

Analysis of Extracellular Vesicles

Study Design Considerations

Protein assays within Olink panels have been optimized for the dynamic range present in human plasma and serum. Results are reported as NPX™ units which are used to compare relative changes in protein abundance between study groups. Identification of true biological differences between study groups is facilitated by reducing technical variability to the fullest extent possible. This includes using the same collection procedure for each sample, keeping the same number of freeze/thaw cycles, and maintaining even storage conditions.

Within a study, all samples should be randomized across all plates and it is best to use a balanced number of samples across the study groups.

In addition to plasma and serum, strategies have been developed to analyze alternative types of samples. Extracellular vesicles (EVs) are membranous vesicles that exist in different sizes (nm to μm). There are different classes of EVs which can be differentiated based on size or cell surface biomarkers (e.g., exosomes, microvesicles, macrovesicles, and apoptotic bodies). EVs contain membrane proteins and luminal proteins (i.e., cargo) that represent important signatures in both health and disease. Olink has analyzed EVs harvested from plasma, urine, cerebrospinal fluid, and saliva.

Biological replicates should be included to account for technical differences in sample preparation. Technical replicates can also be included for better estimation of CVs when using an alternative matrix. To evaluate protein assays at risk for hook, it is recommended to run a few samples from each study group at two additional dilutions. Lysis buffer alone sample(s) can be included to monitor background noise. Special attention should be paid to formulation of the lysis buffer, more information can be found in the document *Running alternative matrices: Buffer compatibility with Olink*.

Normalization of EV samples

There are several approaches for normalizing EV samples, and selection should align with study objectives:

1. For biofluids, it is recommended to normalize by starting volume (e.g., 300 μl of plasma)
2. For *in vitro* studies, samples can be normalized to the number of cells producing EVs (Ref 1)
3. If normalization is performed using total protein concentration, then 0.5 mg/ml is recommended (but less concentrated is possible)
Note: this method is common, but there is the potential to normalize out biological variation if, for example, disease cells are producing more EVs compared to healthy cells
4. EV particle number: There are several methodologies for quantifying EVs, including Nanoparticle Tracking Analysis (NanoSight) and flow cytometry (Ref 2)
5. EV-specific membrane biomarkers: For example, CD63, CD81, or CD9 (Ref 3)

Recommendations for Sample Preparation

Purification of EVs

- There are various methods for isolating EVs including ultracentrifugation, density gradient centrifugation, size exclusion chromatography, polymer-based precipitation, filtration, and immunological separation (Ref 4,5). No single technique is recommended.
- Multiple companies produce kits for isolating intact EVs/exosomes. Follow manufacturer instructions for use of commercial lysis buffers or kits.

General guidelines for lysing purified EVs

- Keep lysis buffer cold, perform lysis on ice, and centrifugations at 4°C.
- Different EV preparation methods will yield varying particle numbers and particle sizes, therefore, the amount of lysis buffer volume to add will have to be determined empirically. It is better to start with a lower volume of lysis buffer such that the final protein concentration will be in the range of 0.5 mg/ml for optimal detectability.

Note: Ref 5 compared different EV isolation techniques from 200 µl of human serum and found that total protein yield ranged from 17-2206 µg.

- If samples are turbid, centrifuge at high speed at 4°C to remove debris.
- If necessary, protein concentration of clarified lysates can be estimated using standard techniques (e.g., BCA, Bradford, Lowry, or Nanodrop assays).

Pre-Dilution Strategies

Target 96:

CAM	CRE	CVDII	CVDIII	DEV	IMO	INF	IRE	MET	NEU	NEX	ODA	ONCII	ONCIII
1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1

Target 48:

1:1

Note: dilutions are denoted as A:B, where A=number of sample units and B=total number of units after dilution, therefore 1:1 = undiluted or 'neat' sample.

References

Ref 1: Lee SS, Won JH, Lim GJ, Han J, Lee JY, Cho KO, Bae YK. A novel population of extracellular vesicles smaller than exosomes promotes cell proliferation. *Cell Commun Signal*. 2019; 17(1):95. DOI: 10.1186/s12964-019-0401-z. [Link](#)

Ref 2: Koritzinsky EH, Street JM, Star RA, Yuen PS. Quantification of Exosomes. *J Cell Physiol.* 2017; 232(7):1587-1590. DOI: 10.1002/jcp.25387. [Link](#)

Ref 3: Kowal J, et al. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc Natl Acad Sci U S A.* 2016; 113(8):E968-77. DOI: 10.1073/pnas.1521230113. [Link](#)

Ref 4: Allelein S, Medina-Perez P, Lopes ALH, Rau S, Hause G, Kölsch A, Kuhlmeier D. Potential and challenges of specifically isolating extracellular vesicles from heterogeneous populations. *Sci Rep.* 2021; 11(1):11585. DOI: 10.1038/s41598-021-91129-y. [Link](#)

Ref 5: Brennan K, Martin K, FitzGerald SP, O'Sullivan J, Wu Y, Blanco A, Richardson C, Mc Gee MM. A comparison of methods for the isolation and separation of extracellular vesicles from protein and lipid particles in human serum. *Sci Rep.* 2020; 10(1):1039. DOI: 10.1038/s41598-020-57497-7. [Link](#)

Publications using Olink

Krishnamachary B, Cook C, Kumar A, Spikes L, Chalise P, Dhillon NK. Extracellular vesicle-mediated endothelial apoptosis and EV-associated proteins correlate with COVID-19 disease severity. *J Extracell Vesicles.* 2021; 10(9):e12117. DOI: 10.1002/jev2.12117. [Link](#) [plasma EVs]

Hyland M, Mennan C, Wilson E, Clayton A, Kehoe O. Pro-inflammatory priming of umbilical cord mesenchymal stromal cells alters the protein cargo of their extracellular vesicles. *Cells.* 2020; 9(3):726. DOI: 10.3390/cells9030726. [Link](#) [EVs from conditioned media]

Sun B, Fernandes N, Pulliam L. Profile of neuronal exosomes in HIV cognitive impairment exposes sex differences. *AIDS.* 2019; 33(11):1683-1692. DOI: 10.1097/QAD.0000000000002272. [Link](#) [neuronal exosomes]

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