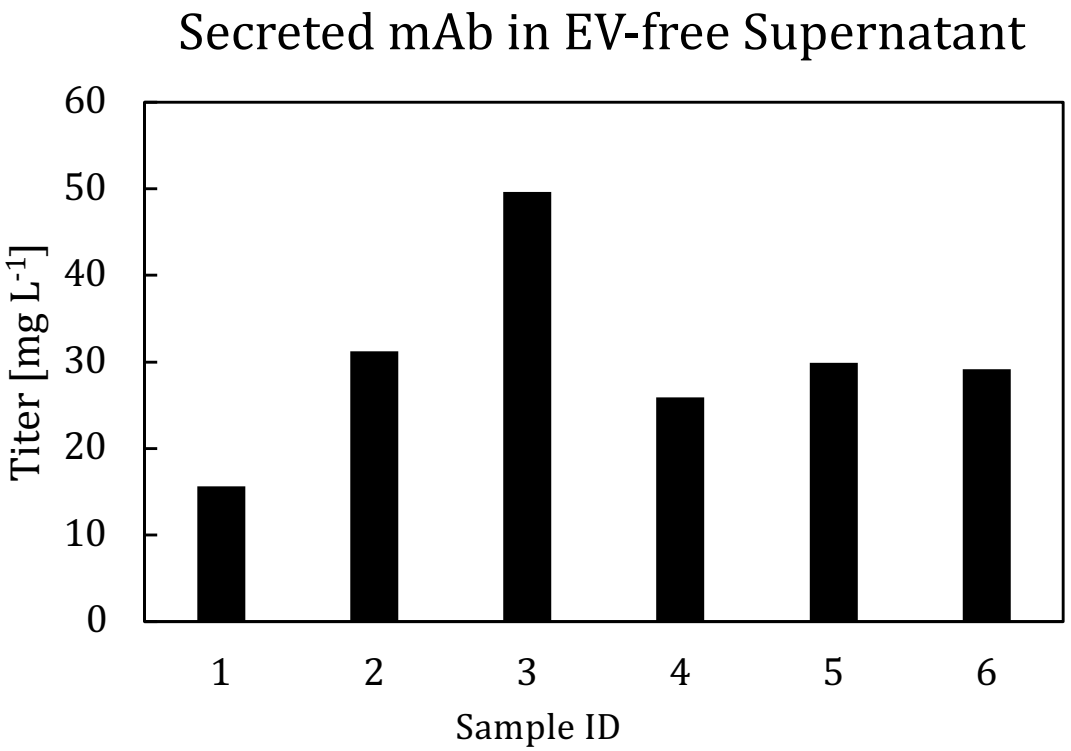
Upon repeating the procedure, resuspended EVs will be further concentrated with spin filters to remove any remaining secreted mAb prior to lysis.

- The EV pellets were partially resuspended in 100 uL of RIPA lysis buffer and 0.5 uL of protease inhibitor. The pellets were highly concentrated and complete resuspension in the lysis buffer was not attainable.

[1] Subedi, P., Schneider, M., Atkinson, M. J., & Tapio, S. (2021). Isolation of Proteins from Extracellular Vesicles (EVs) for Mass Spectrometry Based Proteomic Analyses. *Proteomic Profiling: Methods and Protocols*, 207-212.

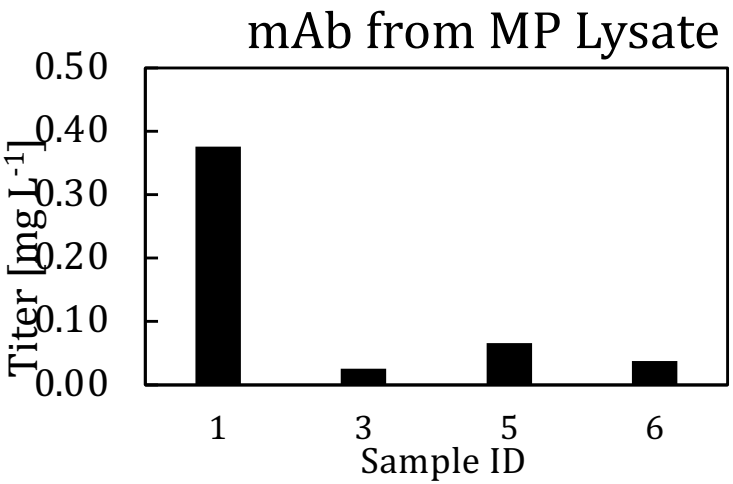
[2] Théry, C., Amigorena, S., Raposo, G., & Clayton, A. (2006). Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Current protocols in cell biology*, *30*(1), 3-22.

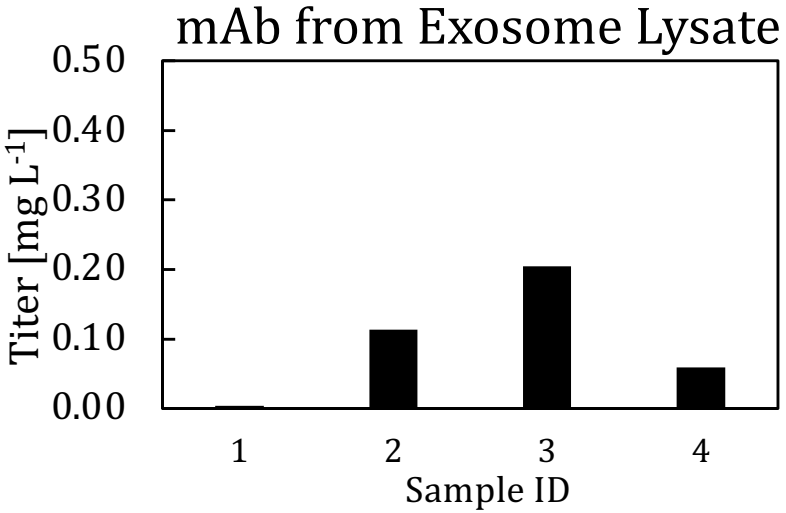
ELISA Assay – EV-free supernatant



mAb in supernatant **prior to and after** EV harvesting will be quantified via Protein A chromatography to corroborate the ELISA assay results and determine if secreted mAb is co-precipitated with EV pellets during ultracentrifugation.

ELISA Assay on MP & exosome lysate verifies presence of loaded mAb





- EV lysate was not diluted prior to ELISA assay.
- Missing sample IDs exceeded the ELISA limit of quantification.
- EV lysate will be diluted in next iteration of the protocol.
- The mAb concentration from the EV lysate is an underestimate as the EV pellets could not be fully resuspended during lysis. However, considering this, the mAb concentration in the EVs is still significantly lower than the EV-free supernatant.

Despite several samples exceeding the limit of quantification, there is an **implication of preferential mAb loading in MPs during earlier days of culture, which then shifts to loading in exosomes.**

Summary

1. Semi-perfusion cultures in spin tubes and T-flasks generate large amounts of EVs. Culture supernatant will be diluted in next iteration to ensure EV pellets can be fully resuspended in preparation for quantification and lysis.

2. The **possibility of secreted mAb co-pelleting with MPs and exosomes** during EV harvest is still a concern which may affect the quantitative results. Next iteration will include an additional spin-filter step to remove secreted mAb after EV harvest.

3. Preliminary ELISA assay verifies ***presence*** of loaded VRC01 mAb in EVs.

4. Harvest and lysis protocols will be refined to provide an improved quantitative measure of loaded mAb in EVs.