qNano

USER MANUAL

IZON

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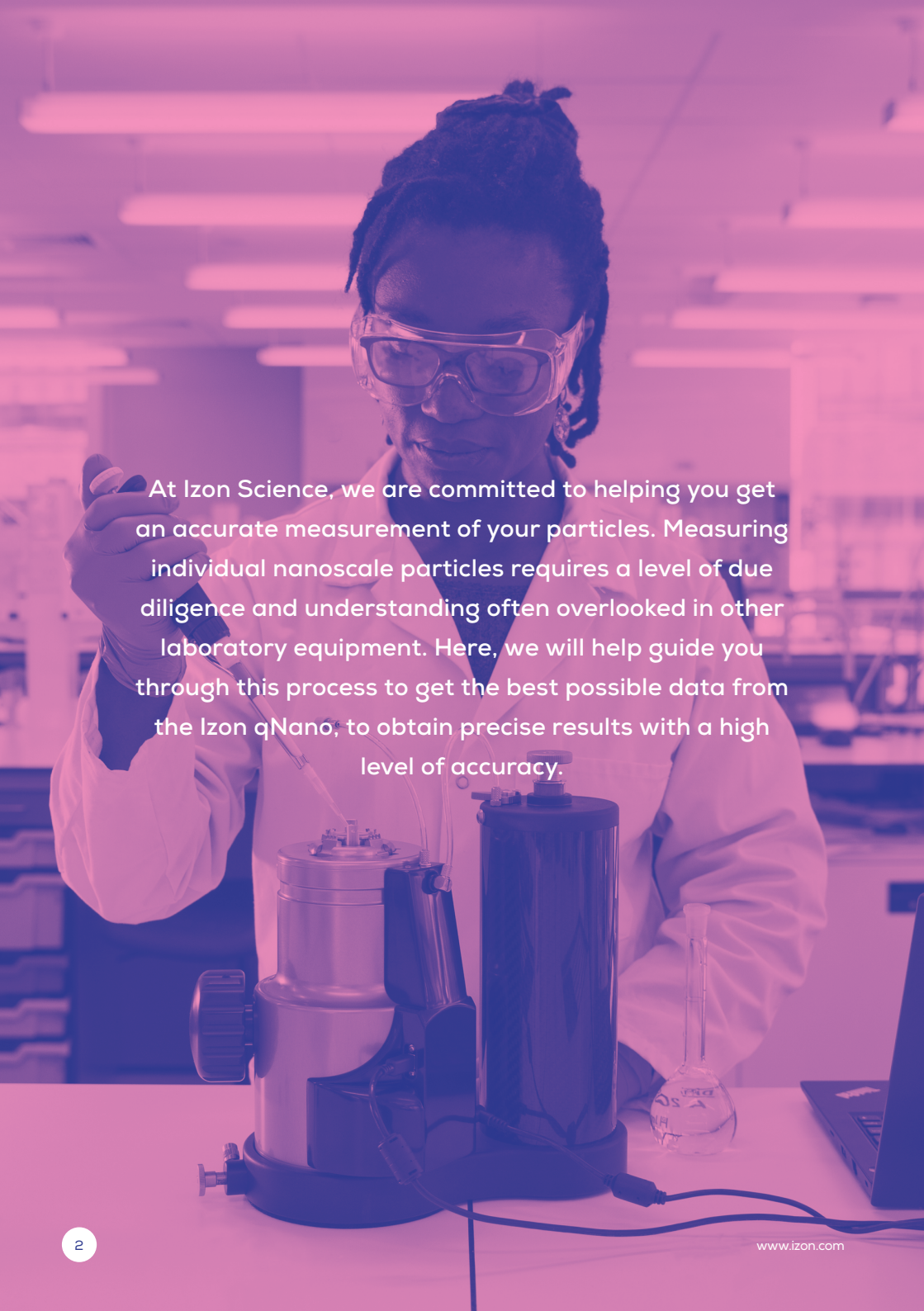


At Izon Science, we are committed to helping you get an accurate measurement of your particles. Measuring individual nanoscale particles requires a level of due diligence and understanding often overlooked in other laboratory equipment. Here, we will help guide you through this process to get the best possible data from the Izon qNano, to obtain precise results with a high level of accuracy.

TABLE OF CONTENTS

GETTING STARTED

1 / READ BEFORE OPERATING IZON INSTRUMENTATION

Follow all directions in this document and complete the Izon Training Module before attempting to operate the instrument, they contain important safety and operational information.

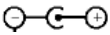
Use of this instrument in a manner not specified by the instructions in this guide and the training module may impair the safety protection provided. Do not operate the instrument outside its rated supply voltages or environmental range.

Failure to read these instructions and use the instrument in the manner specified may result in impaired performance and damage to the equipment.

The following symbols are used on the instrument and in this manual:

Earth (ground) terminal

Direct Current (DC).



Voltage polarity of Jack.

USB Connection.

Caution – risk of danger or impaired operation, if instructions not followed.

2 / CONSUMABLES ORDERING

For ordering of nanopores, calibration particles, and other consumables, please see our online store at store.izon.com

3 / SETTING UP THE SYSTEM

Assembling the Hardware

Unbox your instrument, being careful not to pick anything up by moving parts e.g. the qNano stretch handle, as this will increase the risk of dropping and damaging the equipment. The following is included with your qNano system:

- Callipers
- USB to USB Mini-B Cable
- qNano with Pressure Reading Module (PM2) permanently attached
- Fluid Cell
- Power Supply Unit
- Regional Power Cable (This should be correct for your region, please contact your local Izon office or supplier if this is not the case)
- Izon Training Kit
- Variable Pressure Module (VPM)
- VPM Nozzle Kit

Place the qNano in the recessed area of the base plate on the VPM. Connect the VPM-PM2 and PM2-Fluid Cell tubing by pushing the tubing over the correct barb and screwing the knurled collar on to be finger tight. DO NOT use tools for this operation as there is a risk of damaging the equipment. The correct tubing layout is outlined below:

Check that the fluid cell is connected to the system with the SMA cable screwed in properly by gently pulling the entire fluid cell assembly upwards. Once this has been ensured, click the fluid cell back into place by pushing down firmly in the correct orientation.

Powering Your Instrument

- Plug into Earth Grounded Protected Outlets ONLY.
- Make sure the power supply box is positioned away from fluids.
- To prevent heat build-up, do not cover the power-supply box.
- Position unit so it can easily and quickly be disconnected from mains power.

This instrument has a universal input range and will operate from a nominal 115 V or 230 V mains supply without adjustment. Check that the local supply meets the AC Input requirement given in the Specification.

Izon Instruments are only to be operated with Izon supplied leads and power supplies. Failure to use the correct power supply may result in invalid operation.

Do not place the power supply or operating computer in a position where it can come into contact with spills or moisture. Make sure the power supply is placed away and to the rear of the instrument to avoid spills during operation.

Connecting to a Computer

Devices that can be connected to the equipment should be compliant with a relevant safety standard such as IEC 60950-1 for IT equipment or IEC 61010-1 for laboratory equipment and should provide double or reinforced insulation from hazardous voltage sources. Always use an Izon supplied USB cable to connect to your instrument.

Computer Minimum Specifications:

- Windows (7 onwards) Professional Edition (64-bit)
- i7 processor (i5 minimum)
- 8 GB RAM
- Dedicated graphics processor and memory (1 GB). Intel HD Graphics 620 or higher on an i7 processor is acceptable
- Hard drive with at least 50 GB free space for software installation and data
- USB port

Windows Home Edition is not suitable for the installation of the Control Suite software. Ensure that the computer is installed with Windows Professional Edition.

Install the Izon Control Suite Software (CSS) by inserting the USB drive from the Training Kit into a computer that meets the minimum specifications. View the files and double click on "Install.exe", the installation wizard will then take you through installing the software.

Once the CSS and instrument are both successfully installed, complete reading the User Manual and proceed to undertake the training course described in the "Izon's TRPS Training Module" PDF document on the USB drive.

General Operating Precautions

Izon Instrumentation is designed for indoor use only and is to be used within the rated conditions outlined in the system specification table on the following page.

System Specifications

ITEM		SPECIFICATION	COMMENT
Environment		Indoor use at altitudes up to 2000 m, Pollution Degree 2 20% to 80% RH.	-
Operating Temperature		5 °C to 25 °C	-
RMS Noise Performance		Typical < 15 pA	For +/- 200 nA range NP200
Data Sampling Rate		50,000 samples per second	-
USB	Standard connector	USB Mini-B	Cable must be to a relevant safety standard such as IEC 60950-1 for IT equipment or IEC 61010-1 for laboratory equipment and should provide double or reinforced insulation from hazardous voltage sources
	Max input voltage	5.00 ± 0.25 V	
Electrical Power	Voltage	12 V (DC)	At input jack on instrument
	Current	130 mA	
	Power consumption	1.5 W	
PSU	Input AC	90 to 264 Vac 50/60 Hz ac	Use only Izon supplied TRG45A120-21E11
	Output DC	12 V nom	
	Output current max	3.75 A	
	Output power max	45 W[2]	
Nanopore holding stage extension range		Min. 41 mm Max. 61 mm	Measured across the outside of opposing holding arms
Electrical safety EMC		IEC/EN 61010-1 IEC/EN 61326-1	-

1 Under electrical interference noise may increase but will return to normal operation on end of interference. Position equipment away from electrical switching gear and interfering equipment or noise may increase under sample running conditions.

2 PSU module rating. Izon Instruments running normally are approx. less than 1.6 W.

THEORY OF OPERATION

1 / THE FUNDAMENTALS OF TRPS

What is TRPS?

Tunable Resistive Pulse Sensing (TRPS) enables the characterisation of nanoparticles suspended in electrolytes. TRPS is the only technology that can deliver:

- The concentration of particles in the fluid as number of particles per unit volume of fluid, across a specified detectable particle size range.
- An accurate size distribution of these particles ideally plotted as a histogram of concentration vs. particle diameter (or volume).
- The effective surface charge of individual nanoparticles (up to 800 nm in PBS or KCl based electrolytes).

How does TRPS work?

TRPS technology uses the Coulter particle counting principle on the nanoscale.

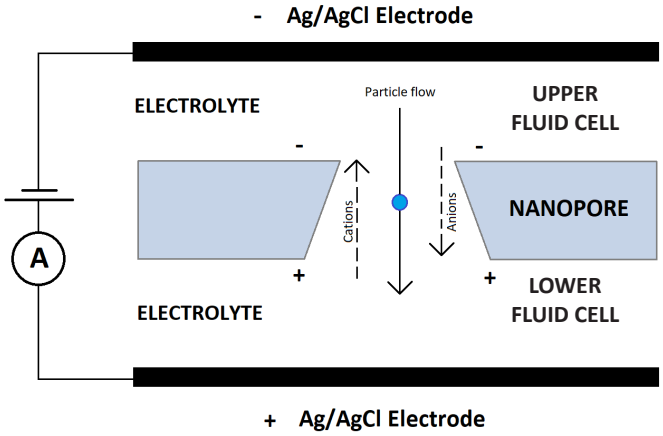


Figure 1. A schematic representation of how TRPS works within the Izon system.

Voltage is applied across the fluid cell via the silver/silver chloride (Ag/AgCl) electrodes. When electrolyte ions move between the electrodes through the nanopore, it creates a baseline current. A temporary decrease in current is detected as particles pass though the nanopore, which allows for the sizing and counting of particles in suspension.

Size and concentration measurements

Sample particles are driven through the nanopore by applying a combination of voltage and pressure (from the variable pressure module, or VPM), and each particle translocation event causes a resistive pulse or "blockade" signal that is detected and measured by the application software.

- Blockade magnitude is directly proportional to the particle diameter.
- Blockade frequency is used to determine particle concentration.

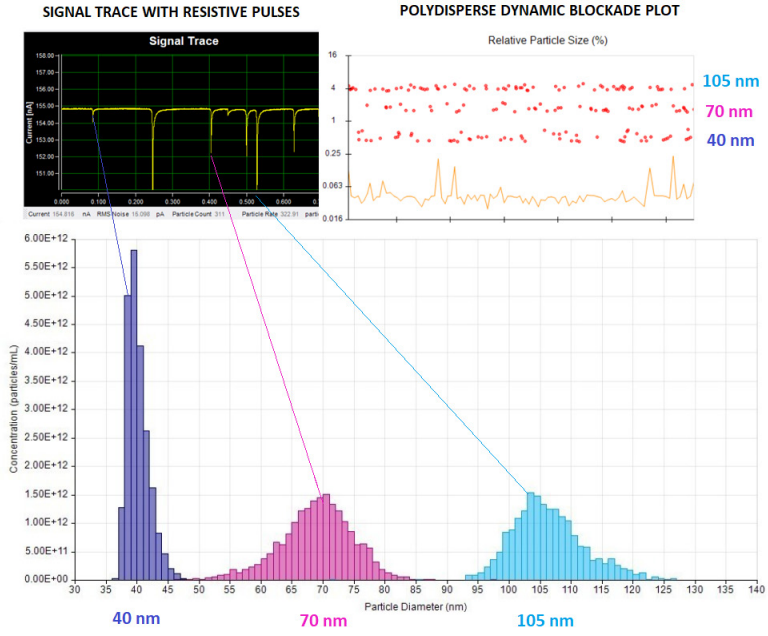


Figure 2. Magnitude and frequency measurements are converted into particle size and concentration by calibrating with particles of known size and concentration.

Charge measurements

The blockade duration changes with the velocity of the particle and can be used to calculate the relative surface charge of each particle.

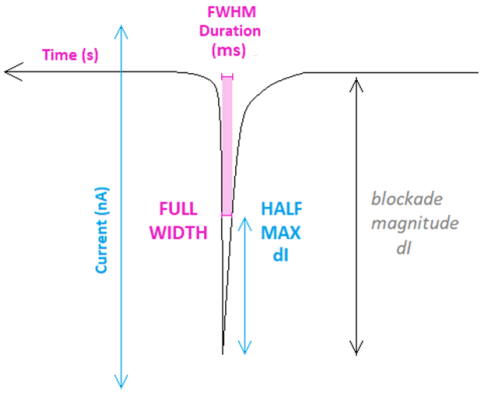


Figure 3. Blockade duration values are converted into particle zeta potential values by calibrating with particles of known size and surface charge.

The FWHM, or "full width at half maximum" is the blockade width (duration in milliseconds) measured at the point of half of the maximum blockade magnitude, shown as the horizontal pink bar in the centre of the blockade above.

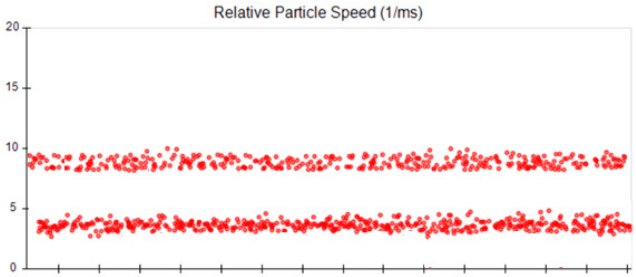


Figure 4. Displaying duration data in the relative particle speed plot using an inverted unit (ms-1) allows for intuitive comparisons of particle speed.

A particle speed plot with two different speed signals implies that the suspension contains particles with different surface charges. A particle with a larger negative surface charge will experience a greater attraction towards the positively charged electrode in the lower fluid cell and will travel through the pore at a greater speed than a more neutral particle.

Summary of dominant forces at play in a nanopore system

Forces influencing the speed at which nanoparticles travel through the nanopore are:

FORCES	EXPLANATION	NOTES
Convection Pressure-dependent force	There is always a static pressure head due to gravitational force on the fluid. An additional pressure or vacuum can be applied with the variable pressure module (VPM)	Convective forces tend to dominate particle velocity in larger pore systems
Electrophoresis Voltage-dependent force	Electrophoretic mobility relates to the movement of charged nanoparticles through an electrolyte solution towards an oppositely charged electrode. It is proportional to particle surface charge (ζ-potential) and the applied voltage	Electrophoretic forces can dominate particle velocity in small nanopore systems, when the applied voltage is increased
Electro-osmosis Voltage-dependent force	The second electrokinetic force is electro-osmosis. Electro-osmosis relates to the fluid flow caused by currents of solvated ions (ions surrounded by water molecules) moving along the surface of the nanopore. It is proportional to the nanopore surface charge (ζ-potential) and the applied voltage	Electro-osmosis is stronger in some nanopore systems (non-zeta capable pores), but weak in others (good zeta capable pores)

What is "tunable" and why?

Nanoparticle suspensions are complex systems. Characterising them fully requires an optimised setup, which involves tuning the applied stretch, pressure and voltage. When a particle passes through the nanopore, it creates a temporary decrease in the baseline current, which results in a blockade event. Each blockade event corresponds to a single particle translocating through the nanopore, from the upper to lower fluid cell.

Blockade magnitude and frequency can be controlled using three parameters:

FORCES	EXPLANATION	NOTES
Applied Stretch, S (mm)	$dI \propto \frac{1}{S}$	Optimise the blockade magnitude (dI) and sample resolution by adjusting the nanopore size (stretch)
Applied Pressure, P (mbar)	$f \propto P$	Optimise the particle rate (blockade frequency, (f) and blockade duration by adjusting the pressure applied by the VPM.
Applied Voltage, V (V)	$I \propto V$ $dI \propto V$	Optimise the signal-to-noise ratio of the system by increasing the electrophoretic force acting on charged particles.

Calibrating particle size

As the software records blockades in nA, calibration particles of a known size are used to convert blockade magnitude (nA) into a diameter (nm).

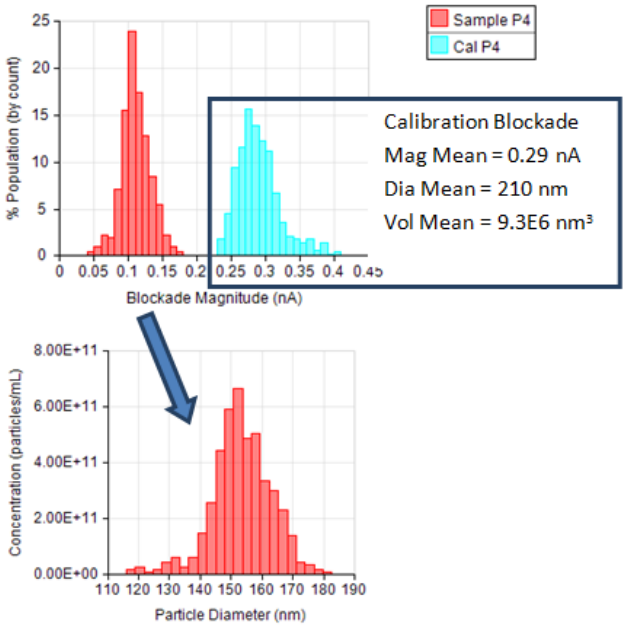


Figure 5. The Izon CSS is able to convert blockade magnitude into a particle diameter by using calibration particles of a known size.

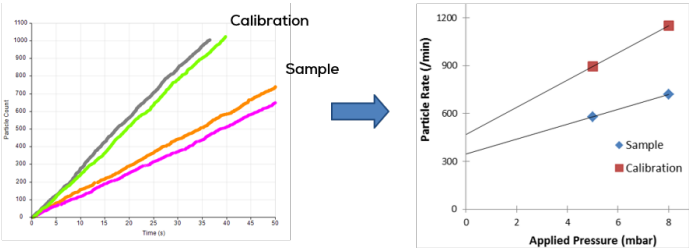
Blockade magnitude is proportional to the volume of the particle passing through the nanopore, giving very high resolution of particle diameter.

For each sample particle passing through the nanopore:

$$Particle\ Volume = \frac{dI\ (sample)}{dI\ (Calibration, mean)}\ x\ Calibration\ \ Volume\ (mean)$$

Calibrating particle concentration

Particle concentration is proportional to the change in blockade rate per unit of applied pressure. Accurate concentration values are typically derived at 2 or more pressure steps using calibration particles of a known concentration.



For each of the particle count v time measurements shown in the plot above, particle rate is calculated and plotted against pressure

Gradient used to calculate sample concentration

Figure 6. The particle rate observed should increase linearly with applied pressure:

This rate plot is used to check for system stability. This is crucial as the concentration value is measured by comparing the gradient of the rate plot of sample to calibration measurements.

$$Sample\ concentration = Calibration\ concentration\ x\ \frac{Sample\ gradient}{Calibration\ gradient}$$

If concentration is calculated at a single pressure the software will calculate a gradient based on the fitted line passing through the origin. This can introduce significant errors, especially with smaller nanopores (NP200 and below) where a large proportion of particles are driven through the nanopore due to applied voltage, V.

Important

For accurate and precise results, the sample and calibration particles must be recorded under identical system settings, which includes the electrolyte characteristics, which affect the baseline current and blockade magnitude. When possible, always use the same electrolyte.

Adjusting any of the parameters between the sample and calibration recordings will affect blockade magnitude and particle rate, invalidating the sample-calibration pairing.

Sample and calibration particles should be recorded at the same time – one immediately after the other, or close together as possible if the system is stable enough to record 2-3 samples before the system needs to be recalibrated.

2 / KEY CONCEPTS WITHIN THE IZON CONTROL SUITE SOFTWARE (CSS)

The Izon Control Suite Software (CSS) has two different data capture modes:

– The Custom Planner tool – automatic data recording

The custom planner capture assistant allows the user to define a sample measurement plan and then guides the user through the measurement process, including sample preparation, system optimisation, and calibration. The CSS assistant accommodates a wide range of measurement needs and can be set up to allow the user to easily and repeatedly obtain consistent measurements.

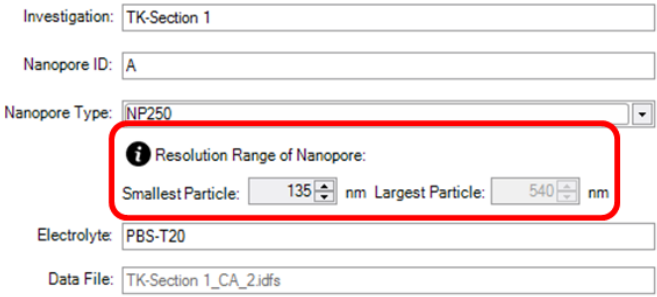
– The Classic Capture tool – manual signal optimisation and recording

The classic capture tool allows the user to tune the system in real time, rapidly respond to changes in the system, remeasure and calibrate different samples. This manual data capture tool is used most often for quick feasibility studies, and as the default tool for experienced instrument users who do not need the measurement process prompts.

There are a few key concepts that the user needs to understand to complete an assistant-based measurement.

Resolution range

When using the custom planner, the user will be asked to enter measurement details, one of which will be the resolution range:



The resolution range is an indication of the size range that can be observed at a single stretch. The default resolution range for each nanopore is calculated at mid-range stretch (e.g. the NP250 default range at 45-46 mm is ~135-540 nm).

The smallest particle can be adjusted within the nanopore size range to suit your sample. Once this has been edited, the largest detectable particle size will be calculated automatically, but cannot be larger than the upper range of the nanopore (e.g. 630 nm for NP250 at mid-range stretches).

The resolution range ensures that the software guides the user to the optimised instrument setting for the selected range of particles. It also allows the user to measure the same size range every time regardless of instrument use or nanopore size.

Analysis size range

The analysis size range is the size range over which the total concentration of the sample will be reported. Only particles in the analysis size range will be used to calculate the sample concentration. If not defined, the custom analysis range will be the same as the resolution range.

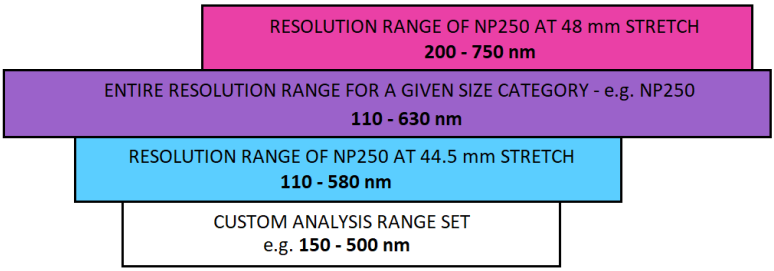


Figure 7. Different resolution ranges possible for a given NP250 pore.

Optimising particle size resolution

The green zone assists users to achieve optimised measurement settings for the quoted resolution range. Calibration particles must lie within the green zone to ensure that the smallest particle can be detected. This can be achieved by changing the applied nanopore stretch. The calculated location of the green zone depends on:

- The smallest particle diameter (set in the resolution range)
- The mean size of the calibration particle used. The software will show the recommended size of calibration particles to use.

The relative particle size (%) is an indication of particle size relative to the nanopore for individual particles. Change the applied stretch to optimise the relative particle size. At a constant stretch, a larger particle size would produce larger blockades.

The green zone is only shown in the custom planner tool and is not available in the classic capture tool.

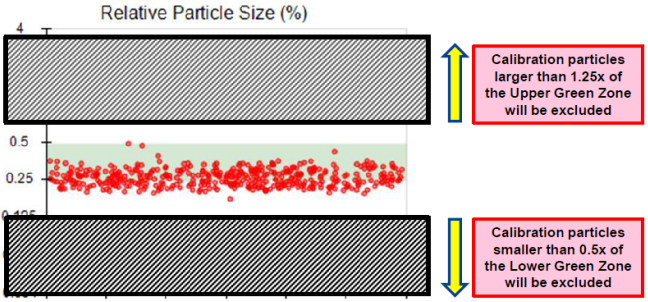


Figure 8. A visual representation of the importance of the green zone and how particles are excluded above and below.

During calibration recording, any particles that are significantly outside of the green zone are assumed to be noise or contamination and are automatically removed from the calibration calculations.

The green zone for the Relative Particle Speed plot (ms-1) is an indication of particle velocity through the nanopore (calculated from the blockade duration of individual particles).

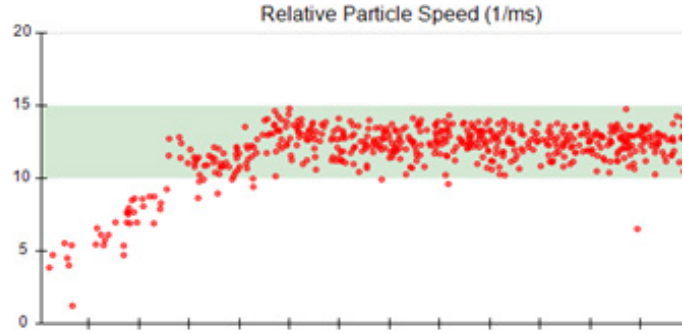


Figure 9. The applied pressure can be altered to optimise the relative particle speed so that particles fall within the green zone.

If using the classic capture tool, refer to the instrument set-up and signal optimisation section for advice.

MEASUREMENT PLANNING

1 / THE IMPORTANCE OF THOUGHTFUL STUDY DESIGN

Any high-quality TRPS study includes the following aims and considerations:

1. Decide what questions you want to answer, what TRPS outputs you need to measure, and which ones you need to control.
2. Include sufficient controls and replicates to ensure accurate, precise, and statistically significant results. Ensure that you have sufficient sample volume available.
3. Ensure that the target particles and suspension conditions are fundamentally compatible with TRPS. Sample particles must be between 45 – 10,000 nm in size, suspended in a buffer, and of sufficient concentration for analysis.
4. Ensure that you understand the properties of your sample and ensure that the structure and function of your particles will not be compromised at any point, by using properly validated sample preparation and storage protocols.
5. Determine how you will optimise the instrument operating conditions to maximise data quality (optimise the signal-to-noise ratio).
6. Ensure that you have the correct consumables.

2 / VARIABLES TO CONTROL AT DIFFERENT ANALYTICAL STAGES

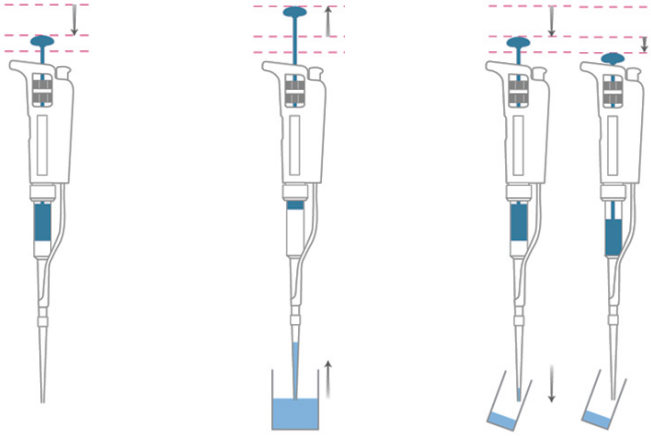
For efficient method development for a TRPS study, breaking up the experimental plan into three stages can be helpful.

STAGE	DETAILS
Pre-analytical	– Develop theoretical understanding – Do some feasibility studies (for novel samples) – Identify suitable protocols and consumables – Method verification – identify controls needed
Analytical	– Optimise instrument operation conditions – Collect high-quality data – Adjust method if needed
Post-analytical	– Analyse data quality – Identify study limitations and suggest improvements

SAMPLE PREPARATION

1 / KEY SKILLS FOR SAMPLE PREPARATION

To reduce the error associated with your concentration results, always use calibrated pipettes and always use the smallest pipette for the job (e.g. use a 2 µL pipette for dispensing 1 µL rather than a 10 µL pipette, and use a 200 µL pipette for dispensing 150 µL rather than a 1000 µL pipette). Always wipe the outside of the pipette tip before dispensing, always dispense the smaller volume into the larger volume, and pump the plunger 3-4 times before discarding the tip.



Rotate the thumbwheel to the correct volume.

Attach pipette tip by firmly pressing the pipette down into the top of a pipette tip in the holder.

Immerse the pipette tip into the fluid.

Smoothly release the plunger to the rest position.

Place tip at an angle in the receiving vessel.

Smoothly depress plunger to the first stop, then to the second stop.

With the plunger still depressed, remove the pipette from the fluid.

Figure 10.Forward or standard pipetting should be used when diluting sample particles.

2 / ESSENTIAL CONSIDERATIONS

1. Ensuring the suspension conditions are suitable for TRPS analysis
2. Excluding contaminants from the suspension
3. Maintaining the structural and functional integrity of target sample particles
4. Enhancing suspension stability

Use the TRPS measurement checklist to ensure you have the equipment you need.

1 / TRPS suspension requirements

General points

As TRPS is a conductance-based technique, all sample solutions must have an ionic strength of at least 0.01 M. Samples dissolved in pure water can be spiked with small amounts of concentrated PBS to rectify this issue. However, the requirement for solutions to be conductive generally excludes samples that are solvent based.

Contamination

Besides preventing sample degradation, mitigating contamination of samples is required to maintain system stability during recordings. Small nanopores especially are easily blocked by large precipitates, aggregates, and microbial contaminants. All samples and reagents that come into contact with nanopores must be filtered with a 0.22 µm syringe filter daily.

2. Excluding contaminants

Contaminants in a TRPS context could include:

- Non-target particles in the same size range (bacterial vesicles, intracellular vesicles, lipoproteins)
- Particulates that could block pores (microorganisms, cells and debris, protein aggregates, dust)
- Pore modification agents (free protein, charged coating agents)
- Destructive or disruptive agents (proteases and nucleases, cells or microorganisms left in samples, harsh detergents that could disrupt membrane particles or membrane-bound proteins).

	CONTAMINANTS EXCLUDED	STEPS TAKEN TO CLEAN SAMPLES OR REDUCE
Reagents and clean calibration samples (DI, electrolyte, wetting and coating solutions)	Dust, pollen, etc.	Use clean glassware rinsed twice with deionised water, and new, dust-free tubes and filters
	Microorganisms and their secretions/ products	Make up electrolyte from powder weekly, filter-sterilise daily, store solutions at 2-8 °C
	Trace chemicals	Use reagent-grade buffers and electrolytes, fresh deionised water, clean glassware
	Precipitates/salt crystals	Filter all solutions that will come into contact with the pore daily with a 0.22 µm filter
Biological samples e.g. plasma	Large particles (cells, debris, etc.)	Centrifuge samples at 2000 g for 10 minutes to remove cells, then spin supernatant at 10000 g for 10 minutes to remove debris and apoptotic bodies
	Small particles and solutes (destructive enzymes, nutrients, etc.)	Remove proteins and solutes via a Size Exclusion Chromatography (SEC) qEV column. Exclude the buffy coat when working with whole blood
	Microorganisms and their secretions/by-products	Store samples at -80 °C only after centrifugation and SEC steps have been completed, flush qEVs in an antimicrobial agent before storage, do not use bench PBS – always make up buffers from powder

3 / Maintaining sample integrity

General points

Nanoparticle surface groups often behave differently in different chemical environments. Changes in ionic strength and pH can cause significant chemical changes at the nanoparticle surface. For biological particles such as viruses and extracellular vesicles, these chemical changes can destroy surface functionalities like surface proteins and RNA. Maintaining the suspension conditions that particles were formed in is relatively simple way of preserving the functionality of all particles.

Biological samples

Immunoassay techniques use membrane-bound proteins as markers to distinguish between specific bio-nanoparticles (messenger or carrier EVs, different types of lipoproteins, etc.). These surface proteins can be destroyed or damaged by significant changes in solution pH, ionic strength and temperature, as well as by the presence of strong detergents (SDS) and destructive enzymes. It is important to maintain the suspension conditions throughout the sample cleaning process. It is also important to remove cells from samples, as both living and dead cells can release destructive agents into the suspension.

4 / Suspension stability

Particle aggregation

The aggregation of small nanoparticles (< 100 nm) in solution is a constant process, involving a complex array of forces and factors. However, there are a few sample preparation and storage techniques that can reduce the rate of aggregation.

- **Ionic strength:** Generally, 1 x PBS (0.15 M) is recommended for suspending particles 100-600 nm in diameter. For nanopores larger than NP600, 1/10th PBS is recommended. For small nanopores (NP80, NP100), using 2 x PBS can significantly improve the signal-to-noise ratio. Higher ionic strength conditions shrink the ion clouds that surround particles in solution, allowing particles to cling closer to each other, where the attractive chemical forces are stronger. By using a more moderate ionic strength, there is a larger cushion of space between particles, and the rate of aggregation is lower.
- **Temperature:** The rate of aggregation for particles < 100 nm is directly proportional to the temperature-dependent rate of diffusion. Keep samples below 25 °C to reduce the diffusion rate, and do not freeze calibration particles. EV samples may be snap frozen and kept at –80 °C.
- **Zeta potential:** Like charges repel each other – the higher the effective particle surface charge (zeta potential), the stronger the repulsive force between particles, and the slower the aggregation rate. Often, balancing suspension stability and maintaining sample integrity can be difficult, as occasionally the pH needs to be adjusted to increase surface charge in order to reduce particle aggregation.
- **Surfactants:** Adding non-ionic surfactants like Polysorbate-20 to a suspension helps to keep particles physically separated and suspended in solution.

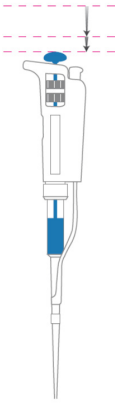
Particle settling

As the particle size and density increases, the rate of settling will also increase. Always vortex samples (except delicate biologicals, which should be mixed with a pipette) before diluting, to avoid concentration errors. For particles larger than 500 nm, we recommend vortexing particles before each recording, and sonicating samples for 5-10 minutes if necessary.

INSTRUMENT OPERATION

1 / KEY SKILLS FOR QNANO OPERATION

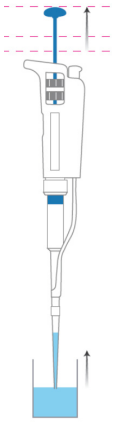
The most common cause of system instability is incorrect pipetting technique. Using the standard forward pipetting technique for dispensing liquid into the fluid cells may introduce bubbles near the pore which can clog the pore and disrupt the baseline current and particle rate. With reverse pipetting, you draw out more fluid than you dispense, minimising the risk of introducing bubbles into the fluid system.



Rotate the thumbwheel to the correct volume.

Attach pipette tip by firmly pressing the pipette down into the top of a pipette tip in the holder.

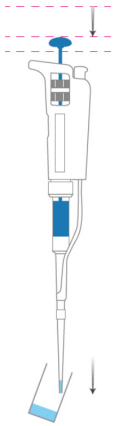
Depress the plunger to the second stop.



Immerse the pipette tip into the fluid.

Smoothly release the plunger to the rest position.

Wipe the tip with a clean tissue to remove any fluid beaded on the outside surface of the pipette tip



Place tip at an angle in the fluid cell.

Smoothly depress plunger to the first stop. Be careful not to push down to the second stop.

Release the plunger to rest position.

Discard the pipette tip.

Figure 11. Reverse pipetting is used when introducing any fluid into the qNano fluid cells.

2 / REFERENCE GUIDE TO NANOPORE SELECTION

The quoted particle detection range for each pore category cannot be detected at a single stretch. For researchers analysing particles between 50 and 200 nm in diameter, we recommend ordering pore sizes slightly smaller than your average estimated particle size and using these smaller nanopores at higher stretches.

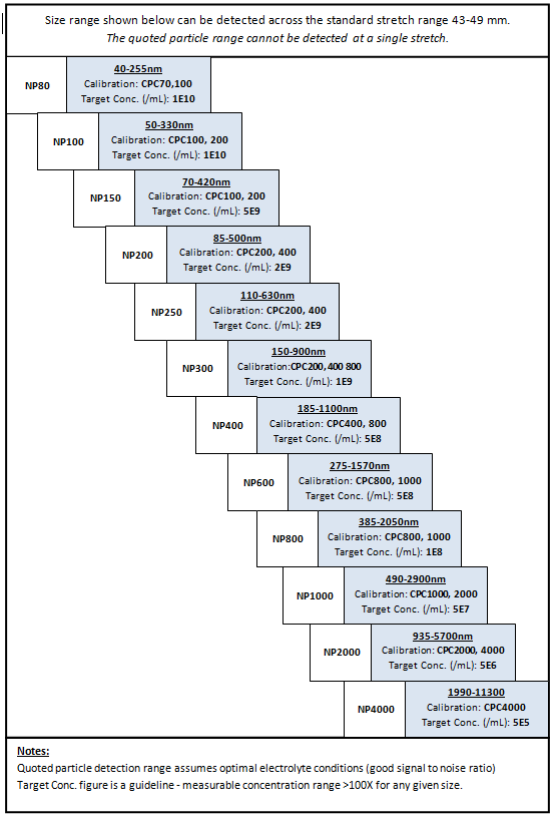


Figure 12. Guide to Izon Nanopore categories and their corresponding appropriate calibration particles sizes and target concentrations.

3 / BEHAVIOUR OF NANOPORES AT DIFFERENT STRETCHES

The stretch of the nanopore being used can be altered by winding the stretching mechanism on the qNano up or down. Rotating clockwise will increase the applied stretch, while anti-clockwise will decrease it. Choosing a suitable operating stretch is critical, as this will have an impact on the stability of the pore.

*Operating at a stretch of 44-45 mm is possible but at low cruciform tension the pore may exhibit less baseline stability and may be more prone to blockages. Operating at a stretch of 47-48 mm and greater may work well but the pore may become problematic for further use at lower stretches. This is because the cruciform has undergone some structural changes at the high stretch. On some occasions, excessive stretch can cause a rupture of the pore.

STRETCH (MM)												
40	41	42	43	44	45	46	47	48	49	50	51	52
Winding below 40 mm can damage the winding mechanism, and pore will not be open		Very unstable recordings, nanopore prone to blocking		*	Optimal operating stretch			*	Very stable recordings, but nanopore is at risk of being stretched out of shape		Very stable recordings, but nanopore at risk of rupturing	

4 / NANOPORE SET-UP AND SYSTEM OPTIMISATION

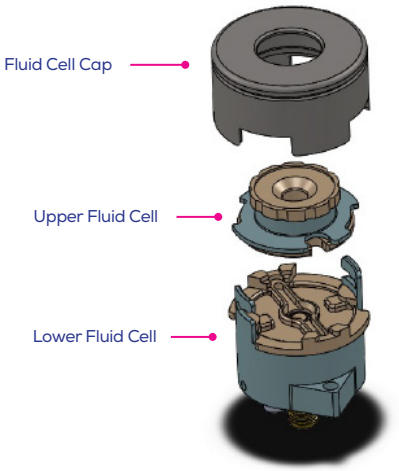


Figure 13.Schematic of the fluid cell assembly and key parts for using the qNano system.

Order of operation:

1 / Prepare the fluid cell and stretch the nanopore

2 / Wet the nanopore

3 / Establish a stable baseline current

4 / Estimate the pore size via a conductance check (change pore if necessary)

5 / Coat the pore (if working with biological samples)

6 / Optimise signal-to-noise ratio for calibration particles

7 / Adjust conditions for the sample if necessary and record data

8 / Re-run calibration measurements to ensure system stability

1 / Prepare the fluid cell and stretch the nanopore

Wetting the porous lower fluid cell paste with 75 microlitres of 70% ethanol for 30 seconds helps prevent bubbles from gathering under the pore.

Remove the fluid in the lower fluid cell before fitting the pore with the serial number facing upwards. Wind the stretching wheel mechanism clockwise and use the callipers provided to calibrate the stretch to 47 mm. Enter the stretch value into the software and click “calibrate stretch”.

The stretch value on the software display should now change as you adjust the stretch manually.

2 / Nanopore wetting protocol

- Make up nanopore wetting solution according the Reagent Kit tech note.
- Add wetting solution to the lower (75 µL) and upper (35 µL) fluid cell and fit the upper fluid cell.
- Fit the fluid cell cap, push in the PM2 nozzle and apply a pressure of 20 mbar for 2 minutes. Apply a voltage of at least 0.1V so that during this time a stable baseline can be observed being established.

If a baseline does not establish, disconnect the PM2 nozzle, remove the fluid cell cap, and apply pulses of pressure with the Izon PAD up to 10 times. If this is still not successful, then try clicking the fluid cell cap by putting pressure down on the nanopore arms and twisting until it clicks into place.

- Remove the upper fluid cell, pipette a droplet of electrolyte on top of the nanopore, and replace the lower fluid with electrolyte (prepared in accordance with the Reagent Kit tech note).
- Wipe away the electrolyte droplet, fit the upper fluid cell and dispense 35 µL of electrolyte into the upper fluid cell.
- Fit the fluid cell cap and check the baseline current is stable, with an RMS noise of <15 pA. Consult the troubleshooting guide at the end of this manual if any difficulties are encountered at this stage.

To ensure that the pore is properly wetted, tap the fluid cell cap gently but firmly a few times to dislodge any bubbles trapped in the aperture once the baseline current has been initially established. The current should slowly increase and reach a stable level once fully wetted. **After wetting, do not click the fluid cell cap at all from this point** and be very gentle when making contact with the pore during pipetting.

3 / Establish a baseline current:

The pore can be considered properly wetted when you can tap the fluid cell cap and the current remains stable and does not jump or drift.

If the current is drifting, unstable, or still zero:

- Ensure that there is a voltage applied.
- Make sure the nanopore is stretched.
- Ensure that you are using the reverse pipetting technique.
- Mix the fluid in the upper fluid cell gently – without introducing more bubbles.
- Apply full pressure on the VPM and tap the fluid cell cap, to dislodge any bubbles trapped in the aperture.
- Repeat the wetting process but use electrolyte instead of the wetting solution and increase the stretch by 0.25 mm. Ensure that your electrolyte contains between 0.03 and 0.1% surfactant.

4 / Estimate the size of the pore:

If the history of the pore is unknown, a quick size check can be easily performed with PBS (with stretch = 47 mm, voltage = 0.1 V).

APPROXIMATE BASELINE CURRENT AT 0.1 V (NA)	NANOPORE SIZE (NP)
7-35	80
10-38	100
13-47	150
17-53	200
20-63	250
27-80	300
65-138	400
97-148	600

5 / Nanopore coating protocol:

When working with biological samples, it is strongly recommended to use the Izon reagent kit and coat nanopores. The Izon coating solution is used to prevent the non-specific binding of protein from biological samples to the nanopore. If left uncoated, the performance of nanopores may rapidly decline, as the pore can become partially blocked and modified, and calibration recordings need to be run more frequently. As the coating solution has a greater viscosity than the electrolyte, the baseline may drift for 30 seconds or so after coating.

- Replace the electrolyte in the lower fluid cell with freshly filtered coating solution, fit the upper fluid cell and dispense 35 μL of coating solution into the upper fluid cell.
- Fit the fluid cell cap, push in the PM2 nozzle and apply pressure of 20 mbar for 10 minutes.
- Disconnect the PM2 nozzle, remove the upper fluid cell, pipette a droplet of electrolyte on top of the nanopore, and replace the lower fluid with electrolyte.
- Wipe away the electrolyte droplet, tap the upper fluid cell dry before refitting it, and dispense 35 μL of electrolyte into the upper fluid cell.
- Fit the fluid cell cap and check the baseline current is stable, with an RMS noise of <10 pA.

6 / Optimise signal-to-noise ratio for calibration particles

The standard sample changeover protocol should be used any time particles or samples are being added or changed.

- Remove upper fluid cell and immediately add a droplet of electrolyte onto the nanopore.
- Rinse the inside of the upper fluid cell with deionised water and dry with compressed air.
- Gently wipe the top of the nanopore dry with a lint free tissue, fit the upper fluid cell and load 35 µL of the next sample before connecting the PM2 nozzle and applying your chosen pressure.

In order to obtain high quality data, certain parameters can be optimised. These are the stretch, the Voltage, and the Pressure.

PARAM-ETER	SIGNIFI-CANCE	DESCRIPTION	SETTINGS FOR HIGH-QUALITY DATA
Stretch S (mm)	$dI \propto \frac{1}{S}$	Optimise the blockade magnitude (dI) and sample resolution by adjusting the nanopore size (stretch)	Average blockade magnitude: Ideally: 0.2–1 nA, (acceptable range: 0.15–2 nA) Stretch range recommended: NP80, NP100: 45–48 mm › NP150: 45–50 mm
Voltage V (V)	$I \propto V$ $dI \propto V$	Optimise the signal-to-noise ratio of the system by increasing the electrophoretic force acting on charged particles.	Baseline current: Ideally: 100–140 nA Applied voltage: Ideally: 0.2–1 V Adjust electrolyte molarity if necessary. Use 1/10 PBS for large pores.
Pressure P (mbar)	$f \propto P$	Optimise the particle rate (blockade frequency, f) and blockade duration by adjusting the pressure applied by the VPM.	Average particle rate: Ideally: 500–1500 p/min (acceptable range: 200–1500 p/min) Pressure ranges recommended: 1–15 mbar Concentrate samples or adjust dilution if necessary.

7 / Adjust conditions for the sample if necessary and record data

Once the system is stable and the resolution is good across the entire sample size range, move onto the recording data stage. Use a lab book to record the voltage, current, blockade magnitude and particle rate readings for each recording, and always monitor the baseline current to track changes in nanopore size. If the baseline current drops or becomes unstable or noisy, or the particle rate drops, consult the troubleshooting guide to restore the system stability.

Use the sample changeover method described on the previous page to introduce the sample particles. To guarantee good resolution for your sample particles AND high-quality data, you must choose a nanopore size that will resolve your sample size distribution properly, AND choose a calibration standard that matches your sample size:

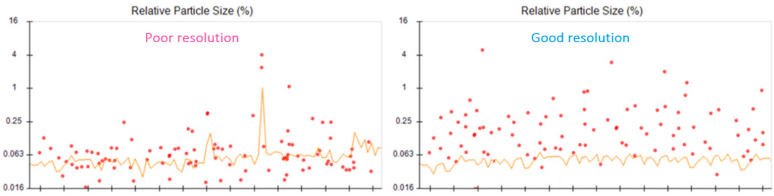


Figure 14. Examples of poor and good resolution of sample particles on a nanopore.

Multiple calibration particles may be resolved on the same pore, but for best results, choose one that gives you an average blockade magnitude of at least 0.25 nA (at a background current of 100 nA) at the stretch value that you can resolve at least 90% of your polydisperse sample.

Use a smaller nanopore if you cannot properly resolve the sample at the lowest recommended stretch (44.5 mm). Concentrate samples if the particle rate is below 200 particles per minute at a pressure of 10 mbar.

8 / Re-run calibration measurements to ensure system stability

A recording is not a meaningful measurement until it has been calibrated with a calibration recording that has been run under the same conditions (stretch, pressure, voltage and baseline current). If the sample recording has not been calibrated properly, the errors for size and concentration can be significant.

For size analysis only, measurements need to only be taken at one pressure. In this case, run one calibration, and then run the sample under identical conditions.

For size and concentration analysis, both the sample and calibration measurements must be taken at a minimum of 2 (ideally 3) pressures. Current and particle rate should be recorded for each run. The measurement plan for multiple samples may look like this:

- Calibration 1 (P10, P6, P3), Current 131 nA
- Sample 1 (P10, P6, P3), Current 131 nA
- Sample 2 (P10, P6, P3), Current 136 nA
- Calibration 2 (P10, P6, P3) Current 135 nA
- Sample 3 (P10, P6, P3), Current 141 nA
- Sample 4 (P10, P6, P3) Current 143 nA
- Calibration 3 (P10, P6, P3) Current 144 nA

Additionally, nanopores are prone to stretching over time, so it is important to use the correct nanopore size and recalibrate the pore often.

If the baseline current increases by more than 10%, this indicates an irreversible change in nanopore size, and the pore must be re-calibrated.

If the baseline or particle rates drop by more than 10%, or the noise increases past the acceptable limit of 15 pA, this indicates a partial nanopore blockage. Consult the troubleshooting guide and restore the system stability before repeating the recording.

9 / Pore cleaning and storage

Once all data has been collected, do not leave the pore on the instrument. Wash the pore before storing in a clean, resealable bag. Nanopores should last in storage for years if they are properly cleaned and dried as below before being packed away.

- Stretch the nanopore to 47 mm.
- Remove the sample from the upper fluid cell with a pipette.
- Wash the upper fluid cell using measurement electrolyte by reverse pipetting three times, then load 35 µL of fresh measurement electrolyte into the upper fluid cell, ensuring no bubbles are present.
- Apply maximum pressure for at least 30 seconds until there are fewer than 10 blockades per minute measured, you may need to wash the upper fluid cell out with measurement electrolyte multiple times to achieve this. If after multiple washes a blockade rate of less than 10 per minute cannot be achieved, remove the fluid from both upper and lower fluid cells, de-stretch and remove the nanopore. Rinse the nanopore and upper fluid cell with DI water and dry.
- Replace the nanopore and repeat with DI water, ensuring that the current goes to <5 nA.
- Remove the nanopore, rinse with DI water and dry with compressed gas. Do the same with the upper and lower fluid cells, and make sure the inside of the shielding cap is also clean and dry.
- Re-assemble the fluid cell components on the qNano and put the clean, dry nanopore in its ziplock bag.

IMPORTANT NOTES:

- Do not submerge the fluid cell – only rinse the fluid chamber.
- Take care not to get any fluid in between the plastic and metal connections in the fluid cell (as fluid trapped between components can cause noise fluctuations and short circuits).
- Always dry the fluid cell with compressed air after it has been cleaned.
- Always ensure the fluid cell is completely dry before fitting back onto the pore.

TROUBLESHOOTING

1 / RECOVERING AN UNSTABLE BASELINE AND MAINTAINING PORE STABILITY

- Gently tap the fluid cell cap to dislodge bubbles on top of the pore, and mix the sample using the reverse pipetting technique, without introducing more bubbles.
- Replace the fluid in the lower fluid cell periodically (once every half-hour) to prevent bubbles from gathering around the pore.
- If the baseline is very unstable, there may be many invisible bubbles beneath the pore. Put a droplet of electrolyte on top of the pore, take the nanopore off the qNano and wipe the bottom gently with a lint free tissue before remounting on the qNano the applying the same stretch as before. Re-establish your baseline current.

2 / RESOLVING COMMON QNANO ISSUES IN DIFFERENT SITUATIONS

Only electrolyte in the pore

ISSUE	ACTIONS THAT MAY BE NEEDED
Cannot establish baseline current	– Unplug and replace USB cable to restore the connection between the qNano and the PC. Ensure voltage is switched on and non-zero. – Use reverse pipetting (as described in the instrument operation section) to prevent bubbles forming on top of the pore and blocking the fluid flow.
Unstable baseline current	– Gently mix the fluid in the upper fluid cell without introducing any bubbles. – If consistently destabilising, wash and dry the upper fluid cell, and gently replace the fluid in the lower fluid cell to remove any bubbles.

Running synthetic or inorganic particles

ISSUE	ACTIONS THAT MAY BE NEEDED
Stable baseline current, but very few particles	– Increase the sample concentration. Use low pressures until you have a stable system, otherwise you risk blocking the pore. – Consult the sample preparation guide and enhance suspension stability. – If your sample particles have positively charged surface/terminal groups, they are likely to stick to and block the pore instead of going through. For concentration and size analysis only, you can suspend particles in Tris buffer, adjusted to pH 10, with 0.03% Triton-X, to mask the charge on the particles to allow them to pass through the pore. Charge analysis will not be possible for particles with a positive surface charge, as zeta potential will be altered with pH.

Running calibration standards or biological sample particles

ISSUE	ACTIONS THAT MAY BE NEEDED
Stable baseline current, but very few particles	– Check the dilution factor (use ~ 1:50 for training particles and ~ 1:1000 for CPC particles ~1:1000). qEV fractions may be very dilute and may need concentrating. – Pore is partially blocked by a bubble or an aggregate. Un-block pores by gently replacing fluid in lower well and flushing the pore with filtered deionised water at 47 mm stretch as shown on the previous page. – If working with a calibration standard above 400 nm, sonicate before use. However, please note that sonication and vortexing can damage biological samples - only mix with pipettes or by gently inverting/ swirling samples by hand. – Make up another CPC dilution with 0.22 µm filter-sterilised measurement electrolyte. All reagents should be filter-sterilised daily before use. – Make sure you have coated the pore if working with biological samples, as non-specific binding of biological macromolecules to the aperture surface can cause partial blockages and instability. – Increase the sample concentration. Use low pressures until you have a stable system, otherwise you risk blocking the pore. – Pore is not the predicted size, try a different calibration standard or pore.
Sample is blocking the pore (unstable baseline current)	– Run highly-polydisperse samples (e.g. EVs) on two different sized pores. First, run the sample (with an appropriate calibration) unfiltered through an NP250 or NP300, and then filter the sample through a 0.22 µm filter and run with a suitable calibration on an NP100 pore. Once calibrated, the results from both pores can be displayed on the same plot. – If only interested in exosomes from polydisperse EV samples, filter the sample through a 0.22 µm filter and run with a suitable calibration on a NP100 pore. – All biological samples should be run through a qEV SEC column before TRPS analysis, to exclude proteins that are likely to interact with and block pores. – Pore is partially blocked by a bubble or an aggregate. Unblock pores by gently replacing fluid in lower well and flushing the pore with filtered deionised water at 47 mm stretch as shown on the previous page.

If you are using the programmable Custom Assistant, and your data quality is not ideal, try switching to the Classic Capture mode instead. Classic Capture allows you to optimise each parameter yourself, so you can get the best possible data out of every pore.

3 / MITIGATING CONTRIBUTING FACTORS TO RMS NOISE

External/environmental noise

50/60 kHz noise from nearby laboratory equipment and ungrounded power supplies can interfere with the baseline current signal.

To reduce influence from external noise, do not operate the qNano in close proximity to large laboratory appliances that draw a lot of power, and ensure the fluid cell cap is used during data recording to minimise noise on the baseline current and obtain better blockade resolution.

Short-circuits

Electrolyte can creep in between metal connection points inside or around the fluid cell, causing significant noise fluctuations.

Remove the upper fluid cell, then wash and dry it, making sure all the metal parts of the fluid cell and fluid cell cap are dry. Then re-establish the baseline current.

Always ensure that you are using the reverse-pipetting technique when introducing fluid to the upper or lower fluid cells, and do not pipette more than 35 µL into the upper fluid cell, or more than 75 µL into the lower fluid cell, or the fluid may leak between connection points and cause the noise to gradually increase.

Partial nanopore blockage

Particle or bubble build up on either side of the aperture can cause partial blocking of the nanopore (indicated by a sudden >10% drop in baseline current or particle rate).

To prevent these blockages, replace the electrolyte in the lower fluid cell every half hour.

If the current becomes unstable and the noise is high, replace the fluid in the lower fluid cell 5 times with filtered deionised water as shown in the troubleshooting protocol.

4 / FURTHER ADVICE ON THE MOST COMMON ISSUES WITH THE QNANO

Measurement planning

Before analysing samples on the qNano, run some quick preliminary tests using other nanoparticle analysis techniques to determine the approximate size and concentration of your particles.

Ensure you have purchased the correct nanopore and calibration particle sizes for your sample. Most optical techniques significantly overestimate particle size. If in doubt, purchase the nanopore size that is slightly smaller than your average particle diameter, and use the larger of two calibration standards if you can resolve two in the same pore.

Sample preparation

When working with small nanopores, and suspensions of particles < 150 nm, extra care must be taken to balance the forces keeping particles in solution. Consult the sample preparation guide for a brief overview, and contact **support@izon.com** for free advice.

If working with biological samples, ensure that the samples are processed thoughtfully, to preserve sample integrity and optimise the yield of target particles.

Pore set-up, signal optimisation, and calibration

Mastering the reverse pipetting technique is essential when working with the instrument. Using normal pipetting techniques can introduce bubbles into the system, which can prevent the baseline current from being established.

Another common issue that can prevent the baseline current from being established is users re-fitting the fluid cell when it has not been completely dried. Whenever the fluid cell is removed it must be firmly patted dry. When washing the fluid cell, only wash the inner chamber, do not immerse or wash the outside. The most common cause of a wandering baseline is a fluid cell that has fluid trapped inside it.

The easiest way to restore the function of a nanopore is to thoroughly wash it with deionised water (using the procedure shown earlier). Flush the pore as shown, then re-coat and re-equilibrate the pore.

If you are working with exosomes or very small particles (> 150 nm), you should filter samples through a 0.22 µm filter.

5 / REPAIR AND SERVICING OF YOUR IZON INSTRUMENT

There are no user-serviceable parts. Return equipment to Izon for service.

All replacement parts, cables, mains lead, power supply, nanopores, fluid cells must be obtained via Izon Science Ltd.

6 / FURTHER SUPPORT

Additional support material is available at **support.izon.com**

If you have any questions that are not answered on the support portal, please contact our support staff via the online support portal or directly by email (**support@izon.com**).

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