EVxplore Kit
USER MANUAL

Content

pico BioScience

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01 Kit Contents and Storage

EVxplore Kit		
Catalog Number:		PICO-0000140
Pouch - store at 4°C	**pcs**	**Item code**
Bovine serum albumin	1	PICO-000010 C
CD9 Antibody P8 (conc 9.64x10^8 cp/µl)	1	PICO-000150 A
CD9 Antibody BL (conc 9.64x10^8 cp/µl)	1	PICO-000150 B
Pouch - store at -20°C		
Coupling dPCR Mix	1	PICO-000010 E

The PICO EVxplore Kit ships at room temperature. Upon arrival, immediately store components at their optimal temperatures (noted on pouches). Note the different required storage temperatures. Expiration dates are on the back of the pouches.

02 Intended Use

The PICO EVxplore Kit is for research use only (RUO) and not for diagnosis, prevention, or treatment of disease. Manage the product with care and adhere to national safety guidelines.

03 Safety Information

Always wear a lab coat, disposable gloves, and protective goggles when handling chemicals. Consult the appropriate material safety data sheets (MSDS) for more information. These are available at: www.actome.de/resources/safety-data-sheets

For reagent spillage, absorb, dispose of, and clean with laboratory detergent and water. If the spilled liquid is potentially infectious, clean first with detergent and water, then with 1% (w/v) sodium hypochlorite.

04 Quality Control

Each PICO EVxplore Kit is evaluated against predetermined specifications to ensure consistent product quality.

05 Introduction

Protein Interaction Coupling (PICO) is an immunoassay utilizing the QIAcuity Digital PCR System to characterize and quantify intact extracellular vesicles (EVs). The EVxplore Kit supports 24-Well QIAcuity Nanoplates (26k), allowing for 24 PICO reactions. The PICO EVxplore protocol is a two-day procedure with minimal hands-on time (< 1 h 30 min) (Figure 1).

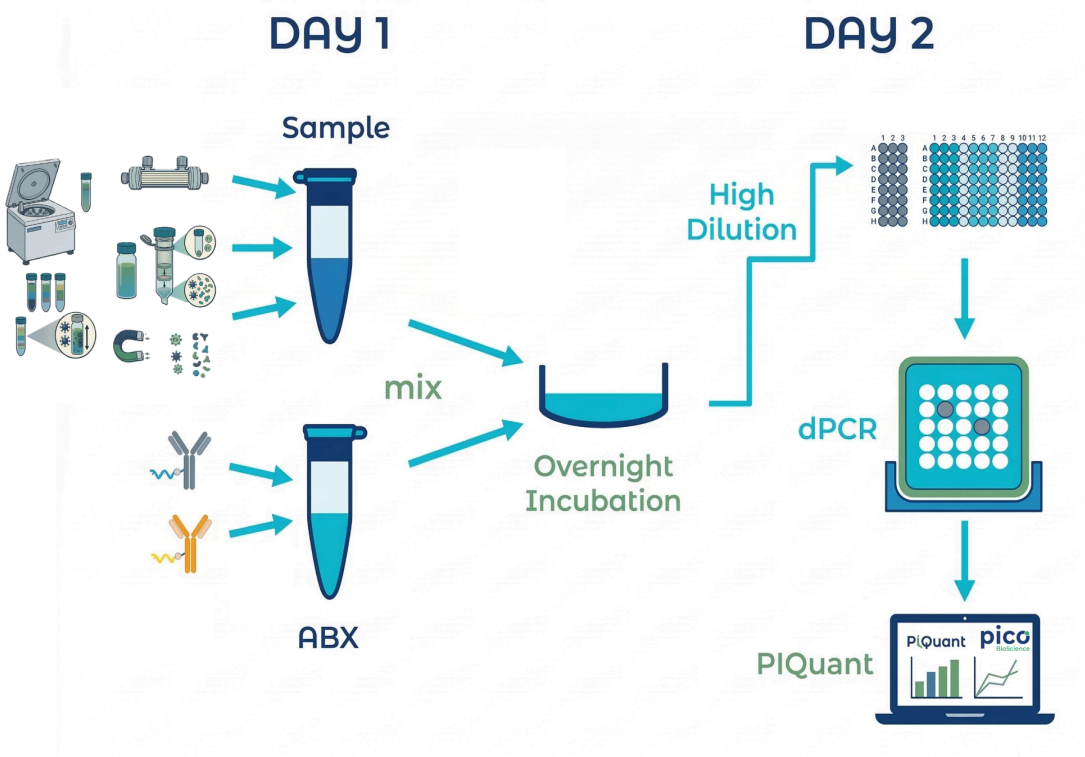


Figure 1 PICO EVxplore Kit Workflow

The EVxplore Kit uses two different labeled antibodies against the same epitope to detect and quantify extracellular vesicles (EV) carrying the CD9 surface protein. PICO quantification also allows for precise counting of intact EVs and, by combining additional labeled antibodies, their sub-populations.

06 Preparation for a PICO Assay

06.1 General Remarks

- The PICO assay requires precise pipetting. Always use calibrated pipettes for accurate results, especially at high dilution factors. Mix gently by pipetting up and down to avoid spills and material loss.
- Change gloves frequently to prevent contamination, which can be detected if the NTC (no-template control) shows positive signals.
- For best results, use four technical replicates per sample. Include both NTC and antibody control (ABC). The ABC contains only antibodies (no sample) and should ideally show zero couplex counts. Any deviation is automatically corrected by the PIQuant software.
- The Lambda value should be below 0.55, with 0.15 being optimal. A lower Lambda improves couplex-detection accuracy by reducing biases, but setting it too low decreases assay sensitivity and limits the detection of statistically significant couplexes.

06.2 Concentration of Antibodies in the Binding Reaction

Each target in a PICO assay necessitates a minimum of two antibodies, which must recognize distinct copies of the CD9 surface protein on an individual vesicle. For optimal performance with the EVxplore Kit, it is suggested to maintain an antibody working concentration of **40 pM** during the binding phase.

06.3 Considerations Regarding Biological Material

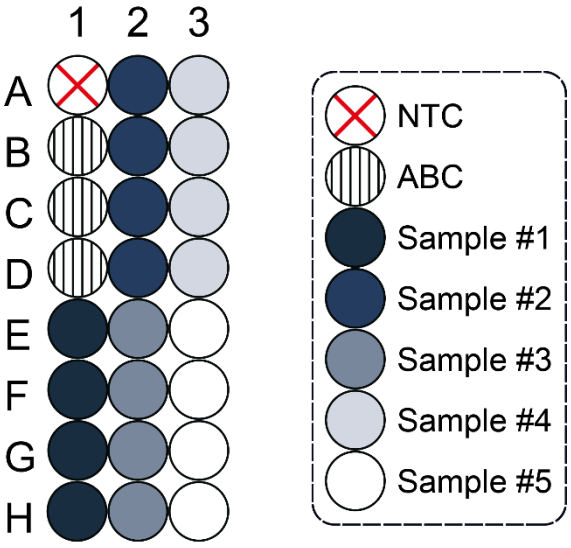
Biological Material		
Feature	Main criteria	Additional criteria
Sample type	Isolated EV samples	Choose your preferred method for EV isolation
Sample preparation	EV Sample dependent	Intact EVs suspended in PICO BioScience's EV buffer (EVB) to quantify EVs based on surface markers

- For EV samples, we recommend performing a Dilution Curve (DC) to titrate the optimal EV concentration that allows robust detection while saving biological material. For instance, a 5-fold dilution series with five dilution steps can be prepared in EVB. This results in a dilution series of undiluted, 5-, 25-, 125-, and 625-fold diluted samples.

06.4 Exemplary Setup of a Binding Reaction for a 24-well dPCR Nanoplate

Plate layout of an example binding reaction for a 24-well PICO experiment. In this example, five samples, with four technical replicates each, were used. For the ABC, three technical replicates are recommended.

Binding reaction



Notes

07 Equipment and Reagents to be Supplied by User

Note: Equipment and reagents required to prepare biological material are not listed below and depend on the input material.

Devices/Equipment

- QIAcuity Digital PCR System (QIAGEN, Cat. #: 911001)
- Table-top mini centrifuge for quick spins (~1,000 rcf)
- Plate centrifuge (e.g., Megafuge 8 (Thermo Fisher, Cat. #: 75007210)
- Vortex mixer
- Multichannel pipette, 8-channel (1-10 µl)
- Multichannel pipette, 8-channel (10 – 100 µl)
- Multichannel pipette, 8-channel (30 – 300 µl) (optional)
- Regular 1-channel pipettes (1 – 1,000 µl)
- Electronic Multichannel pipette

Consumables

- QIAcuity Nanoplate 26k 24-well (QIAGEN, Cat. #: 250001)
- PCR microplates, 96-well, Polypropylene, V-bottom or similar
- Sealing Foil Adhesive Film (e.g., Thermo Fisher, Cat. #: 10696771)
- 1.5 ml reaction tubes
- 0.5 ml reaction tubes
- 0.5 ml low protein binding tubes (e.g., Eppendorf, Cat. #: 0030108094)
- 10 µl, 200 µl, 1,000 µl standard pipette tips
- 15 ml Falcon tubes

Chemicals and Kits

- QIAcuity Probe PCR Kit (QIAGEN, Cat. #: 250101) (~ 1.5 ml)
- PICO Probes (PICO P8, BL Probe, Cat. #: PICO-000070-71)
- Phosphate-Buffered Solution (PBS), without calcium or magnesium ions (e.g., Thermo Fisher Scientific, Cat. #: 12037539)
- Ultrapure water (e.g., Thermo Fisher Scientific, Cat. #: 15667708)

08 Protocol of PICO EVxplore Kit

08.1 Buffer Preparation for intact EV characterization based on surface markers

1. Prepare the chemicals and buffers as listed below. The volumes can be adapted to the needs (e.g., if required, more EV Buffer can be prepared for the dilution series).
   The buffers can be kept for specified periods of time and used for further experiments. Prepare the buffers directly when they are used.

BSA (5x stock)
Add 400 µl PBS
Stable for 3 days at 4°C

EV Buffer (EVB)
100 µl BSA
400 µl PBS
Prepare the EV Buffer fresh

08.2 EV Isolation and Storage

Use any preferred EV isolation method or commercial kit. If a commercial kit is used, the isolated EVs must be re-buffered before the binding reaction, though they may remain in the isolation elution buffer beforehand.
Immediate analysis of the isolated EVs is recommended. For short-term storage, keep the EVs at 4°C for a maximum of 4 days. For long-term preservation, aliquot the EVs and freeze them at -80°C.

08.3 Binding Reaction

2. Calculate the volume of antibody stocks and EVB required for the antibody mix (ABX) using the PICO Calculator (Step 2 in the 'PICO Calculator' tab).
3. Prepare the ABX using EVB. Then add the calculated volume of the antibody solutions.

   Prepare 100 µl of ABX for a QIAcuity Nanoplate 26k 24-well. Recommended antibody working concentrations is 40 pM.

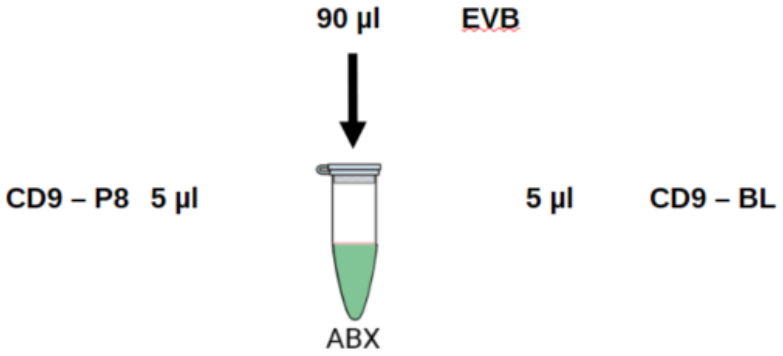


Figure 3 Example ABX pipetting scheme for 2 anti-CD9 antibodies

4. After preparation of the ABX, the EV samples prepared in *section 08.2* can be diluted or used directly for the following binding reaction.

5. Set up the binding reaction in a 96-well PCR microplate (v-bottom) (see example in **Section 06.4**). Add 4 µl of EVB to a dedicated well for the NTC.

6. Combine 2 µl of EVB with 2 µl of ABX and make at least three technical replicates for the ABC control
   Due to the small volume of the binding reaction, do not mix by pipetting. The sample volume and ABX can be adjusted. This must be considered in the calculations of the ABX and the pre-dilution.

7. Combine 2 µl of the EV sample with 2 µl of ABX. Good laboratory practice recommends using at least four replicates.

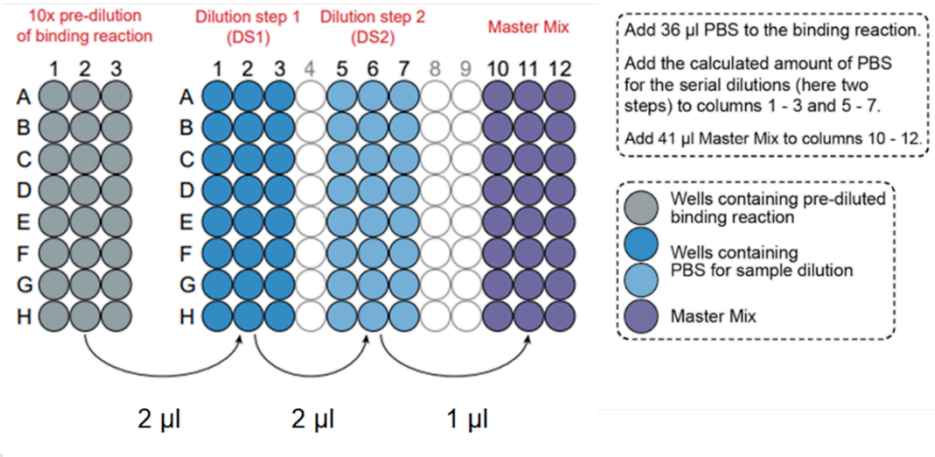
8. Seal the plate (without the lid) with an adhesive foil. Vortex the plate for thorough mixing. Centrifuge the plate to collect the liquid at the bottom (~1,000 rcf, 30 s) and incubate at 4°C overnight.
   The incubation time can be varied between 12-24 hours.

08.4 Pre-dilution and Digital PCR

9. Prepare the Master Mix, ensuring it contains the required PICO Probes. Vortex for 10 s and spin down (~1,000 rcf, 5 s). The Master Mix can be prepared up to three days in advance.

Master Mix	
Reagents	**24-well plate**
Ultrapure water	696 µl
QIAcuity Probe Master Mix	284 µl
PICO Probe P8	45 µl
PICO Probe BL	45 µl
Coupling dPCR Mix	36 µl

10. PICO Assay Preparation Instructions



a. Prepare a new 96-well plate for the dilution steps.
b. Add 33 µl of PBS to columns 1-3 (for Dilution Series 1, DS1) and columns 5-7 (for DS2).
c. Add 41 µl of Master Mix to columns 10-12.

11. Remove the adhesive foil from the incubated sample plate. Add 36 µl of PBS to the 96-well plate containing the binding reactions (represents the recommended first 10x pre-dilution). Mix thoroughly by pipetting up and down.
    Perform the following steps without unnecessary breaks, as the dilution disrupts the equilibrium binding conditions and induces antibody dissociation.

12. Transfer 2 µl volume from each pre-diluted sample into the corresponding wells of the dilution plate (DS1), mix by pipetting up and down 10 times.

    When performing the second dilution step, transfer 2 µl volume from the first dilution step into the corresponding wells of the dilution plate (DS2). Mix thoroughly by pipetting up and down.

13. Finally, transfer **1 µl** from the last dilution step into the wells containing the Master Mix. Mix thoroughly by pipetting up and down.

14. Transfer 40 µl (24-well) of the diluted sample Master Mix to the QIAcuity Nanoplate. Seal and insert the Nanoplate into the QIAcuity dPCR System. Run the dPCR program using the following dPCR settings:

Priming			
QIAGEN Standard Priming Profile			
PCR conditions			
Step	**Temperature**	**Time**	
Hot-start	95°C	2 min	
Denaturing	95°C	15 s	Cycle 40 times
Annealing	58°C	30 s	

Imaging conditions			
PICO Probe	**QIAcuity channel**	**Integration time**	**Gain**
P8 Probe	FAM, green channel	500 ms	6
BL Probe	HEX, yellow channel	400 ms	6

09 Evaluation

For the Evaluation with the PIQuant software, please use this link. Do not open the Multiple Occupancy File with Excel before uploading to prevent changes in the data structure. To get more information, consult the PIQuant User Manual or contact our support team here.

10 Troubleshooting Guide

If you encounter any issues, please refer to the troubleshooting guide provided below. Additional support is available here.

Troubleshooting	
Issue	Comments and Suggestions
Lambda value in PICO assay not in range (0.01-0.55)	
Antibody concentration determined during quality control of labeled antibodies was not correct	Recalculate antibody concentrations using the data of the PICO assay and repeat the assay with the new concentrations. For this, the antibody concentration of each antibody found in the 'Current Results' file of the PICO assay is multiplied with the dilution factor back to the antibody stock for the corresponding antibody (which can be found at the bottom of the PICO Calculator).
No couplexes or small numbers of couplexes detected	
Antibody concentration determined during quality control of label loaded antibodies was not correct	Check if lambda value is in range (0.01-0.55), if not recalculate concentrations based on the data of the PICO assay and repeat the assay with the new concentrations.
Wrong default threshold of fluorescence intensity (RFU) was set in the QIAcuity software suite	Select '1D Scatter Plot' in analysis mode of QIAcuity software suite and adapt the thresholds.
Sample concentration outside the dynamic range of the PICO assay	Perform a longer dilution curve to find the optimal concentration of target detection. Too high target concentration can result in lack of couplex detection.
Sample buffer incompatible with the PICO assay	While PICO is generally compatible with common EV elution buffers, high salt concentrations (exceeding 0.5M) can interfere with antibody binding efficiency. For such samples, re-buffering in EVB is highly recommended to ensure optimal assay performance.

11 Ordering Information

PICO kits can be purchased directly from pico-bioscience.myshopify.com, or a quote can be requested from sales@PICO-BioScience.eu. Supporting materials are available on https://www.pico-bioscience.eu/resources or can be requested from our Customer Support (support@PICO-BioScience.eu).

Ordering		
Product	Description	Cat. #
PICO EV Q Kit	dPCR detection for PICO assays for intact EV detection and quantification (5 x 24 reactions; 5 x 96 reactions)	PICO-000140
PICO Probes	P8 (FAM), BL (HEX), N6 (TAMRA), O7 (ROX) for detection in dPCR (5 x 24 reactions; 5 x 96 reactions)	PICO-000070 - 73