



## **NCL Method PCC-20**

### **Particle Concentration and Size Using the Spectradyne nCS1**

**Nanotechnology Characterization Laboratory**  
Frederick National Laboratory for Cancer Research  
Leidos Biomedical Research, Inc.  
Frederick, MD 21702  
(301) 846-6939  
[ncl@mail.nih.gov](mailto:ncl@mail.nih.gov)  
<https://ncl.cancer.gov>



This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

Method written by:

Alison Vermilya

Jeffrey D. Clogston

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## 1. Introduction

This protocol describes the use of the Spectradyme nCS1 instrument to measure the particles per mL concentration and size of nanoparticles. The Spectradyme nCS1 is a particle analyzing instrument that uses microfluidic resistive pulse sensing rather than optical measurements to determine the concentration and size of samples. Microliters (typically 5  $\mu$ L) of a sample are placed in a microfluidic cartridge that is inserted into the instrument for analysis; thus, low sample volumes are needed for analysis and the instrument is relatively easy to use. The size and concentration of a sample is determined by measuring the changes in voltage as particles travel through a nano-constriction in the microfluidic cartridge. This method also has the advantage over optical techniques in that measurements are not dependent on the type of material being measured (e.g., refractive index of the sample itself is not needed for accurate analysis).

The lower size limit for this technique is approximately 50 nm; however, a wide range of cartridges are available through the manufacturer that allow measurement of particles of up to 2000 nm. Most samples examined at the NCL use a 300 nm cartridge, meaning that the cartridges measure from 50-300 nm. The instrument requires that the running buffer and sample dilutions contain a low concentration of surfactant. Spectradyme recommends PBS with 1% Tween as the standard running buffer and sample diluent. Note though, this may cause some samples to be incompatible with this technique and will be discussed later.

Detailed explanations of how the instrument works, use of the instrument, and data analysis are available in the instrument handbook and on the company website; the intention of this protocol is to provide guidance and detail for the types of nanoparticles typically analyzed at the NCL. Our experience with this instrument has provided us with some additional helpful techniques and insight for the samples frequently examined in the NCL.

## 2. Measurement Principles

Full details on the theoretical basis of the Spectradyme nCS1 instrument can be found in the instrument handbook and on the company website.

In brief, this instrument relies on resistive pulse sensing (RPS), which is also the basis of Coulter counting instruments. In the device, there is a nano-constriction across which a voltage is applied. The sample reservoir is on one side of this constriction, and running buffer (typically PBS + 1% Tween) flows on the opposite side of the constriction. As particles move through this

constriction, the measured voltage across the nano-constriction will be disrupted each time a particle travels through it. The magnitude of this disruption correlates to the size of the particle traveling through the nano-pore. The rate at which these voltage disruptions occur correlates to the concentration of the sample.

Spectradyne manufactures the disposable cartridges in various size ranges so that the user can choose the most applicable size cartridge for the particles being analyzed; each batch of cartridges is assigned a unique identifier which is used in the particle size calculation after data collection. External calibration is not necessary using this technique; however, particle standards can be spiked into any sample and used as an internal standard.

### **3. Reagents and Equipment**

*Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.*

#### **3.1 Reagents**

##### **3.1.1 Tween-20**

##### **3.1.2 PBS (see section 4.3.2 for preparation of running buffer)**

##### **3.1.3 DI H<sub>2</sub>O for instrument shutdown**

#### **3.2 Equipment**

##### **3.2.1 Spectradyne nCS1 instrument and software. Instrument will also need a nitrogen connection.**

##### **3.2.2 Spectradyne nCS1 sample cartridges. Various sizes are available through the company; choose the size that best suits your sample of interest.**

### **4. Experimental Procedure**

#### **4.1 The Spectradyne nCS1 manual should be referenced; below is a brief summary of instrument use and operation.**

#### **4.2 Instrument Set Up**

##### **4.2.1 The instrument's power should be turned on before the nCS1 software is loaded on the computer; turn on nitrogen gas connection and check that**

power supply, gas supply, and USB connection are in proper working order. The waste bottles should be empty and the buffer bottles should be filled with at least 50 mL each of running buffer. All five bottles should be tightly attached via both the bottle lid and the quick connects.

#### 4.3 Preparation of nCS1 Running Buffer

4.3.1 The recommended buffer is PBS + 1% Tween (polysorbate 20) filtered through a 0.2 µm filter. The running buffer does not interact with the sample until after the sample has been analyzed.

4.3.2 At the NCL, this running buffer is typically made in either 100 mL or 200 mL batches. Filter the running buffer through a 0.2 µm filter prior to use.

4.3.2.1 99 mL PBS + 1 mL Tween = 100 mL running buffer

4.3.2.2 198 mL PBS + 2 mL Tween = 200 mL running buffer

**Note:** Tween does not immediately mix or dissolve into the PBS. Use a stir bar/stir plate to achieve complete dilution of the Tween into PBS. Be careful to use a low/medium stir speed to avoid foaming of the Tween.

4.3.3 Spectradyne recommends discarding buffer after approximately two weeks. Running buffer should be stored in the refrigerator when not in use.

#### 4.4 Cleaning Cartridge

4.4.1 The provided cleaning cartridge is used to clean the instrument between sample runs and is used in the shutdown procedure.

4.4.2 At the NCL, when starting to use the instrument, we run a cleaning cartridge cycle prior to running any samples. We find that this can reduce sample cartridge prime time by making sure that fluids are flushed throughout the instrument.

#### 4.5 Sample Preparation and Sample Loading

4.5.1 Spectradyne recommends a sample concentration of approximately  $10^8$  -  $10^9$  particles/mL. For many samples run at the NCL, 100-fold dilutions are often sufficient for nCS1 analysis.

4.5.2 For standard samples that are stable in the running buffer, samples should be diluted from stock in the running buffer.

- 4.5.3 For dilute samples that are compatible with the running buffer (for example, fractions collected from AF4 separation), the samples can be spiked with Tween.
  - 4.5.3.1 For example, add 0.5  $\mu$ L Tween to a 50  $\mu$ L aliquot of sample.

Assure that the Tween is fully dispersed in the sample before running the sample.
- 4.5.4 Section 5.2 addresses samples that are incompatible with the running buffer, specifically the Tween component.
- 4.5.5 Allow the diluted sample to sit at room temperature for approximately 15 minutes so that the sample can equilibrate in the running buffer solution. During this time the instrument can be turned on, running buffer prepared, or cleaning cartridge can be run.
- 4.5.6 Add 3-5  $\mu$ L of prepared sample to the microfluidic cartridge. Ensure that there are no bubbles in the sample chamber. Gentle tapping can remove bubbles but be sure to not break the glass cartridge base. Pipetting the sample aliquot into the chamber at an angle often reduces bubbles.
- 4.6 Loading the Cartridge into the Instrument
  - 4.6.1 Load the cartridge into the front of the instrument with the glass lip edge first. When the cartridge is loaded properly, the back edge of the glass cartridge base will be flush with the edge of the cartridge holder and the indicator light on the front panel will light.
  - 4.6.2 Press the front button to close the instrument and raise the cartridge stage into position for sample analysis. The white light will light up to indicate that the sample cartridge has been loaded correctly and sample analysis can begin.
- 4.7 Software Settings for Analysis
  - 4.7.1 The software has various “run details” that should be filled out before priming the cartridge and beginning sample analysis. Most are required to be filled out before the software will permit you to begin the priming process. A screenshot is shown in Figure 1.

4.7.1.1 Mold ID – this identifies the size and mold of the cartridge being used, this can be found on the box of cartridges. **Note:** Not all cartridges of the same size (i.e. TS-300) will have the same mold ID.

4.7.1.2 Save Location – where the files will be saved on the computer

4.7.1.3 Data file prefix – name of your data files

4.7.1.4 Optional - description – can include any other information as desired

4.7.1.5 Optional – calibration size and concentration – these are used if you are using a calibrant particle spiked into your sample (At the NCL, we typically do not use a spiked internal standard during Spectradyme nCS1 measurements.)

The screenshot shows a software window titled "Run Details". It contains several input fields: "Cartridge Mold ID:" and "Box Number:" are at the top with red rectangular highlights; "Save Location:" is set to "C:\Spectradyme\Data" with a folder icon to its right; "Data File Prefix:" is a text box; "Sample Description:" is a larger text area; and at the bottom, "Calibration:" is followed by "Size (nm):" and "Conc. (1/mL):" each with its own text box.

**Figure 1.** Screenshot of “Run Details” section of Spectradyme nCS1 experiment software. These items are filled out prior to the sample run.

## 4.8 Priming the Cartridge

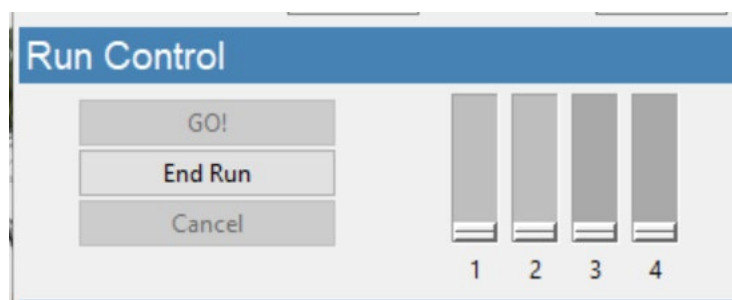
4.8.1 Figure 2 shows the instrument control options for priming and ending a sample run. Click “GO” to begin the cartridge priming process.

4.8.1.1 While the instrument manual notes that priming should only require 1-2 minutes, we have typically found that it takes much longer for the instrument to prime. Running a cleaning cartridge prior to running a sample seems to encourage faster priming but is still typically longer than just a few minutes.

4.8.1.2 It is helpful to watch the four fill monitors on the instrument panel (sliders with red/yellow/green background) to monitor the progress of the priming.

4.8.1.3 On occasion, you might just have a flawed cartridge and it might be better to use a new cartridge entirely and start over; other times, if you end the priming and restart it, it will end up priming properly.

4.8.2 The instrument will begin collecting data immediately after the priming is complete.



**Figure 2.** Screenshot of “Run Control” section of nCS1 software. The buttons on the left control the instrument priming and sample termination processes; the sliders on the right indicate priming progress.

## 4.9 Acquisition Settings

4.9.1 Figure 3 shows a screenshot of the acquisition settings of the nCS1 software. Acquisition time can be chosen in the drop-down menu and there are buttons to pick “single” or “continuous” data acquisition.

4.9.1.1 At the NCL, we typically collect 60 second runs continuously.

4.9.1.2 Each run will be numbered sequentially beginning with  
“prefix\_date\_00000.h5”

4.9.1.3 Partial acquisitions (runs stopped before completion) are NOT saved.

## 4.9.2 Voltage Balance



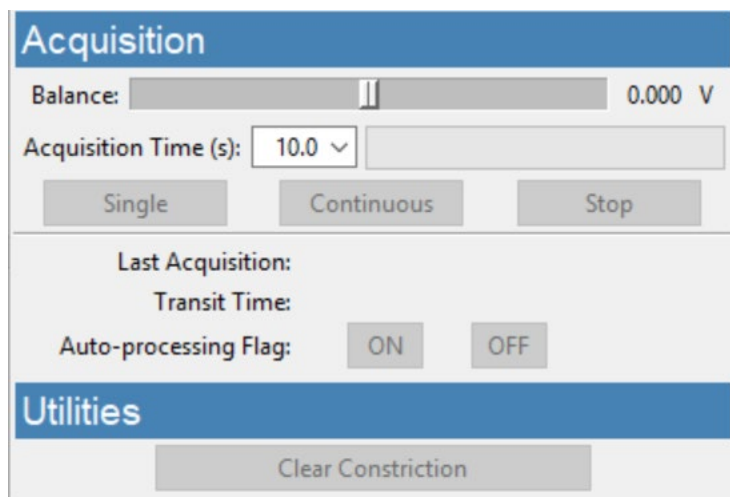
4.9.2.1 Voltage should be between  $\pm 0.8$  V and preferably between  $\pm 0.2$  V.

4.9.2.2 The voltage balance can often indicate the presence of a clog in the chamber.

#### 4.9.3 Clear Constriction Function

4.9.3.1 Should the voltage balance be out of range or sample traces appear to have multiple clogs or no sample, the clear constriction function can be used.

4.9.3.2 If running continuous runs, stop collecting runs and then click “clear constriction”. The clear constriction program will run to flush the cartridge of any clogs. Once the program is complete, allow the voltage to return to the normal range and resume collecting runs.



**Figure 3.** Screenshot of “Acquisition” and “Utilities” sections of the nCS1 experimental software.

#### 4.10 Ending a Run

4.10.1 To end a run, stop collecting data and then click “end run”. This will begin the instrument flush procedures to clean the fluid lines after running the sample and prepare the instrument for another sample run. The instrument

will indicate when it is ready to have you lower the stage and remove the cartridge.

4.10.2 Throw away the cartridge in an appropriate refuse container – remember that the cartridge has glass, so a broken glass box or sharps container may be the best place to dispose of nCS1 cartridges.

4.10.3 While a run is ending, it is a good time to start preparing your next sample; you can choose to run another sample immediately or run the cleaning cartridge process between sample cartridges.

#### 4.11 Shutdown cleaning procedure

4.11.1 The shutdown procedure requires two instrument compatible bottles of DI water and the cleaning cartridge. At the NCL, we have separate running buffer and DI water bottles to avoid rinsing the bottles during the shutdown process.

4.11.2 Click “Instrument Shutdown” to begin. Follow the prompts on the software to progress through the shutdown procedure.

4.11.3 The instrument will note when it is safe to turn the instrument off. At this time, the running buffer should be placed in the refrigerator, the waste bottles emptied, the air supply turned off, the power supply turned off, and the computer software closed.

## 5. Sample Considerations

### 5.1 Choosing a Cartridge Size

5.1.1 Cartridges come in a variety of sizes from Spectradyne. Their naming convention is that the number listed for the cartridge model (for example, TS-300) means that measurement in nanometers is the maximum diameter that the cartridge can measure. A TS-300 cartridge can measure particles up to 300 nm; it is recommended that your particle of interest have a nominal diameter approximately 20% smaller than the maximum size for the cartridge. Regardless of the maximum size, the lower size limit for the instrument is 50 nanometers.

### 5.2 Sample Incompatibility with Running Buffer

- 5.2.1 Some samples that appear to be unstable with the addition of 1% Tween – either when diluted in the running buffer or when a dilute sample is spiked with 1% Tween.
- 5.2.2 Comparing the DLS traces of samples with and without the addition of Tween can be helpful to determine if the sample will work under standard operating conditions for the nCS1. For some samples, we have slightly lowered the amount of tween (from 1% to 0.5%) and the DLS trace did not indicate disruption of the sample while dilution in a 1% Tween solution indicated significant sample disruption.

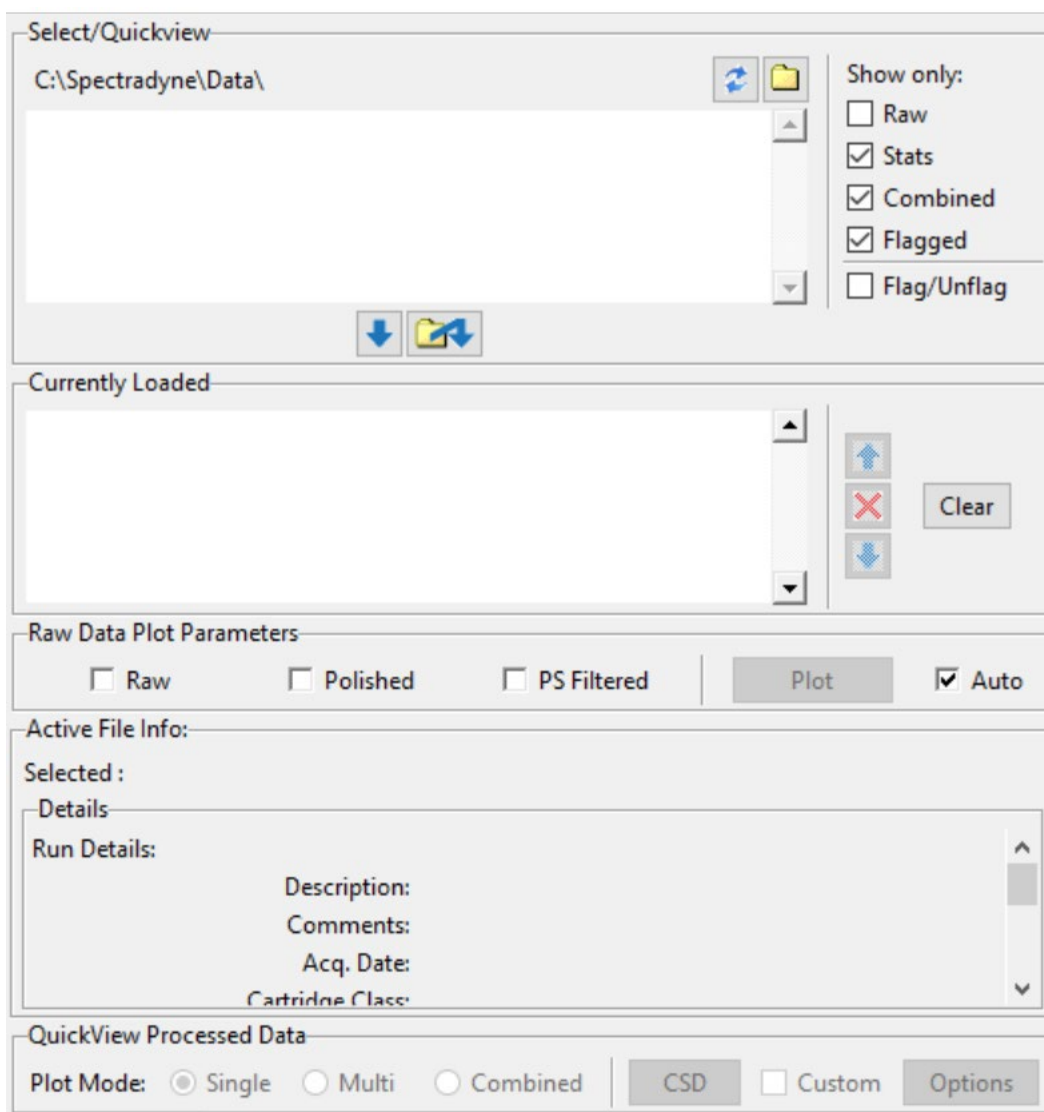
## **6. Data Analysis**

### **6.1 Spectradyne nCS1 Data Analysis Software**

- 6.1.1 A detailed explanation of all software functions will not be given here, please refer to the Spectradyne nCS1 manual and website for thorough explanations.
- 6.1.2 The Spectradyne instrument control software and data analysis software are two different programs; it is recommended that you close the instrument control software before running the data analysis software.
- 6.1.3 All data analysis functions can be completed using the data analysis software including (but not limited to) data processing, data filtering, peak fitting, size and concentration determination, and graph creation.

### **6.2 Basic Data Processing**

- 6.2.1 To begin, all collected raw data files must be processed to begin data analysis. Figure 4 contains a screenshot of the main data processing screen in the nCS1 data processing software.



**Figure 4.** Screenshot of the main data processing software screen (without files loaded for analysis)

- 6.2.1.1 Using the file explorer, select the files to be processed; files can be added one at a time or in a batch.
- 6.2.1.2 Once the files to be processed are added to the queue, the processing settings can be chosen and adjusted. Figure 5 contains a screenshot of the processing settings portion of the software.
- 6.2.1.3 Finally, click “process all” to process the raw data files into analyzed data files. This process can take a significant amount of time; it can be helpful to set up the raw data files to process overnight.

6.2.1.4 Processed data files will be saved in the same location as the raw data files being processed.

The screenshot displays the 'Process Raw Data' window of the nCS1 software. It features a tabbed interface with 'Process Raw Data', 'Data Visualization', 'Quantification', 'Peak Filtering', and 'Data Output'. The 'Process Raw Data' tab is active, showing several sections: 'Signal Processing Options' with a 'PS Filtered' checkbox and an 'Update Measurements' button; 'Measurements of Selected Raw Data' with fields for 'Baseline Noise (1-sigma):' and 'Large Peak Transit time:', both with units '(Normalized Voltage)' and '(us)'; 'Processing Parameters' with 'Peak Detection Threshold' options (Relative and Absolute), a 'Peak Fit Width (us):' field, and checkboxes for 'Auto' and 'Apply Default Peak Filters'; and 'Processing Actions' with 'Process Selected', 'Process All', and 'STOP' buttons.

**Figure 5.** Data processing options in the nCS1 data analysis software.

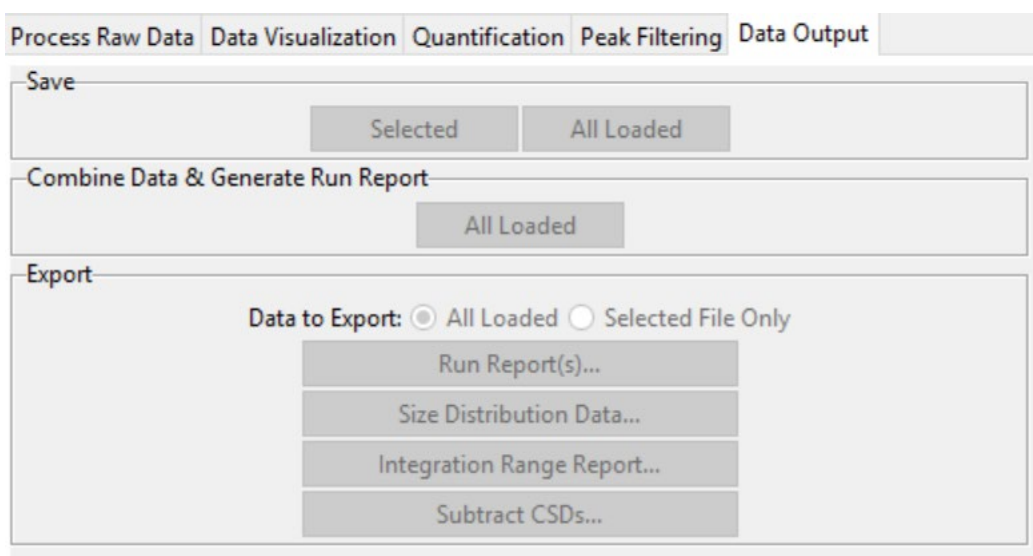
6.2.2 Once raw data is processed, data files containing the concentration spectral density (CSD) data can now be viewed, combined, and analyzed.

6.2.2.1 There are three options to view CSD data – single, multiple, or combined. The single option will only pull up the CSD file highlighted. The multiple option will pull up all loaded CSD files and overlay them. The combined option will combine all loaded CSD files into a single trace. These options can be seen in Figure 4.

6.2.2.2 It is helpful to review each CSD file to determine if there are runs without any particles detected; these files should be removed and not used in the determination of particle size nor concentration.

The multiple view is helpful in identifying if any runs are outliers or if the sample is degrading over time.

6.2.2.4 Once data is reviewed, the individual run files can be combined into a single data file. Using the software, click “combine all loaded” to create a single file from the individual CSD data files loaded on the software. This new file can be saved with the file name of your choice. The data output portion of the software is shown in Figure 6.



**Figure 6.** Screenshot of Data Output section of the nCS1 data analysis software

6.2.2.5 Using the combined final data file, use the software tools to determine particle size and concentration; this is found in the “quantification” tab, as shown in Figure 7. The simplest manner to determine size is to select the particle peak using the software and use the “Gaussian fit” function. This will fit a simple Gaussian function to the graphed data and is used to determine the average particle size and coefficient of variation of the data. The Gaussian fit will also report a particle concentration and standard deviation; this concentration corresponds to the Gaussian peak fit not the integrated concentration under the graphed data. The integrated

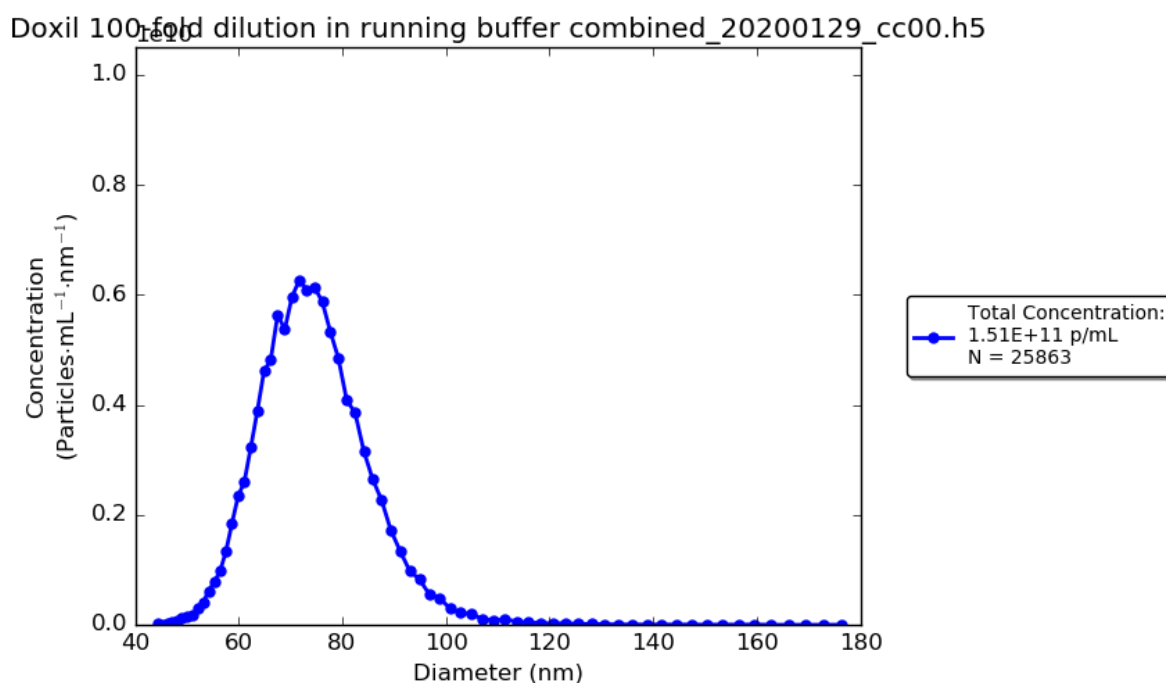
concentration is given at the right of the graph. For samples with a single particle population the integrated concentration is typically very close to the Gaussian fit concentration.

The screenshot displays the 'Quantification' tab of the nCS1 software. It features a top navigation bar with tabs: 'Process Raw Data', 'Data Visualization', 'Quantification' (active), 'Peak Filtering', and 'Data Output'. Below this, the 'CSD Measurements' section contains a 'Range Selection' area with 'Graphical' selected and 'Manual' (with 'Min' and 'Max' input fields) as an option. Below the range selection are three buttons: 'Concentration', 'Gaussian Fit', and 'D10, D50, D90'. The 'Auto Scaling' section below it has two rows: 'Diameter' with 'Rescale last fit to' and 'nm' unit, and 'Concentration' with 'Rescale last fit/measured to' and 'particles/mL/nm' unit. Each row has an 'Apply' button.

**Figure 7.** Screenshot of Quantification options in the nCS1 data analysis software.

## 7. Example

Doxil is an FDA approved liposomal formulation of doxorubicin. A sample of Doxil was diluted 100-fold in filtered running buffer and run using a TS-300 cartridge. The data was collected, processed, and analyzed as described above. According to the analyzed data, Doxil had an average diameter of 73.3 nm (SD: 13.5 nm) and a concentration of  $1.5 \times 10^{13}$  particles per mL. Note that the “total concentration” reported in the graph reflects the concentration of the measured diluted sample, not the concentration of the original sample.



**Figure 8.** Graph depicting particle size by particle concentration for Doxil.

## 8. References

Spectradyne nCS1 user manual, Revision 2.5, 2019. Available online in the Spectradyne Library:

<https://nanoparticleanalyzer.com/xYqeRgYx.php>

For more information, visit the Spectradyne website: <https://nanoparticleanalyzer.com/>

## 9. Abbreviations

CSD	concentration spectral density
DI	deionized
DLS	dynamic light scattering
PBS	phosphate buffered saline
RPS	resistive pulse sensing