

Quantification of Extracellular Vesicles Using PICO Technology

Pablo Sánchez-Martín¹, John Atanga², and Tobias Gross¹

¹PICO BioScience GmbH, Freiburg im Breisgau, Germany

²Extracellular Vesicles Group, Institute for Infection Prevention and Control, University

Medical Center Freiburg, Freiburg im Breisgau, Germany

Introduction

Extracellular vesicles (EVs) are minute lipid bilayer-encased particles that are actively secreted by nearly every cell type. Both in their membrane and in their content, EVs contain biomarkers representative of their parental cells. Originally considered just a debris subproduct of maturing cells (1), intensive research in the last two decades has established their role in a complex cell-to-cell communication mechanism, influencing biological processes through the transfer of functional cargos between cells (2). For that reason, fluctuations in the total number of circulating EVs can be indicative of physiological changes. They can be considered as potential diagnostic tools, as their isolation from liquid biopsies is both less invasive and even more informative than traditional screening methods. Thus, both in discovery-phase research and diagnostics, there is a growing interest in the study and characterization of these small vesicles.

However, better analysis methods are still required. Their small size, heterogeneity, and low abundance are the factors that make their analysis difficult. ELISA, the conventional technology employed in the analysis of EVs, requires high volumes of many precious samples, and the ELISA procedure exposes the EVs to nonspecific adsorp-

tion. It also requires a calibration standard, which is not commonly available. Other techniques, like western blot, cannot discriminate between the different subpopulations of EVs that are purified together.

Therefore, there's a need for a more robust and quantitative approach to measure EVs. The **Protein Interaction Coupling** (PICO) technology is a highly sensitive immunoassay for the detection of proteins, protein interactions, and post-translational modifications using digital PCR as a readout. Here, we present a PICO workflow for the analysis of intact EVs, using as little as 1 µl of starting material, and without the requirement of external calibration standards enabling absolute quantification. In addition, the PICO assay offers the possibility of measuring not only a single protein, but also several protein targets simultaneously.

Highlights

- PICO enables the precise quantification of EVs with picomolar sensitivity.
- Robust quantification of EVs independent of EV origin.
- Several EV surface proteins (e.g. CD9, CD63) can be measured by PICO, individually or in combination.

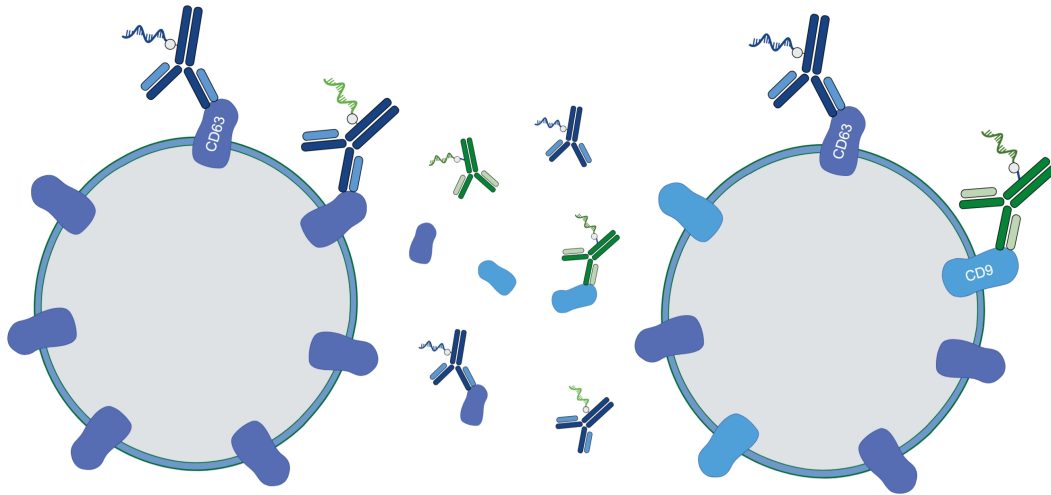


Figure 1. Detection strategies for intact EVs. (1) The same CD63 antibody labeled with two different PICO Labels targeting the same surface protein (CD63, left) or (2) different proteins (CD9 and CD63, right) are used. The PICO assay's detection unit, known as a 'couplex,' comprises two distinct antibodies labeled with PICO Labels, bound to the partitioned intact EV, in the example of (1) the differently la-beled CD63 antibody are linked through the EV membrane forms the complexes, while example (2) shows the experimental setup of using two different antibodies (also labeled differently). This enables the specific detection of intact EVs, while soluble proteins with only one antibody bound are not quantified.

Results

Detection of EVs by any technique presents a series of challenges. First, the extremely low abundance of these vesicles is in the lower picomolar range even after enrichment and purification. Second, to properly characterize the different subpopulations, the surface epitopes need to be detected in detergent-free conditions to preserve the integrity of the vesicles, a requirement extremely challenging in immunoassays with large adsorption surfaces, like ELISA. The PICO technology employs two or more DNA-labeled antibodies against each EV target or targets. A target EV bound by two different DNA-labeled antibodies, called 'couplex', is the detection unit of PICO. PICO samples are analyzed using dPCR, partitioning the individual EVs into separate partitions, with subsequent amplification of the different DNA labels in the partitions detecting the presence of the antibodies. Through the distribution pattern of the negative, positive, and double positive partitions a statistical model allows to determine the number of complexes and subsequently the EVs.

To evaluate the suitability of PICO in the detection of intact EVs, we aimed first at the detection of a single EV surface marker in detergent-free conditions using EVs derived from cell culture. A single monoclonal antibody targeting the extracellular domain of CD63, a common EV surface marker, was divided into two stocks and each labeled using a different single-stranded DNA label (PICO Labels). This allows the detection of double-positive signals only in the presence of at least two copies of the CD63 in the partitions, as occurs in the surface of intact EVs, preventing the detection of free single copies of the protein (**Fig 1**). To preserve the integrity of the EVs during the analysis, both the EVs dilution series and the antibody mix were prepared in PBS.

Using EVs isolated from cell culture supernatants, we were able to detect CD63 in the surface of these vesicles (**Fig 2A**). CD63 positive EVs could be measured in all the dilutions tested, down to 3×10^5 vesicles/ μ l. In all cases, the number of complexes were significantly different from the negative control (ABC - the antibody mix without EVs). ABC has a reading of zero, as PICO has a proven zero-background (3), which allows sensitive measurements even at low concentrations of the target.

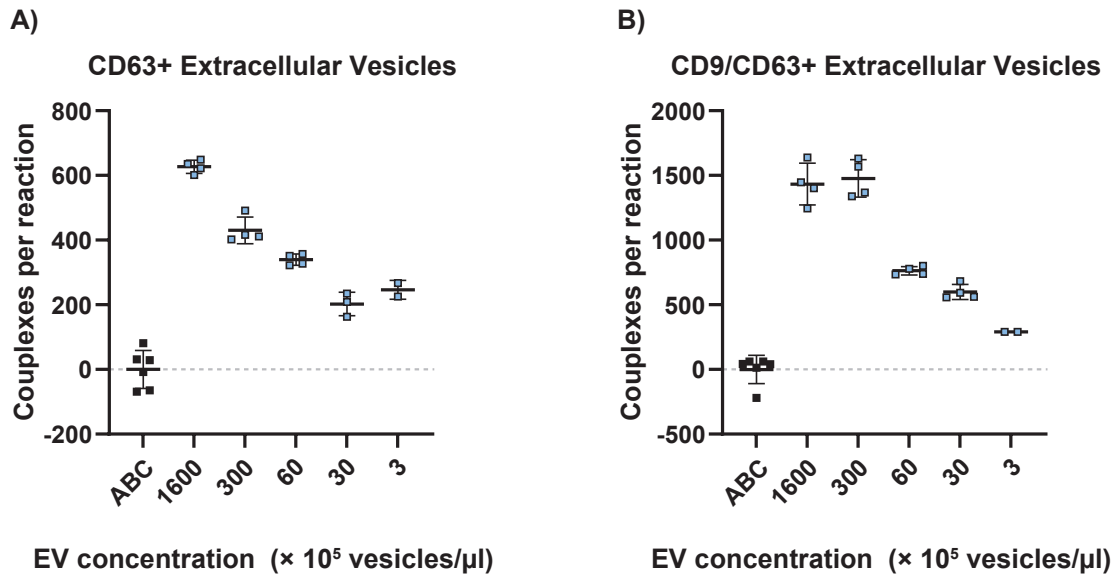


Figure 2. Detection of EVs from cell culture using PICO with CD63-N6 and CD63-O7 (A) or CD9-BL and CD63-O7 (B) antibodies. In both cases, the number of EV complexes per reaction was measured using a dilution series of the EV sample. The results were normalized to a BSA negative control (ABC - antibody binding control). The mean and SD of the individual replicates are indicated with lines.

The identification of the EV subpopulation of interest may depend on the co-occurrence of two or more membrane biomarkers on their surface, which may critically distinguish them from other EVs. Thus, analysis of EVs requires the detection of not only individual markers, but also the co-occurrence of several markers on the surface of intact EVs. For that, we combined the CD63 antibody employed in **Fig 2A** with a monoclonal antibody targeting the extracellular domain of CD9, another common EV surface marker. During the detection of CD63+ EVs, both CD63 antibodies were competing for the same binding sites. During the detection of CD9/CD63+ vesicles, the antibodies are not competing for the binding of their targets, explaining the two-times higher complex count observed in the detection of CD9/CD63+ EVs (**Fig 2B**) also indicating that almost all EVs in this sample are expressing both markers. Interestingly, the highest complex count was observed at the concentration of 3×10^7 vesicles/ μl , and in-

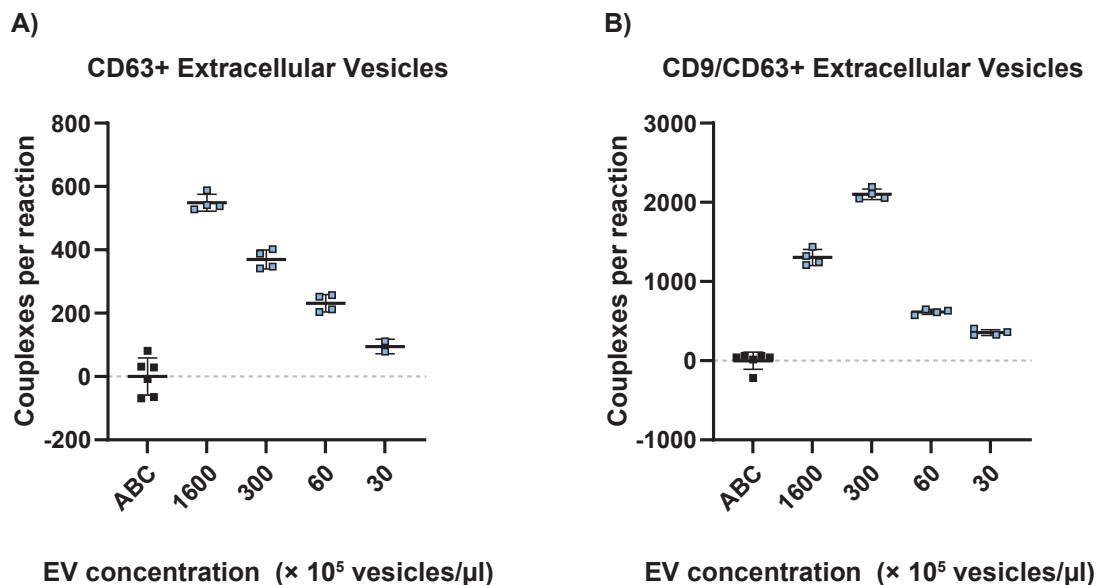


Figure 3. Detection of EVs from human blood plasma using PICO with CD63-N6 and CD63-O7 (A) or CD9-BL and CD63-O7 (B) antibodies. In both cases, the number of complexes per reaction was measured using a dilution series of the EV sample. The results were normalized to a BSA negative control (ABC - antibody binding control). The mean and SD of the individual replicates are indicated with lines.

creasing the EV concentration further did not yield higher complex counts. This phenomenon is the oversaturation of the PICO reaction and happens at high EV concentrations. EV isolation protocols easily achieve concentrations of 10^7 vesicles/ μl (4), this means that PICO can achieve the detection of EVs (LOD is at 10^5 vesicles/ μl) using just a fraction of microliters of EVs.

Having proven that PICO is suitable for the detection of single and multiple surface markers in EVs isolated from culture cells, we decided to test our assay against clinically-relevant samples. With that aim, EVs isolated from the blood plasma of healthy human donors were subjected to the same analysis. As with the case of samples derived from cell culture, we obtained high detection levels both for CD63+ and CD9/CD63+ EVs (**Fig 3A and B**). Similar results were obtained also from EVs derived from primary immune cells (data not shown).

Figure 3B shows that the sample with a higher EV concentration of 1.68×10^8 vesicles/ μl has a lower complex count per reaction compared to the seemingly lower concentrated sample of 3×10^7 vesicles/ μl , due to the so-called oversaturation (5). The oversaturation is a phenomenon that occurs when there is an excess of analyte relative to the antibody. This reduces the likelihood of two antibodies binding to the same antigen, resulting in a lower complex concentration. PICO assay's forthcoming absolute quantitative version accounts for these phenomena and allows to maintain linearity over the entire detection range of the assay.

Conclusion

So far, PICO has only been applied to the detection of solubilized cytosolic or membrane proteins (3). Here, for the first time, we prove that PICO is also able to detect proteins in the surface of intact membranous structures. The easy multiplexing (up to 4 antibodies) of PICO allows the quantification of individual EV markers, as well as their co-occurrence on the surface of the same EV. This critical feature allows the distinction of EVs from similar origin (e.g. healthy vs tumorigenic cells), unlocking the analytic potential of our method. Indeed, PICO has been successfully employed for the detection of EVs derived from diverse sources and using different purification methods. After the incubation of the EVs with the antibodies, the samples are heavily diluted during PICO, which prevents the interference of contaminants present in the original sample. This is specially interesting in the study of EVs, as the fluids from which these vesicles are enriched are complex mixtures containing multiple contaminants (6).

Traditional technologies applied to the detection of EVs, such as ELISA or flow cytometry, require large volumes of samples to achieve sensitive detection. In contrast, PICO has been established to employ even volume of sub microliter samples. In our hands, this led to the detection of EVs at concentrations of 10^5 vesicles/ μl , which is, to our knowledge, one to two orders of magnitude more sensitive than traditional assays (7). Another advantage of the dPCR technology employed by PICO, is that it does not require the pooling of the sample, as happens in bulk methods like western blotting. Instead, each individual EV is analyzed separately through sample partitioning, allowing the distinction and quantification of different EV subpopulations. For all these reasons, combined with the minimal hands-on time and simple workflow, we propose PICO as a strong competitor in the study of EVs.

Materials and Methods

Monoclonal antibodies targeting the extracellular domains of CD9 (EV-Lab Anti-CD9, #PICO-001011, PICO BioScience) and CD63 (EV-Lab Anti-CD63, #PICO-001012, PICO BioScience) were used. For oligonucleotide attachment on unlabeled antibodies with PICO Labels (PICOglue BL, N6, and O7 Labels; #PICO-000120, 122, and 123 respectively, PICO BioScience) PICOglue Antibody Labeling Kit (#PICO-000110, PICO BioScience) was used. For the quantification of CD63+ vesicles, a single monoclonal antibody was selected, divided into two pools and each of them were labeled with different PICO Labels (e.g. CD63-N6 and CD63-O7).

For the binding reaction, 2 μl of purified EVs were mixed with 2 μl of labeled antibodies in PBS and incubated overnight. The antibody concentration in the binding reaction was 5×10^{-11} M.

For the dPCR amplification step, we aimed for an average lambda of 0.15, as recommended in the PICO Amplification Core Kit (#PICO-000010, Actome) manual. The dPCR was performed using QIAGEN's QIAcuity Digital PCR System according to the PICO Amplification Core Kit manual, using the matching PICO Probes (PICO BL, N6, and O7 probes; #PICO-000070, 72, and 73, respectively, Actome). The raw dPCR data was analyzed using Actome's AMULATOR software. The raw complexes were processed as described in the PICO Protein Detection Trial Kit manual, incorporating both ABC correction and labeling efficiency correction. ABC correction accounts for any offsets in the dPCR data, such as signal dropouts or incorrect clustering, while labeling efficiency correction adjusts for the actual number of formed complexes. Applying the appropriate statistical test, it was assumed that the data followed a normal distribution based on the theoretical statistical distribution of complexes.

Acknowledgments

The Actome team is very thankful to Sophie Gabriel and Heiko Pfister (Institut für Laboratoriumsmedizin, Munich) for the isolation and purification of the EVs employed in this report.

References

1. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem*. 1987 Jul;262(19):9412–20.
2. Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczak MZ. Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. *Leukemia*. 2006 Sep;20(9):1487–95.
3. Gross T, Dewes P, Szórádi T, Lübbert P, Jeney C. Highly sensitive HER2 detection in BT474 and MCF7 cells using the PICO technology.
4. Lang JB, Buck MC, Rivière J, Stambouli O, Sachenbacher K, Choudhary P, et al. Comparative analysis of extracellular vesicle isolation methods from human AML bone marrow cells and AML cell lines. *Front Oncol*. 2022 Oct 3;12:949261.
5. Amarasiri Fernando S, Wilson GS. Studies of the 'hook' effect in the one-step sandwich immunoassay. *J Immunol Methods*. 1992 Jul;151(1–2):47–66.
6. Ramirez MI, Amorim MG, Gadelha C, Milic I, Welsh JA, Freitas VM, et al. Technical challenges of working with extracellular vesicles. *Nanoscale*. 2018;10(3):881–906.
7. Bragina VA, Khomyakova E, Orlov AV, Znoyko SL, Mochalova EN, Paniushkina L, et al. Highly Sensitive Nanomagnetic Quantification of Extracellular Vesicles by Immunochromatographic Strips: A Tool for Liquid Biopsy. *Nanomaterials*. 2022 May 6;12(9):1579.