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# Determination of particle size distribution — Electrical sensing zone method —

## Part 2: Tunable resistive pulse sensing method

*Détermination de la distribution granulométrique — Méthode de détection de zones électrosensibles —*

*Partie 2: Méthode par détection d'impulsions résistives accordable (TRPS)*



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# Foreword

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This document was prepared by Technical Committee ISO/TC 24, *Particle characterization including sieving*, Subcommittee SC 4, *Particle characterization*.

A list of all parts in the ISO 13319 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

# Introduction

Monitoring particle size distributions and particle concentrations are required in various fields, where particle dispersions in liquid play a role. The electrical sensing zone technique has, since its discovery by W. H. Coulter around 1950, been widely employed for size and count analysis of (blood) cells, bacteria and other fine particles. Over the last decades, the application range has expanded to nanoparticles, such as liposomes, exosomes, and nano- and micro-bubbles, as a result of improved electronics and aperture fabrication. The tunable electrical sensing zone technique is useful for the determination of the size distribution, concentration and zeta potential of micro- and nanoparticles suspended in a liquid. The purpose of this document is to provide the background and procedures for application of tunable electrical sensing zone equipment for particle size distribution and concentration measurements, so as to improve the reproducibility and the accuracy of the acquired results.

# Determination of particle size distribution — Electrical sensing zone method —

## Part 2: Tunable resistive pulse sensing method



## 1 Scope

This document specifies the measurements of particle size distribution and concentration of suspended particles, ranging from 40 nm to 100 µm, using tunable resistive pulse sensing (TRPS). This document provides a comprehensive overview of the methodologies that are applied to achieve reproducible and accurate TRPS measurement results. This document also includes best practice considerations, possible pitfalls and information on how to alleviate or avoid these pitfalls.

## 2 Normative references

There are no normative references in this document.

## 3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

**3.1**
**aperture**
small diameter hole through with suspension is drawn

[SOURCE: ISO 13319-1:2021, 3.2]

**3.2**
**sensing zone**
volume of electrolyte within and around the aperture in which a particle is detected

[SOURCE: ISO 13319-1:2021, 3.3]

**3.3**
**pulse frequency**
number of pulses per duration

**3.4**
**detection range**
size range between the smallest and largest detectable particle diameter

**3.5**
**dynamic range**
ratio between the largest and smallest detectable particle diameter

**3.6**
**electrokinetics**
phenomena that are associated with the tangential liquid motion in respect to a charged surface

[SOURCE: ISO 26824:2022, 3.17.16]

**3.7**
**electrophoresis**
movement of charged colloidal particles or polyelectrolytes, immersed in a liquid, under the influence of an external electric field

[SOURCE: ISO 13099-1:2012, 2.2.4]

**3.8**
**electroosmosis**
motion of liquid through or past a charged surface, e.g. an immobilized set of particles, a porous plug, a capillary or a membrane, in response to an applied electric field, which is the result of the force exerted by the applied field on the countercharge ions in the liquid

[SOURCE: ISO 13099-1:2012, 2.2.1]

**3.9**
**electrophoretic mobility**
electrophoretic velocity per unit electric field strength

Note 1 to entry: Electrophoretic mobility is expressed in metres squared per volt second.

[SOURCE: ISO 13099-3:2014, 3.2.5, modified — the symbol "$\mu$" and the former Note 1 to entry have been deleted.]

**3.10**
**zeta potential**
difference in electric potential between that at the slipping plane and that of the bulk liquid

Note 1 to entry: Slipping plane is the abstract plane in the vicinity of the liquid/solid interface where liquid starts to slide relative to the surface under influence of a shear stress.

Note 2 to entry: The zeta potential is expressed in volts.

[SOURCE: ISO 13099-1:2012, 2.1.8]

## 4 Symbols

For the purpose of this document the following symbols apply.

| | |
|---|---|
| $A_i$ | pulse height of particle $i$ |
| $C$ | particle number concentration |
| $C_5$ | particle concentration at which coincidence probability is 5 % |
| $D$ | aperture diameter |
| $d$ | particle diameter |
| $E$ | electric field |
| $f_{CM}$ | Clausius-Mossotti factor |
| $F_{dep}$ | dielectrophoretic force |

| | |
|---|---|
| $f_p$ | pulse frequency |
| $K_C$ | calibration constant of concentration |
| $K_d$ | calibration constant of diameter |
| $L$ | aperture length |
| $N$ | true count of particles |
| $n$ | observed count of particles |
| $P$ | pressure |
| $S$ | applied stretch |
| $U$ | voltage |
| $V_m$ | analysis volume |
| $V_{sens}$ | sensing volume |
| $\epsilon_0\epsilon_{fl}$ | absolute permittivity of the fluid |

## 5 Principles

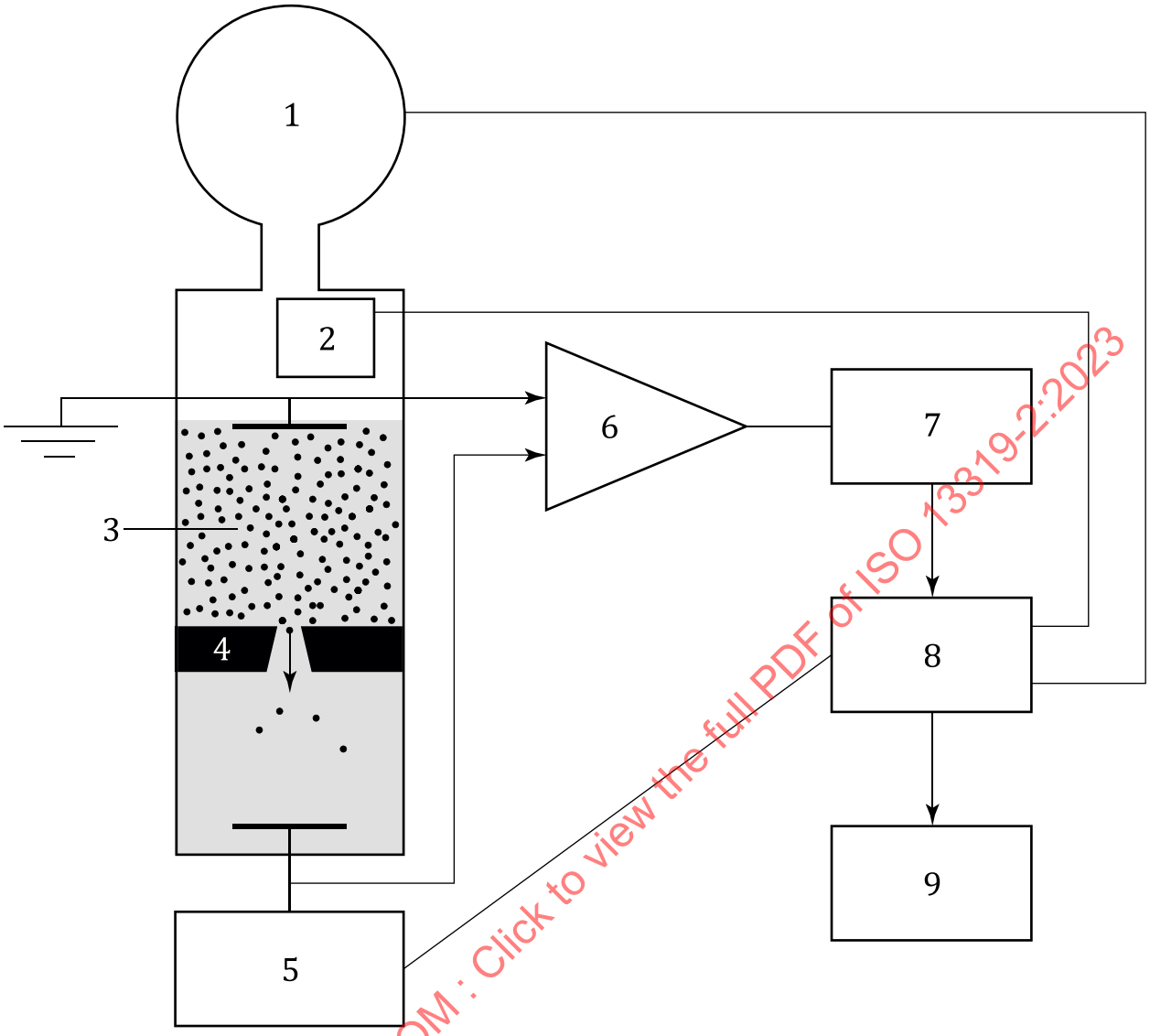
TRPS is an electrical sensing zone technique that can be used for characterization of the particle size distribution, concentration and zeta potential of synthetic (e.g. metallic, polymeric or ceramic particles), biological particles [e.g. nano-pharmaceuticals or extracellular vesicles (EVs)] and naturally occurring organic and inorganic nano- and microparticles suspended in liquids. A dilute suspension of particles in an electrolyte passes through an aperture in a membrane. There is an Ag/AgCl electrode on both sides of the membrane, between which an electric potential is applied, which causes a stable ionic current passing through the aperture. When a particle translocates the aperture, it causes a resistive pulse due to the replacement of conductive electrolyte solution by a non-conductive solid particle[1]. The height, width and frequency of these pulses provide all the information required to determine particle size, concentration and zeta potential[2]. Particle passage through the aperture is caused by:

— a pressure difference across the aperture for particle size determination and concentration;

— a voltage difference between the two electrodes across the aperture for zeta potential measurement;

— both a voltage and a small pressure difference between the two electrodes across the aperture for simultaneous measurement of particle size and zeta potential.

More background and a schematic of the instrumentation is given in ISO 13319-1 and Figure 1. Pressure can be monitored directly via a pressure sensor as shown in Figure 1 or indirectly via a flow rate meter.






**Key**

1 pressure module
2 pressure sensor
3 nanoparticle/microparticle suspension
4 aperture
5 voltage source
6 amplifier
7 analogue to digital converter
8 computer
9 output device

**Figure 1 — Schematic representation of TRPS instrument**

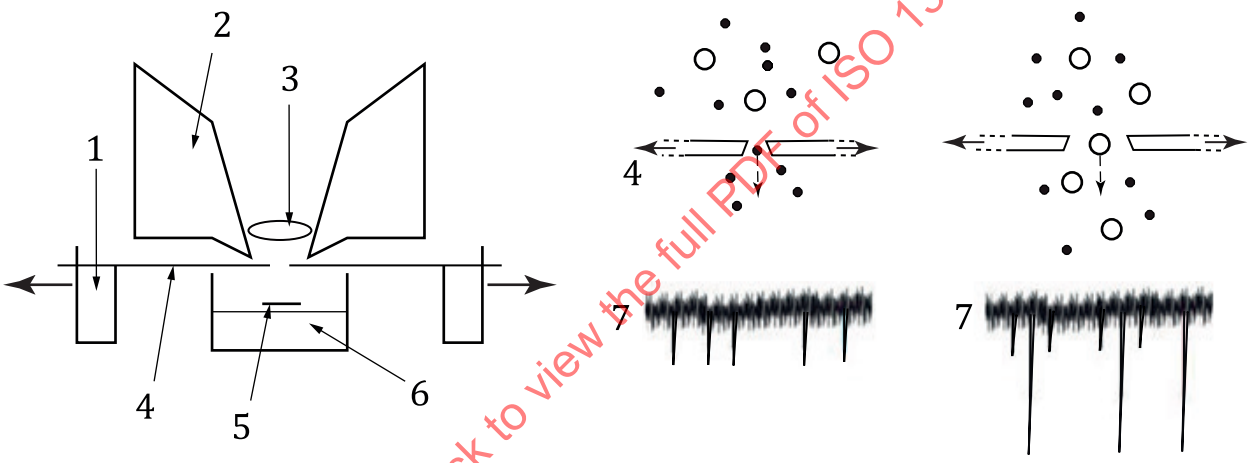
There are three main differences between conventional electrical sensing zone and TRPS equipment. Firstly, calibration standards are typically used to calibrate the aperture and provide traceable and accurate TRPS measurements. However, measurements can also be done without the use of calibration standards, in particular when fixed aperture geometries are applied.

The second difference is that pressure (pressure module) and voltage (voltage source) are tunable to allow for full control of convective and electrokinetic velocity contributions of single particles translocating the aperture, a prerequisite for measuring particle size, concentration and zeta potential.

The third difference is the use of both fixed and tunable apertures for TRPS application. While there are several chip and aperture providers using 3D printed microfluidic and glass-based fixed geometry apertures that can be used, there are also tunable apertures, for example, made in an elastic thermoplastic polyurethane (TPU) membrane. Despite having several aperture options, the focus is on tunable TPU aperture based TRPS operation.

TPU apertures are formed by generating a micron-sized hole into an elastic TPU membrane, which can be stretched mechanically to the desired size for measurement. Thus, the aperture can be tuned to the optimum size for the particles at hand. A schematic of the setup is given in Figure 2. For very polydisperse samples, a range of apertures can be required for the full measurement of the sample size distribution and concentration (see example in Figure C.1).

NOTE In Figure 2, jaws are used for clamping and stretching/relaxing the membrane.




**a) TRPS with a tunable TPU aperture** **b) Detail of a TPU aperture**

**Key**

1 stretching device
2 top fluid cell
3 ground electrode
4 tunable aperture
5 signal electrode
6 bottom fluid cell
7 current

**Figure 2 — Schematic representation of TRPS with a tunable TPU aperture**



## 6 General operation

### 6.1 Determination of particle size

As in the conventional electrical sensing zone technique, a pressure drop over the aperture causes the suspension to flow through it and the pulse height resulting from particle passage is regarded directly proportional to particle volume (see ISO 13319-1). TRPS is a particle by particle, as opposed to an averaging ensemble measurement technique, with each pulse corresponding to a single particle. Calibration, if required, is executed through the application of certified reference materials with

traceable certified values[3]. The calibration factor relates the height of the measured pulses to volume-equivalent diameters, as shown in Formula (1).

$$d_i = \mathrm{K}_d \sqrt[3]{A_i} \tag{1}$$

where

$d_i$ is the size of particle $i$ (volume-equivalent diameter);

$\mathrm{K}_d$ is the calibration constant;

$A_i$ is the pulse height of particle $i$.

## 6.2 Determination of particle concentration

At sufficient pressure drop over the aperture, convection is the dominant transport mechanism. Then, the stochastic pulse frequency ($f_p$), which is equal to the particle count rate at 0 coincidence probability, is proportional to the product of the particle concentration ($C$) and the fluid flow rate ($Q$)[4]. Since the fluid flow rate is proportional to the applied pressure drop ($P$), the particle concentration can be calculated as the slope of the linear relationship of pulse frequency versus applied pressure, with $\mathrm{K}_C$ being a calibration constant [see Formula (2)]. Formula (2) also applies to scenarios where convection is not the dominant transport mechanism, with the slope (equal to $\frac{\Delta f_p}{\Delta P}$) of the linear $f_p$ versus $P$ relationship determining the particle concentration.

$$C = \mathrm{K}_C \left( \frac{\Delta f_p}{\Delta P} \right) \tag{2}$$

The use of a calibration standard of known concentration allows the determination of the concentration of a sample at a single pressure, if convection is the dominant transport mechanism. This is typically the case for larger particles and larger apertures. Particle concentration and size can be determined simultaneously.

However, concentration measurements over a defined particle size range are typically obtained with a multi-pressure calibration procedure, where particle rates for sample and calibration are analysed at two or more pressures[5],[6]. Particle concentrations are calculated from the slope of the linear particle rate versus pressure dependence, while particle sizes are determined individually from the respective resistive pulse heights, which are linearly related to particle volumes [see Formula (1)]. This includes information about the size distribution, i.e. the number concentration of each size population within a sample, given by the number of particles per ml and per nm (bin-size). An example of a typical TRPS concentration measurement is shown in Figure C.1.

A summary of recommended setting for TRPS size distribution and concentration measurements using TRPS instrumentation with a tunable TPU aperture is shown in Table 1, and reference guidance to aperture selection can be found in Table 2.

**Table 1 — Typical settings for various TRPS measurements using TRPS instrumentation and TPU apertures**

| Parameter | | | | |
|---|---|---|---|---|
| **Applied voltage** | **Applied pressure** | **Sample size range** | **Sample concentration** | **Reference** |
| ≤5 V | ≤2,5 kPa | 40 nm to 100 µm | Size dependent | [3] and [7] |

## 6.3 Calibration

TRPS instruments are preferably calibrated with monodisperse polystyrene particles, whose certified mean diameter is traceable to the International System of Units (SI), however that does not exclude

the use of quality control materials such as silica particles or liposomes, depending on the application at hand. For particle TRPS concentration measurements, using elastic TPU apertures, the knowledge of the aperture characteristics is generally unavailable. It is therefore required to use a calibration standard with known particle size and number concentration to obtain the concentration information of the analytes. However, it is possible to calculate particle concentration by predicting the size and geometry of the aperture through the measured background current at a given voltage. Nevertheless, calibration increases measurement reproducibility and traceability and hence it is predominantly used[6]. For TRPS concentration measurements, calibration and sample are typically measured in alternation in order to virtually eliminate the impact of any change in aperture geometry occurring during the measurement process.

The concentration of the standards is determined gravimetrically (mass fraction of solids) with the knowledge of the mean particle diameter and particle density. Concentration standards are typically bare polystyrene particles, whose certified mean diameter is traceable to the international system of units (SI) or alternatively carboxylated polystyrene standards. Ideally, the standards should have concentration values that are traceable to SI, but unfortunately such standards are not available yet. The linearity of the counting system can be tested by obtaining three repeat measurements of the total counts (across all channels) at an arbitrary concentration. The concentration is then reduced and three further repeat total counts obtained (see ISO 13319-1).

Table 2 gives reference guidance to TPU aperture selection, target calibrant and sample particle concentrations. The size range shown can be detected across the standard stretch of 3 mm to 7 mm under optimal setting conditions. Note that target concentrations lie well below the particle concentration $C_5$, at which the coincidence probability is 5 % (see Table 3) for respective apertures.

**Table 2 — Guidance on TPU aperture size selection, calibrant and particle concentration**

| Average tunable TPU aperture diameter<br>nm | Range of measured particle diameters<br>nm | Range of possible polystyrene standard particle diameters<br>nm | Target particle concentration<br>$ml^{-1}$ |
|---|---|---|---|
| 300 | 40 to 225 | 70 to 100 | $1 \times 10^{10}$ |
| 400 | 50 to 330 | 100 to 200 | $1 \times 10^{10}$ |
| 800 | 85 to 500 | 200 to 400 | $2 \times 10^{9}$ |
| 1 500 | 185 to 1 100 | 400 to 800 | $5 \times 10^{8}$ |
| 4 000 | 490 to 2 900 | 1 000 to 2 000 | $5 \times 10^{7}$ |
| 15 000 | 2 000 to 11 300 | 4 000 | $5 \times 10^{5}$ |

## 6.4 Dynamic range

The dynamic range is the ratio between the largest and smallest diameter of spherical particles that can be detected. The detection range at its lower end is determined by the electrical noise of the system, that originates from thermal motion of charge carriers, dielectric noise due to the energy dissipated by the dielectric aperture substrate, amplifier noise and flicker noise[8]. Hence, it depends on many factors, including the aperture material, aperture thickness, the choice of electronic components, temperature, etc. In practice, the lower limit of the detection range is defined as the diameter of a spherical particle that causes a relative current change of 0,05 %.

The upper limit of the detection range has been defined as half of the smallest constriction of an aperture, which has been experimentally proven to be an appropriate value, with an aperture blocking kept at a low level. The dynamic range for a typical conical TPU aperture is ranging from 4 (for a fixed stretch) to 15, with the upper limit achievable over the full aperture stretch range.

## 6.5 Coincidence events

Coincidence events occur, when two or more particles translocate the sensing zone at the same or slightly different time. Consequences and calculation of coincidence events have been dealt with in ISO 13319-1. In order to keep such events at a minimum, particle concentration needs to be kept under

a certain level. Table 3 shows estimated particles per ml for the coincidence probability to be 5 %. The probabilities that a given number of particles are found within sensing zone of the aperture follows the Poisson distribution. Formula (3) is derived from the Poisson distribution[9]:

$$n = \frac{V_m}{V_{sens}}\left(1 - e^{-\frac{V_{sens}}{V_m}N}\right) \quad (3)$$

where

$n$ is the observed count of particles;

$N$ is the true count of particles;

$V_m$ is the analysis volume;

$V_{sen}$ is the sensing volume.

**Table 3 — Particle number concentrations with 5 % coincidence probability and analysis volumes for typical TPU aperture diameters — Small aperture opening of conical apertures**

| Aperture diameter<br>$D$<br>nm | Particle concentration with 5 % coincidence[a]<br>$C_5$<br>$ml^{-1}$ | Analysis volume[b]<br>$V_m$<br>ml |
|---|---|---|
| 4 000 | $3{,}7 \times 10^8$ | $1 \times 10^{-4}$ |
| 1 500 | $6 \times 10^9$ | $1 \times 10^{-5}$ |
| 400 | $4 \times 10^{11}$ | $5 \times 10^{-7}$ |

[a] Calculated from 90 % (of total aperture resistance) sensing zone of a conical aperture with a cone angle of 10°.

[b] Analysis volume equivalent to 5 000 counts.

## 6.6 Off-axis particle transport

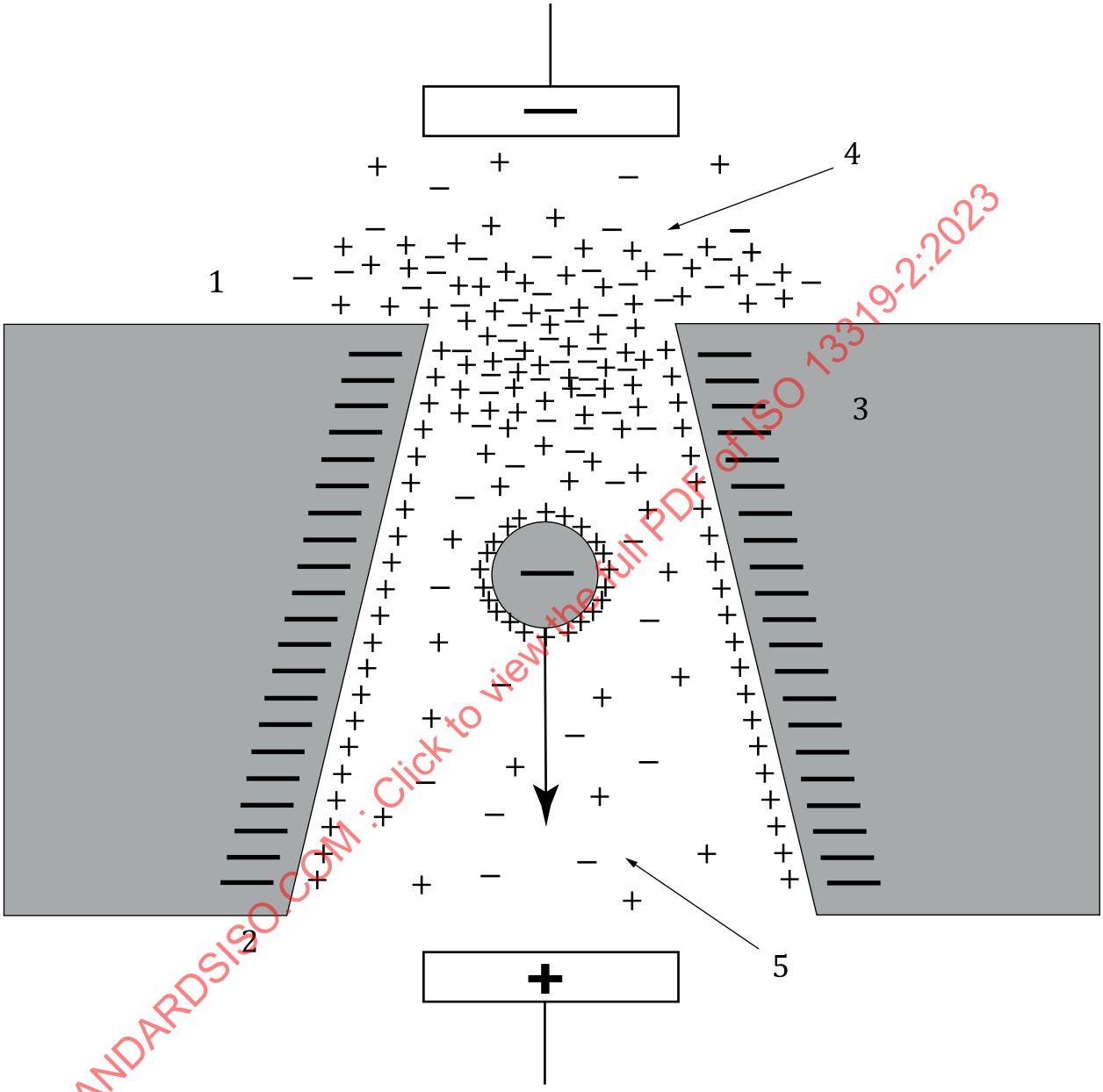
As detailed earlier, the sizing method can be summarized, using the simple relation that the pulse height is proportional to particle volume, while applying calibration particles of known diameter. Size histograms are constructed under the assumption that each pulse height accurately reflects the size of a single particle according to this relationship. However, the size distribution width is not solely determined by particle size dispersity. It also depends on different trajectories particles can take through the aperture, with resistive pulses increasing in size with the distance of the particle from the central axis of cylindrical apertures[10]-[13]. Distribution broadening and associated size shifts can be reduced significantly by working with relatively (compared to the particle sizes) small apertures and using a calibration-based methodology.

## 6.7 Polarization

In TRPS, a transient pulse in ionic current is observed, when an individual colloid particle passes through an aperture which separates two fluid reservoirs. The pulse is typically resistive, but under certain experimental conditions conductive pulses as well as biphasic pulses, with both resistive and conductive components are observed[14]. Factors, that impact on the type and height of the pulse are the ionic strength and type of the electrolyte, aperture size and shape asymmetry, aperture surface charge, applied voltage and particle surface charge.

The ionic distribution in a conical TPU aperture is affected by concentration polarization, that also causes ion current rectification of asymmetric nanopores[15]. When a positive voltage is applied across a negatively charged aperture, positive ions move freely from one fluid cell to the other, while negative ions are electrostatically repelled by the membrane. This results in a net increase in ion concentration near the cathode and a net decrease in ion concentration near the anode. Figure 3 shows the schematic cross-section through a membrane, showing a conical aperture with a pressure gradient ($P_2 - P_1$)

applied across and one particle translocating it. Both the particle and the aperture are negatively charged. Ionic concentration polarization is qualitatively indicated by the distribution of "+" and "–" signs. The polarization leads to a zone that is enriched with ions and another one that is depleted of ions on opposite sides of the conical aperture.






**Key**

1 pressure at small pore entrance
2 pressure at large pore entrance
3 aperture
4 ion enrichment
5 ion deficiency

**Figure 3 — Concentration polarization**

Concentration polarization as shown in Figure 3 can lead to underestimation of measured particle size, with the size shift increasing with increasing particle surface charge, increasing particle size for a given aperture size, decreasing applied pressure, increasing voltage, and decreasing aperture stretch and aperture size. The increase of pressure will disrupt the polarization profile of the ions and hence reduce the size shift effect, while increased voltage will increase the electric field, increase the polarization and

hence increase the size shift effect. Through the application of pressure and using a calibration-based methodology, this polarization dependent size shift effect can be minimised and rendered negligible.

In TRPS, using conical TPU apertures, biphasic pulses are not observed at electrolyte concentration of phosphate buffered saline above approximately 50 mM[14]. Biphasic pulses are consistent with concentration polarization, where the conductive component is generated as the particle passes through the ion depletion region. In case of biphasic pulses, the resistive component is used to calculate the diameter of the particle, applying the conventional calibration-based methodology.

## 6.8 Dielectrophoresis

Dielectrophoresis is caused by the force on a dielectric particle in an electric field gradient. It has been frequently applied for particle focusing, trapping and separation in microfluidic devices[16]-[18]. In brief, when an external electric field is applied across a particle suspended in a liquid, both the particle and the surrounding fluid medium are polarized. The result are effective surface charges that are cumulated at the interface between particle and medium and, in turn, distort the original electric field. Conductivity and permittivity of the particle and medium are crucial for this charge separation[19]. The associated force on the polarized particle in an inhomogeneous electric field is the dielectrophoretic force.

Under certain conditions dielectrophoresis can play a role in TRPS, becoming comparable with forces associated with electrophoresis, electroosmosis and convection. For a conical aperture, the highest electric field gradients in both, the axial and radial directions are located at the aperture orifice and its edge respectively. For a homogeneous sphere in a medium, the analytic expression for the dielectrophoretic force is defined in Formula (4).

$$F_{\mathrm{dep}} = 2\pi\left(\frac{d}{2}\right)^3 \epsilon_0\epsilon_{\mathrm{fl}} Re[f_{\mathrm{CM}}]\nabla|E|^2 \overset{Re[f_{\mathrm{CM}}]=-0,5}{\rightarrow} F_{\mathrm{dep}} = -2\pi\left(\frac{d}{2}\right)^3 \epsilon_0\epsilon_{\mathrm{fl}} E\nabla E \tag{4}$$

where

$f_{\mathrm{CM}}$ is the Clausius-Mossotti factor that depends on the complex permittivities of the particle and surrounding fluid;

$d$ is the particle diameter;

$E$ is the electric field;

$\epsilon_0\epsilon_{\mathrm{fl}}$ is the absolute permittivity of the fluid.

Depending on the relative permittivities of the particle and the solution, $Re[f_{\mathrm{CM}}]$ can be positive or negative and determines in which direction the force is directed. For a perfectly insulating particle, it becomes −0,5[20] and the resulting force and particle motion is directed away from regions of high electric field density towards regions with low electric field density.

The radial component of the force pushes the particle closer to the middle of the aperture, which leads to a focusing effect in conical apertures, with the effect being proportional to the square of the applied voltage. The component parallel to the aperture axis of the dielectrophoretic force will slow down the particles on their way into the conical aperture, entering from the small end of the aperture. It will speed up particles once they have fully entered the aperture and passed the point of maximum resistance.

Due to the quadratic relationship of the force with the applied voltage, the dielectrophoresis becomes more important with increasing voltage, when compared with electrophoresis and electroosmosis, which have a linear relationship with the applied voltage. The quadratic relationship comes about since a linear increase in voltage leads to a linear increase in both the electric field and the electric field gradient. Also, the smaller the aperture, the higher the field gradient becomes at the same applied voltage. Finite element modelling has shown that for a conical TPU aperture with a small opening of approximately 400 nm at an applied voltage of 1 V the velocity along the aperture axis due

to dielectrophoresis becomes comparable with the other transport mechanisms (electrokinetic and convection).

### 6.9 Drag

The magnitude of the drag force in a confined space, such as nano- or micro-apertures is vastly different from the drag force in free space. The drag force increases significantly with increasing particle diameter relative to the aperture diameter. The magnitude of the drag force impacts on the measured particle velocity and hence needs to be considered for TRPS-based electrophoretic mobility and zeta potential measurements.

In case of 'fuzzy particles', drag can affect the velocity of particles translocating the aperture and slow them down. Such 'fuzzy particles' can be particles with biomolecules, such as DNA being attached to the particle surface. Pertinent examples are discussed in References [21] and [22].

## 7 Operational procedure

### 7.1 General

Clause 7 predominantly focuses on the instrumental components of TRPS instrumentation. Method development considerations are detailed in a fishbone diagram in Annex D.

### 7.2 Instrumental components

The full TRPS system consists of the actual tunable resistive pulse sensing device, a variable pressure module and TRPS software to control and analyse the measurements.

At the heart of the sensing device is the aperture, which can be fixed or tunable. TPU apertures are typically fabricated by automated puncturing the membrane with a chemically etched tungsten needle. The size and shape of the needle determines the size and shape of the fabricated aperture, with conical apertures being produced in the approximately 200 µm to 300 µm thick circular septum located in the centre of a membrane [see Figure 2 b)]. Tunable apertures are clamped on the instrument above the lower fluidic cell and then symmetrically stretched [see Figure 2 a)]. Both fluid cells are filled with electrolyte [see Figure 2 a)] and measurements are ready to commence, controlled by TRPS software.

Alternatively, two tunable TPU membranes glass apertures of controlled size can be used in TRPS applications. Glass-based membranes with fixed geometry are commercially available and have been proven a viable option[23]. 3D printed microfluidic apertures are another option for TRPS applications[24]-[25]. These apertures are part of another commercial approach, where a series of resistive pulse sensors are combined within a microfluidic channel, with each sensor being tuned to a specific size range. Despite having several aperture options, the focus is on tunable TPU aperture based TRPS operation.

Pressure control is achieved with a variable pressure module. Applied pressure typically can be altered between –2,5 kPa and +2,5 kPa. In a vertical fluid cell set-up, the hydrostatic pressure between top and bottom fluid cell due to gravity needs to be considered in the total applied pressure. The pressure module allows users to apply pressure to tune convection forces in comparison with electrokinetic forces to control particle motion. The pressure module is also used to optimize pulse frequency during analysis, with recommended particle rates typically between 100 pulses per minute and 3 000 pulses per minute.

### 7.3 System set-up and optimization

#### 7.3.1 General

The system set-up and optimization procedures include the following important steps:

a) preparing fluid cell and stretching the TPU aperture,

b) wetting the aperture,

c) establishing stable baseline current and estimating the TPU aperture size,

d) coating the aperture,

e) optimising measurement parameters and running calibration,

f) adjusting conditions for the sample and recording data,

g) re-calibrating, to ensure system stability.

The TRPS set up procedure is well documented. Some studies[7],[26],[27] elaborate on system set-up and contain some useful tips. Users can also find some trouble-shooting methods available in Annex B.

### 7.3.2 Preparing fluid cell and stretching the TPU aperture

Before the tunable aperture is fitted, the porous Ag/AgCl electrode of the lower fluid cell is wetted with the buffer in use, in order to remove bubbles and prevent them from gathering under the aperture during the measurement process. Then, the tunable aperture is mounted by a symmetric clamping mechanism on the instrument above the lower fluidic cell and stretched. It has been demonstrated that stretching the elastomeric apertures can increase the aperture size by more than 50 %[28]. However, overstretching a membrane can cause rupture of apertures that can adversely affect the TRPS measurements. Hence, recommended stretching limits are typically between 2 mm to 8 mm.

### 7.3.3 Wetting the TPU aperture

Fully wetting of the aperture, without any trapped air bubbles, is essential to guarantee accurate and repeatable TRPS measurements. A full protocol of the recommended wetting procedure is detailed in Annex A. Addition of surfactant to the buffer and membrane agitation will facilitate the wetting process.

### 7.3.4 Establishing stable baseline current

Once the aperture is fully open/wetted and a voltage is applied, the baseline current should be stable. If a stable baseline current cannot be achieved, refer to Annex B.

### 7.3.5 Coating the TPU aperture

When working with biological samples, it is strongly recommended to coat the apertures (using a TRPS reagent kit, specifically developed for TPU based membranes), in order to prevent the non-specific binding (NSB) of biomolecules and particles to the aperture wall that can interfere with sample measurements. In TRPS, NSB can interfere with measurements in two ways. Firstly, contaminating molecules can bind to the aperture, altering the membrane properties. Secondly, samples such as EVs themselves can bind to the membrane, causing it to block and preventing further measurements. It has been shown, that aperture coating, as provided in the reagent kit, significantly reduces NSB[29]. If left uncoated, the performance of the apertures can rapidly decline, as the aperture can become partially blocked and modified. A full protocol of the recommended coating procedure is detailed in Annex A.

### 7.3.6 Optimising measurement parameters and running calibration

Table 2 gives guidance on aperture selection, target calibrant and sample particle concentrations. The size range shown can be detected across the standard stretch range under optimal setting conditions. Please note that target concentrations lie well below $C_5$ (see Table 3) for respective apertures.

All calibration particles shall be diluted from concentrated stocks in measurement electrolyte immediately before use. The use of calibrated pipettes for preparing dilutions and the gentle mixing of samples to avoid introduction of bubbles that can interfere with TRPS measurements are essential for accurate, reproducible TRPS measurements. Each aperture size has an associated target particle concentration. Table 2 summarizes typical particle size ranges and particle concentrations for various

aperture sizes. Membrane stretch, voltage and pressure are varied to optimize measurement conditions. Typical settings for a calibration measurement are shown in Table 4.

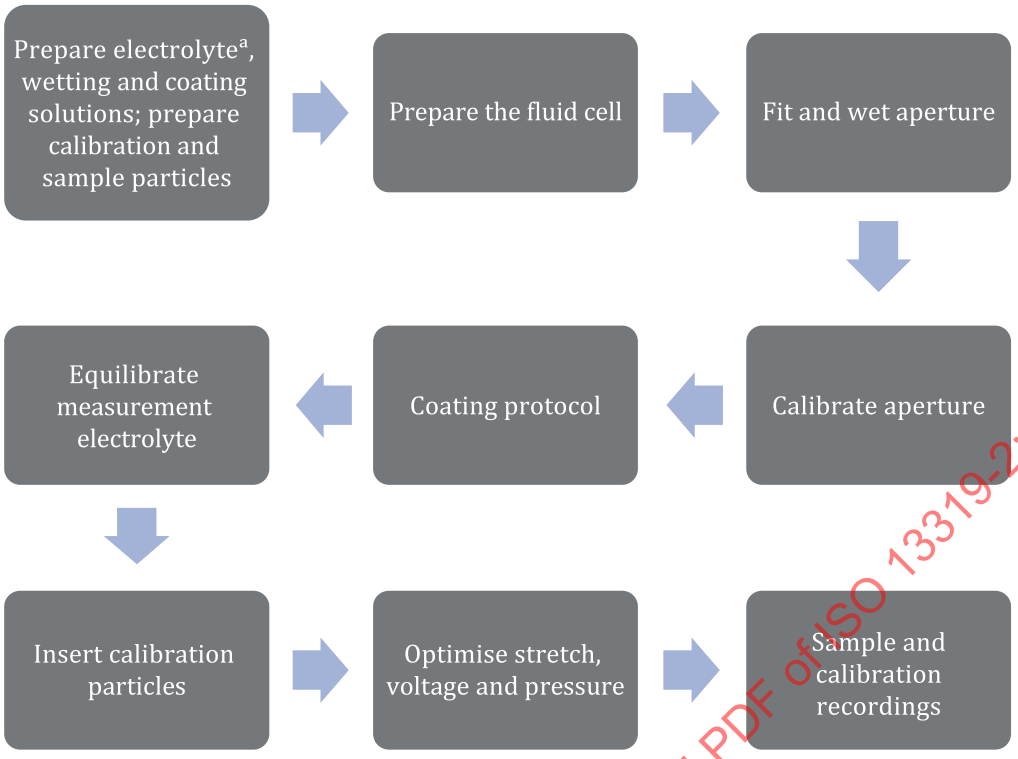
**Table 4 — Optimization of measurement parameters for calibration**

| Parameter | | | |
|---|---|---|---|
| **Average pulse magnitude**<br>nA | **Recommended applied stretch range**<br>*S*<br>mm | **Applied voltage**<br>*U*<br>V | **Recommended applied pressure range**<br>*P*<br>Pa |
| Ideally: 0,2 nA to 1 nA<br>(acceptable range: 0,15 nA to 3 nA) | For 300 nm < *D* < 600 nm: 3 mm to 7 mm<br>For *D* > 600 nm: 3 mm to 8 mm | Ideally: 0,2 V to 1 V<br>Adjust electrolyte molarity if necessary (dilute phosphate buffered saline for large apertures). | 0,1 kPa to 2,5 kPa<br>Concentrate samples or adjust dilution if necessary. |

### 7.3.7 Adjusting conditions for the sample and recording data

Once the system is stable, the data are recorded. Since the system was optimized using calibration particles, it is faster and more efficient to first measure the calibration and then the sample. A calibrant is selected at a membrane stretch that can resolve all or most of the sample's size distribution. When changing over between calibration and sample or between samples, the upper fluid cell needs to be cleaned, to avoid crossover contamination. To guarantee useful and high-resolution measurements, an appropriate aperture size shall be selected, that will properly cover the sample size distribution. The appropriate calibration standard shall be chosen with its size being comparable with the average sample size.

A recording is not meaningful until it has been calibrated with a calibrant that has been recorded under the same conditions (stretch, pressure, voltage and baseline current) as the sample. Measurements of particle size are typically taken at one pressure only. For simultaneous size and concentration analysis, both the sample and calibrant measurements are typically taken at more than one pressure. The same applies for simultaneous size and zeta potential measurements.




**Key**

[a] Electrolyte is typically phosphate buffered saline.

**Figure 4 — Flow chart of typical TRPS preparation and measurement procedures**

Correct sample preparation will provide faster and more accurate results. Each sample can require slightly different preparation, which is discussed in 7.4. Figure 4 shows typical TRPS preparation and measurement procedures.

### 7.3.8 Re-calibrating to ensure system stability

When running more samples at more than one pressure, a complete measurement set can be quite lengthy in time. Apertures can change their size through processes like stretching over time or biomolecule adsorption onto the aperture. Hence, it is important to re-calibrate the aperture. If the baseline current increases significantly, this can indicate an irreversible change in aperture size, and the aperture shall be re-calibrated. If particle rates drop or the noise increases past the acceptable range, this can indicate partial aperture blockage. Troubleshooting, including alleviation of partial blockage, is further discussed in Annex B.

## 7.4 Sample preparation

### 7.4.1 General

Points that need to be considered when preparing samples are:

— ensuring the suspension conditions are suitable for TRPS analysis,

— excluding contaminants from the suspension,

— maintaining the structural and functional integrity of target sample particles,

— enhancing suspension stability,

— careful sample preparation.

Table 5 gives basic sample preparation guidance.

**Table 5 — Basic sample preparation guidance**

| | Contaminants excluded | Steps taken to clean samples or reduce this type of contamination |
|---|---|---|
| Reagents and clean calibration samples (deionized water, electrolyte, wetting and coating solutions) | Dust, pollen, etc. | Use clean glassware rinsed several times with deionised water, and new, dust-free tubes and filters. |
| | Microorganisms, and their secretions and products | Make up electrolyte from powder weekly, filter-sterilize daily and store solutions at 2 °C to 8 °C. |
| | Trace chemicals | Use reagent-grade buffers and electrolytes, fresh deionised water and clean glassware. |
| | Precipitates and salt crystals | Filter all solutions that will come into contact with the aperture daily with a 0,22 µm filter. |
| Biological samples (e.g. plasma) | Large particles (cells, debris, etc.) | Centrifuge samples at 2 000 g for 10 min to remove cells, then spin supernatant at 10 000 g for 10 min to remove debris and apoptotic bodies. |
| | Small particles and solutes (destructive enzymes, nutrients, etc.) | Remove proteins and solutes via size exclusion chromatography (SEC) 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 SEC columns in an antimicrobial agent before storage. Do not use bench phosphate buffered saline – always make up buffers from powder. |

### 7.4.2 TRPS suspension requirements

As TRPS is a conductance-based technique, all sample solutions shall have an ionic strength of at least 0,01 M. Samples dissolved in pure water can be spiked with small amounts of concentrated phosphate buffered saline to rectify this issue. However, the requirement for solutions to be conductive generally excludes samples that are dispersed in organic solvents.

### 7.4.3 Preventing contamination

Besides preventing sample degradation, mitigating contamination of samples is required to maintain system stability during recordings. Small apertures especially, are easily blocked by large precipitates, aggregates and microbial contaminants. All reagents that are used for sample and calibration standard preparation and are measured with apertures shall be filtered with syringe filters (e.g. 0,22 µm). Contaminants in a TRPS context can include:

a) non-target particles in the same size range (e.g. for EVs these can be bacterial vesicles, intracellular vesicles or lipoproteins);

b) particulates that can block apertures (e.g. microorganisms, cells and debris, protein aggregates, dust);

c) aperture modification agents (e.g. free protein, charged coating agents);

d) destructive or disruptive agents (e.g. proteases and nucleases, cells or microorganisms left in samples and harsh detergents).

### 7.4.4 Removing proteins and solutes

Size exclusion columns may be used to isolate particulates from biofluids, for example exosomes, microvesicles, viruses, etc., to ensure quality TRPS data on biological particles of interest. When isolating EVs prior to TRPS analysis from sources, such as blood-derived samples (serum, plasma), urine, cell cultured media or cerebrospinal fluid, a large amount of free protein is removed from EV samples.

### 7.4.5 Maintaining sample integrity

Nanoparticle surface groups often behave differently in different chemical environments. Changes in ionic strength, pH, temperature, as well as the presence of strong detergents and destructive enzymes can cause significant physical and chemical changes at the particle surface. For biological particles such as viruses and extracellular vesicles, these changes can destroy surface functionalities like surface proteins and RNA. Maintaining the suspension conditions that particles were formed in is a straightforward way of preserving the functionality of all particles. It is also important to remove cells from samples, as both living and dead cells can release destructive agents into the suspension.

### 7.4.6 Enhancing suspension stability

#### 7.4.6.1 Particle agglomeration

The agglomeration of dispersed nanoparticles is a constant process, involving a complex array of forces and factors. However, there are sample preparation and storage techniques that can reduce the rate of agglomeration:

a) Ionic strength: The effective surface charge of particles decreases with increase in ionic strength and hence repulsive forces between particles in suspension are lowered, reducing the suspension stability. Hence, the lower the ionic strength of a specific electrolyte the larger the suspension stability. TRPS measurements are typically done in phosphate buffered saline (approximately 0,15 M). When measuring smaller nanoparticles (<100 nm), using concentrated phosphate buffered saline (approximately 0,25 M to 0,3 M) can significantly improve the signal-to-noise ratio, as long as suspension stability is guaranteed.

b) Temperature: The rate of agglomeration for particles <100 nm is directly proportional to the temperature-dependent rate of diffusion, and hence most particulates can be kept in the fridge. However, do not freeze calibration particles. EV samples may be snap frozen and kept at –80 °C.

c) Zeta potential: Like charges repel each other – the higher the effective particle surface charge and related zeta potential, the stronger the repulsive force between particles, and the slower the agglomeration 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 agglomeration.

d) Surfactants: Adding small amounts of surfactants like Tween 20[1)] to a suspension helps to keep particles physically separated and suspended in solution.

#### 7.4.6.2 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 size and concentration errors. For particles larger than 500 nm, it is recommended to vortex particles before each recording and to sonicate samples if necessary.

1) Tween 20 is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

# Annex A
(informative)

# TRPS best practice

The best practice has been laid down in protocols for aperture wetting, coating, equilibration and re-coating (see Table A.1).

**Table A.1 — TRPS best practice protocols**

| Step | Procedure |
|---|---|
| **Wetting protocol** | a) Load aqueous surfactant solution in the lower and upper fluid cells.<br>b) Agitate the membrane and then apply a positive pressure of up to 2,5 kPa and voltage of at least 0,1 V for several minutes.<br>c) Verify that the aperture is wetted by applying an appropriate voltage and check for a stable aperture current. If this is not the case, repeat steps a) to b). |
| **Coating protocol**[a] | a) Load filtered coating solution in the upper and lower fluid cells.<br>b) Apply maximum pressure for 10 min. |
| **Equilibration** | a) Flush the coating solution out of the upper and lower fluid cells two to three times with phosphate buffered saline solution.<br>b) Apply positive pressure of 2 kPa to 2,5 kPa for approximately 1 min. |
| **Recoating aperture** | a) The aperture coating is stable for at least 2 h. After that, the aperture needs to be recoated.<br>b) Wash out aperture with measurement electrolyte at pressure of 2 kPa to 2,5 kPa for at least 1 min after replenishing both fluid cells with measurement electrolyte.<br>c) Repeat the coating protocol.<br>d) After equilibrating with measurement electrolyte, the aperture is ready for sample measurements again. |

[a] Aperture coating is only required for biological samples, where biomolecules, such as proteins, in solution otherwise would stick to the aperture.

# Annex B
(informative)

# Troubleshooting factors that affect TRPS measurements

## B.1 General

Partial wetting, presence of bubbles, blockages and electrolyte leakage are major factors that can detrimentally affect the accuracy of TRPS measurements; this annex discusses how to deal with these factors. Please note, that this is by no means exhaustive and comprehensive troubleshooting guidance. More information can be found in Reference [7].

## B.2 Wetting

Full wetting of the aperture, without any trapped air bubbles, is essential for accurate and repeatable TRPS measurements. If the baseline current is low and unresponsive when applying voltage and stretch, it suggests that there can be air bubbles trapped within the aperture. Use of correct wetting procedures, including buffers containing surfactants, application of pressure and membrane agitation will facilitate the full wetting of the aperture. Stretching the membrane to the maximum recommended stretch, while applying pressure and membrane agitation, either done manually or automatically can facilitate complete aperture wetting.

## B.3 Bubbles

Bubbles can be introduced when pipetting samples into the fluid cells. They can also originate from the porous electrode or incomplete wetting of the aperture. Once introduced, these bubbles can build up on either side of the aperture and cause partial blockage of it, indicated by a drop in baseline current or particle rate and increased RMS noise. Appropriate pipetting techniques and pre-treatment of the electrode will alleviate the issue. Replacement of electrolyte in both fluid cells can reverse the partial blockage.

## B.4 Blockages

When dealing with very polydisperse, agglomerated particles and complex biological samples, it is not uncommon for aperture blockage to occur during TRPS measurements. In this case, it is important to identify its occurrence and unblock the aperture in a timely manner. A blocked aperture manifests itself in a nonlinear particle rate and a significant decrease in the baseline current and increase in RMS noise. To dislodge trapped particles, the sample in the fluid cell is gently mixed with a pipette. The application of pressure and/or membrane agitation can also resolve blockages. For an unblocked aperture, particle rate will linearly increase with increasing applied pressure. In case of biological samples with high protein content using small apertures, it is recommended to coat the aperture. Coating the aperture will reduce non-specific binding and minimize particles and biomolecules binding to the aperture walls.

## B.5 Electrolyte leakage

Electrolyte leakage can manifest itself in increased RMS noise and increased background current. To avoid this, it needs to be ensured that both lower and upper fluid cells are free of spillage.