

Tunable Resistive Pulse Sensing for the Characterization of Extracellular Vesicles

Sybren L.N. Maas, Marike L.D. Broekman, and Jeroen de Vrij

Abstract

Accurate characterization of extracellular vesicles (EVs), including exosomes and microvesicles, is essential to obtain further knowledge on the biological relevance of EVs. Tunable resistive pulse sensing (tRPS) has shown promise as a method for single particle-based quantification and size profiling of EVs. Here, we describe the technical background of tRPS and its applications for EV characterization. Besides the standard protocol, we describe an alternative protocol, in which samples are spiked with polystyrene beads of known size and concentration. This alternative protocol can be used to overcome some of the challenges of direct EV characterization in biological fluids.

Key words Extracellular vesicles, Exosomes, Microvesicles, Characterization, Quantification, Size distribution, qNano, Resistive pulse sensing

1 Introduction

Due to their small size (50–1000 nm), accurate characterization of extracellular vesicles (EVs) is technically challenging. Over time, different techniques have been developed to overcome these challenges. Most of these techniques are based on bulk analysis of EVs. For instance by total protein quantification, western blotting, bead-based flow cytometry [1] or modified protein microarrays [2]. However, alternative techniques, that allow for single particle analysis of EVs, have become recently available [3–8]. One of those techniques, provided by the qNano platform (Izon Science Ltd), is tunable resistive pulse sensing (tRPS) (Fig. 1).

In tRPS, a non-conductive membrane (“nanopore”) separates two fluid cells [9] (Fig. 2). This nanopore is punctured to create a single conical shaped opening (Fig. 2, top-left). Once a voltage is applied, a current of charged ions through the nanopore is established. This baseline current is distorted, as observed by the appearance of peaks or “pulses,” as particles move through the nanopore (Fig. 2, bottom). Once a particle enters the sensing zone of the

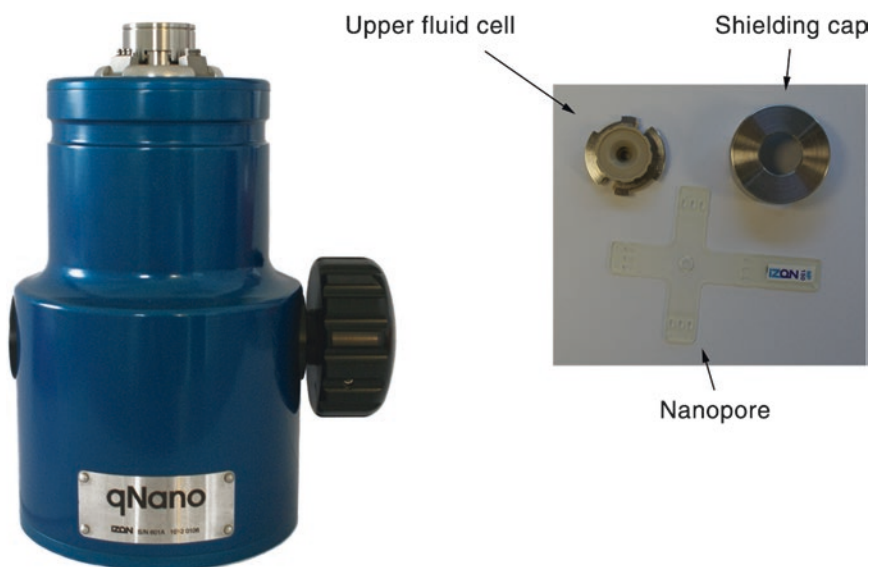


Fig. 1 Photographs of the qNano instrument and instrument parts

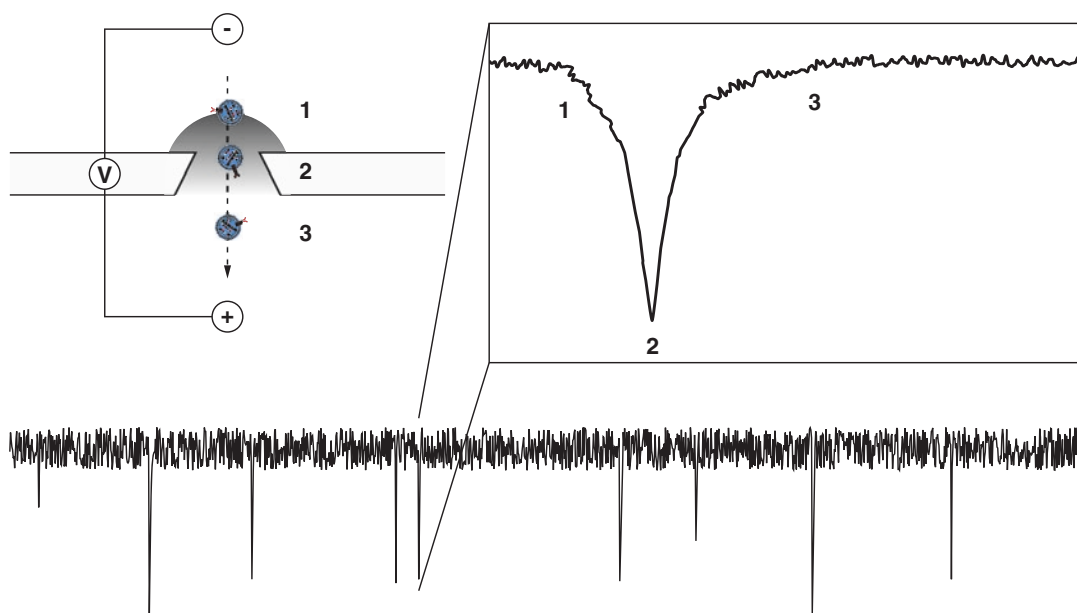


Fig. 2 The working mechanism of tunable resistive pulse sensing (TRPS). A membrane ("nanopore") with a nanosized, stretchable pore is separating two fluid compartments (top-left). After applying a voltage across the nanopore, a baseline current is established (bottom) which is disrupted by the movement of particles through the nanopore. As a particle moves towards the opening (timing 1), it starts to reduce the flow of ions through the nanopore (top-right) which will be maximum as the particle enters the nanopore opening (timing 2). This disruption reduces as the particle moves across and exits the nanopore (timing 3)

nanopore [10] (Fig. 2, timing 1), the flow of charged ions, and thus the baseline current, will be altered (Fig. 2, top-right). As the particle enters the conical opening, the relative blockade of the baseline current will be maximum (Fig. 2, timing 2). This blockade will gradually decrease to baseline levels as the particle moves further through the nanopore (Fig. 2, timing 3). To characterize particles in a sample, a calibration sample of (polystyrene) beads of known volume and concentration is measured first. The magnitude of pulses and the particle rate induced by this reference sample can subsequently be used to calculate the size profile and concentration of the particles in the measurement sample [11, 12].

The movement of particles through the nanopore is based on several independent forces, being electrokinetic (electrophoretic and electro-osmotic) and fluidic forces [10]. The variable pressure module (VPM) can be used to apply additional external force and should be used (≥ 0.8 kPa) to minimize interfering electrokinetic forces when analyzing particles using the smaller (NP100-NP200) nanopores [13].

Characterization of EVs using tRPS is technically challenging. Due to the heterogeneous nature of EVs a large size range of particles is usually present in a sample. Larger-sized EVs may clog the nanopore, thereby obstructing the measurement. Secondly, the sample with calibration beads should consist of the same buffer components as the EV sample. This may be technically unfeasible, as the buffer components are regularly unknown when measuring EVs, especially when measuring EVs directly in a biological sample. This problem can be overcome by using a “spiking” approach, in which the calibration beads are added to the measurement sample [3].

Here, we describe two different approaches for the characterization of EVs using tRPS. First, the standard protocol is described, which often suffices for the characterization of purified EVs. Secondly, we describe the alternative spiking approach, which could be of benefit when characterizing EVs in biological samples.

2 Materials

2.1 qNano Specific Equipment/Materials

1. qNano instrument (Izon Science Ltd, Christchurch, New Zealand).
2. Variable Pressure Module (Izon Science Ltd, Christchurch, New Zealand).
3. Polystyrene calibration particles (Izon Science Ltd, Christchurch, New Zealand) (*see Note 1*).
4. Nanopores (Izon Science Ltd, Christchurch, New Zealand) (*see Note 2*).

2.2 General Laboratory Equipment/Materials

1. Filter-tip pipette tips (*see* **Note 3**).
2. Sonication bath (*see* **Note 4**).
3. Lint-free tissues (*see* **Note 5**).
4. Phosphate buffered saline (PBS).
5. Digital calipers (supplied with the qNano instrument).

2.3 Software for Data Recording and Analysis

1. Izon Control Suite (Izon Science Ltd, Christchurch, New Zealand).
2. Spreadsheet software (*see* **Note 6**).

3 Methods

3.1 Standard Protocol

The standard protocol of tRPS-based EV quantification involves separate measurement of a (polystyrene bead-containing) calibration sample and the EV-containing sample.

1. Connect the qNano instrument to a computer running the Izon Control Suite Software. Make sure no sources of electrical interference are located close to the instrument (*see* **Note 7**).
2. Wet the lower fluid cell by introducing 75 μ l PBS and immediately removing it again (*see* **Note 8**).
3. Place the nanopore of choice (*see* **Note 2**). To calibrate the stretch, use the digital calipers to measure the distance between two opposing arms of the qNano.
4. Stretch the nanopore to 47 mm and reapply 75 μ l to the lower fluid cell. Prevent the formation of air bubbles in the lower fluid cell. If air bubbles are formed, remove and reapply the PBS.
5. Place the upper fluid cell and the shielding cap (which creates a “Faraday cage”) on the nanopore. Add 40 μ l PBS into the upper fluid cell and apply a voltage. Make sure a stable baseline current is established (*see* **Note 9**).
6. Dilute the calibration particles in PBS to the target concentration of the used nanopore (*see* **Note 10**).
7. Remove the PBS from the upper fluid cell and apply 40 μ l of the calibration particles into the upper fluid cell. Make sure a stable baseline current is established (*see* **Note 9**). Reduce the applied stretch slowly towards 43 mm and observe the blockades caused by the calibration particles. Stop reducing the stretch when the mode blockade caused is at least 0.1 nA, but preferable >0.3 nA (*see* **Notes 11 and 12**).

8. Apply ≥ 0.8 kPa pressure using the VPM and click “record” (*see Note 13*). Make sure that a particle rate (*see Note 14*) of $>100 \text{ min}^{-1}$ and a mode blockade height of $>0.1 \text{ nA}$ is recorded (*see Note 12*).
9. If the baseline current suddenly drops or keeps drifting during recording, pause the recording and try to reestablish a stable current (*see Note 9*).
10. Record >500 particles, for at least 30 s (*see Note 14*). Fill out the details of the calibration sample in the pop-up form.
11. Optionally, multi-pressure measurement can be performed (*see Notes 13 and 15*). Hereto, add at least 0.2 kPa and record a second measurement (more steps could increase accuracy).
12. Remove the calibration sample and wash the upper fluid cell by resuspending 100 μl PBS in the upper fluid cell 3–4 times. Remove residual PBS by usage of the lint-free tissue (*see Note 16*).
13. Introduce the EV sample and make sure the baseline current is within 3% of the baseline for the calibration sample (*see Note 17*).
14. Record the sample at the same VPM pressure(s) as applied for the calibration sample.
15. Click the “Analyse data” tab and right-click on “Unprocessed files” and select “Process files”.
16. Click on the checkbox in the “calibrated” column next to one of the sample files. This will initialize the calibration pop-up menu. Select the “multi-pressure measurement” tab if applicable and select the sample files and calibration file(s).
17. Once calibrated, an EV sample file will display a size distribution in nm instead of nA (Fig. 3, right). Click on “Preview” to generate a .pdf file containing statistics such as the concentration (measured and raw if a diluted sample was used).

3.2 Spiking the Sample with Polystyrene Beads of Known Size and Concentration

The standard protocol for tRPS-based EV quantification relies on usage of appropriately formulated calibration samples (i.e., with the diluents resembling the fluid of the EV sample). This may be unfeasible for biological fluids, since their exact composition may be unknown rendering their simulation impossible. Secondly, the volume of the biological sample (e.g., only 100 μl of plasma) may be insufficient for preparation of calibration fluid (which usually can be done by removal of small particulate matter by ultracentrifugation or filtering). In such cases, an alternative is provided by performing a spiking protocol, in which calibration beads are introduced in the EV sample [3]. This methodology can also be used when samples are measured over a prolonged period of time and stable nanopore conditions cannot be guaranteed due to nanopore clogging.

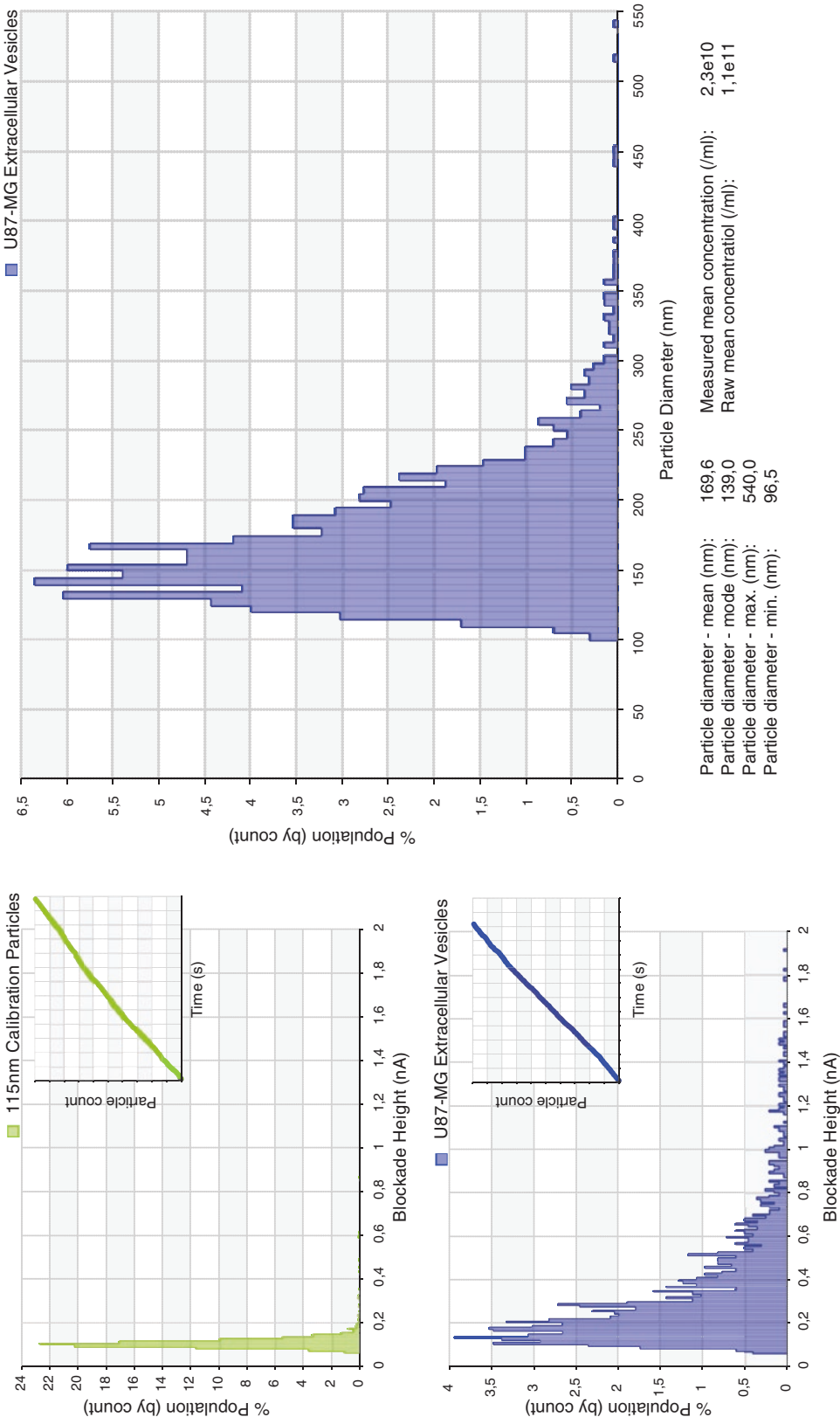


Fig. 3 Characterization of extracellular vesicles (EVs) by calibrating the EVs to polystyrene beads of known size and concentration. A sample of 115 nm polystyrene beads is measured, resulting in a size distribution and particle rate plot (top-left). Secondly, the EVs, purified by sequential ultracentrifugation, are measured resulting in a second size distribution and particle rate (bottom-left). Once the EV sample is calibrated to the reference beads, the recorded blockades in nA, can be calculated to absolute sizes in nm (right-side). The particle rates are used to calculate the concentration of the EVs

1. Setup the qNano instrument as outlined in Subheading 3.1 steps 1–5.
2. Check the approximate particle rate of the EV samples.
3. Dilute the EV sample in PBS (*see* **Note 18**).
4. Determine the dilution of polystyrene beads that is needed to obtain a count rate that resembles the count rate of the EV samples (*see* **Note 19**), and check for the ability to distinguish EVs and polystyrene beads (*see* **Note 20**).
5. Prepare the samples by diluting polystyrene beads into the samples (*see* **Note 21**). Also prepare a “beads-only” sample (*see* **Note 22**).
6. Record the beads-only and sample measurements, preferable in triplicate (*see* **Note 23**).
7. Process all files as outlined in Subheading 3.1 step 15.
8. Display the size distribution graphs (uncalibrated) of the beads-only samples and sample files (Fig. 4, left). Determine at which nA value a cutoff can be set to distinguish the two populations (Fig. 4) (*see* **Note 24**).
9. Obtain the total particle count (in sample details window) for each sample and put this into a spreadsheet software program (Table 1).
10. Click the “filter options” button to obtain the filter settings. Enter the cutoff obtained in **step 8** and filter the samples. Make sure to select the “apply to all samples in group” checkbox to filter all samples directly.

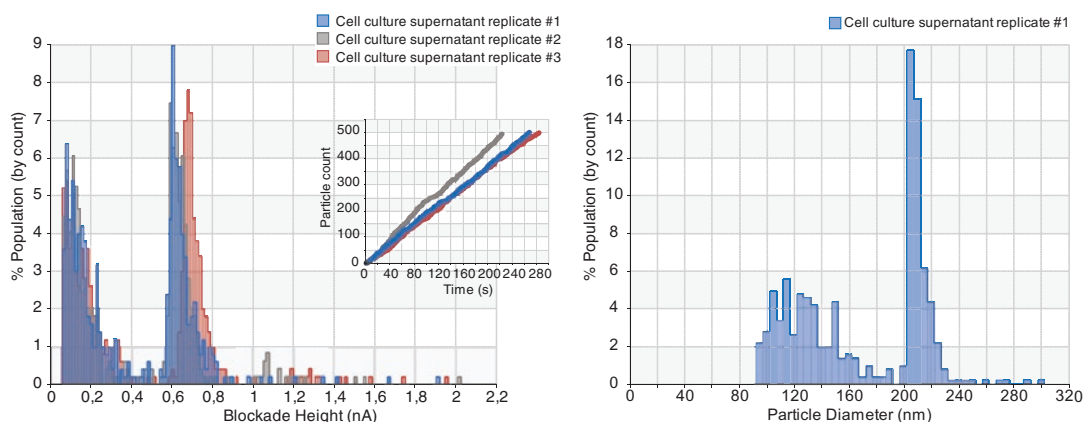


Fig. 4 Quantification and size estimation of EVs by spiking the sample with polystyrene beads of known size and concentration. Three replicates of glioblastoma cell culture supernatant spiked with 203 nm polystyrene beads are measured (left). All particles smaller than 0.48 nA were determined EVs. The EV-to-beads ratio is used to calculate the concentration of EVs. The spiked polystyrene beads can be used to obtain an accurate size distribution without the need of an external calibration sample (right)

Table 1
Example calculation of EV concentration using the alternative spiking method

Sample	Beads-only #1	Beads-only #2	Replicate #1	Replicate #2	Replicate #3
Average current	63	60	62	61	61
Rate	57	74	112	133	105
Cutoff used	0.48	0.48	0.48	0.48	0.48
Total particles	297	299	502	495	500
Extracellular vesicles (EVs)	27	46	254	240	246
Beads + multimers	270	253	248	255	254
EVs/beads	0.100	0.182	1.024	0.941	0.969
Sample—background			0.88	0.80	0.83
EVs (10^8 /ml) in sample			8.83	8.00	8.28
Dilution factor of EVs			2.5	2.5	2.5
EVs (10^8 /ml) raw			22.08	20.01	20.69

11. Obtain the total particle counts for each sample after the filter step. Fill out these numbers into the spreadsheet software (*see* Table 1 for an example calculation).
12. Subtract the EV counts from the total counts to obtain the amount of calibration particles. Subsequently, divide the number of EVs by the number of polystyrene particles to obtain the EV-to-bead ratio.
13. To account for background particles, subtract the average ratio obtained for the beads-only samples from each EV-to-bead ratio.
14. Multiply the EV-to-bead ratio to the concentration of polystyrene beads in the sample. Secondly, multiply this value by the dilution factor of the EVs (*see* Note 25) to obtain the raw concentration of EVs.
15. Optionally: introduce a correction when overlap of EVs and polystyrene beads is observed (*see* Note 26).

3.3 Obtaining an EV Size Distribution from a Spiked Sample

The above-described spiking procedure can also be utilized to obtain a proper size distribution profile of EVs in case the preparation of appropriate calibration samples is impossible.

1. Prepare, measure, and process the EV samples as outlined in Subheading 3.2 steps 1–8.
2. Once processed open the sample of interest twice in the Izon Control Suite.

3. For one of the files, filter the sample to display particles larger than the determined cutoff only. Set this sample as “calibration” and enter the mode size of the calibration particles.
4. Couple the sample file and the newly create calibration file as outlined in Subheading 3.1 step 16.
5. Once successfully coupled, the unknown sample can now be displayed as a size distribution in nm based on the spiked calibration particles (Fig. 4, right). This graph will display two populations, one for the EVs and one for the reference particles.

4 Notes

1. For EV characterization different polystyrene beads are used: CPC100, CPC200, and CPC400, with mode diameters of 115, 203, and 335 nm, respectively (these numbers may vary based on the batch used).
2. Different sizes of nanopores are used: the NP100 nanopore (optimal size range 70–200 nm), NP150 (80–300 nm), and NP200 (100–400 nm). Due to heterogeneity of EV samples, the NP150 and NP200 are most often used for characterization of EVs.
3. To minimize background particle detection, we use filter-tip pipette tips.
4. To homogenize the calibration particles a basic tabletop sonicator can be used.
5. To completely remove any residual liquids between measurements, lint-free tissue can be used. To minimize contamination of background particles, lint-free tissue is preferred over regular tissues.
6. For almost all data analyses the Izon Control Suite can be used. However, all data-points can be exported for analysis in other software packages. For EV quantification using the spiking method, a spreadsheet software program is required.
7. Electronic devices used in close proximity of the instrument can significantly interfere with the detection signal. This interference is observed as identical, quickly repeating short pulses. We have most often observed this interference caused by mobile phones.
8. This is done to decrease the risk of air-bubble formation in the lower fluid cell. Air bubbles can be a major source of instable baseline current.
9. The baseline current depends on the applied buffer, stretch and voltage. The current should be stable and the root mean square

(RMS) noise should be <10 pA. If these conditions are not met, air bubbles or (partial) nanopore blocking may be causative. To solve this, resuspend the sample in the upper fluid cell and check if the baseline becomes stable. If not, remove both the sample and the PBS in the lower fluid cell. If (after reapplication of PBS) no stable baseline current is established, the nanopore may be (partially) blocked. Tap the shielding cap (using the supplied plunger) to vibrate the nanopore and to disrupt particles. Clogging may also be solved by induction of a brief pressure by pushing down and pulling out of the plunger. Alternatively, the shielding cap can be put in place whilst pressing on the nanopore, which will vibrate the nanopore. Also, the nanopore can be maximally stretched (i.e., 47 mm), combined with applying maximal external pressure. If still unsuccessful, remove the nanopore and rinse heavily using deionized water. Re-place the nanopore on the instrument.

10. Each nanopore has a target concentration. For the NP100 and NP150 nanopores the target concentration is $10E10$ per ml and for the NP200 the target concentration is $10E9$ per ml.
11. The blockade height caused by a particle moving through the nanopore is based on the stretch, the applied voltage and the buffer used. If the nanopore opening is reduced (less stretch applied) the relative blockade by the particle will increase. This also implies that smaller particles surpass the detection threshold. Larger particles, on the other hand, will block the nanopore more frequently. By increasing the voltage applied, the flow of ions will increase and so will the (relative) blockade caused by particles moving through the nanopore. However, an increased voltage can also result in increased RMS noise. The flow of ions, and thus a higher baseline current, can also be established by using a buffer with increased salt concentration. However, this may influence the EV characteristics, for instance caused by changes in osmosis.
12. For accurate detection of particles a mode blockade of at least 0.1 nA is required. However, the mode blockade set for the calibration particles will also determine the range of EVs detectable by the instrument. For instance, a mode blockade of 0.1 nA for 203 nm calibration beads indicates that the instrument will only be able to detect particles that are slightly smaller than 203 nm. Reducing the stretch or increasing the voltage (*see Note 11*) could be needed to decrease the lower detection limit.
13. External pressure needs to be applied to counteract the influence of electrokinetic forces. These electrokinetic forces are not negligible when using small pore sizes (NP100-NP200) [14], which is often the case upon EV quantification. Since EVs display a modest zeta potential (i.e., the potential

difference between the dispersion medium and the stationary layer of fluid attached to the particle) [15, 16] the influence of the electrokinetic forces is low and can be completely abolished by applying >0.8 kPa external pressure [13].

14. The particle rate recorded (particles per minute) will depend on the concentration of the particles, the applied pressure and applied stretch (the rate will decrease by decreasing the stretch). Since at least 500 particles should be recorded, a particle rate of >100 per minute is advised but not required. In our experience particle rates >2000 per minute will be less reliable.
15. Multi-pressure measurement is advisable when measuring EVs with increased surface charge (e.g., as a result of coupling highly charged ligands to the surface). In such cases, difference in surface charge between EVs and polystyrene calibration beads will result in inaccurate concentration estimations as one of the particle sets is more likely to move through the nanopore than the other [14]. Measurement of the calibration bead sample and EV sample at multiple pressures provides additional data that is used to accurately calculate the concentration of EVs.
16. Residual PBS in the upper fluid cell can dilute the measurement sample. To prevent this, remove the upper fluid cell and gently wipe lint-free tissue in the bottom-opening of the cell.
17. To accurately compare a calibration sample with an EV sample, the baseline current should not differ more than 3%. If unable to reach a comparable baseline current, apply the strategy outlined in Note 9. Alternatively, dilution of the sample in PBS could make the EV sample more comparable to the calibration sample.
18. Dilution in PBS may facilitate EV counting by the qNano instrument. However, to guarantee appropriate counting of EVs, try to keep the particle rate above 70–100 particles per minute (*see* Note 14).
19. Although not strictly necessary, an EV-to-bead ratio of approximately 1 will make the measurements most reliable. If EVs or beads outnumber their counterparts the calculation of concentrations will be more prone to variation.
20. To distinguish EVs from polystyrene beads, both populations should be identifiable based on blockade sizes. For EV quantifications in biological samples we tend to use a NP200 nanopore in combination with CPC400 (mode 335 nm) polystyrene beads or an NP150 nanopore in combination with 203 nm beads. To maximize the population of EVs detected, try to obtain settings where the polystyrene beads induce blockades of at least 0.5 nA. By increasing the blockade height caused by the polystyrene particles, the detection limit for the EVs will decrease (*see* Note 12).

21. Example sample preparation:
 - (a) 40 μl cell culture supernatant (after 5 min $300\times g$ centrifugation to remove cells).
 - (b) 40 μl PBS.
 - (c) 20 μl 1:200 diluted 203 nm polystyrene beads (stock $1\text{e}12\text{ ml}^{-1}$).
22. A beads-only sample is used to quantify background particles and to identify the population of polystyrene beads. For this sample “EV free cell culture medium” should be used that has received the same treatments as the samples of interest, but lacks EVs.
23. To spread variation in nanopore conditions, each set of samples should be measured once before recording duplicates and triplicates. Prepare fresh samples (i.e., addition of PBS and beads) directly before each measurement.
24. Setting the cutoff remains arbitrary. Make sure each sample has the same bin-size setting (ViewSettings panel, accessible by clicking the popup button in the View panel). We choose to set the cutoff at 0.48 nA (Fig. 4, left). All particles smaller than the cutoff are determined EVs.
25. Since the EVs are diluted (upon mixing with calibration beads and addition of PBS) the obtained concentration should be corrected for this. For the example setup outlined in note 21, the EVs are diluted 2.5 times.
26. A correction can be introduced when the detection of EVs and polystyrene beads overlaps. Measure the EV sample without polystyrene beads and determine the “Bead-to-EV ratio” based on the cutoff determined in Subheading 3.2 step 8 (here the term “bead” refers to the fraction of EVs that are detected within the spiked-bead-detection range). Usually this ratio is insignificant, but if not add this Bead-to-EV ratio to the EV-to-bead ratio as determined in Subheading 3.2 step 13. This new ratio should be used for the remaining steps in the protocol.

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