Fluorescent nanoparticle flow cytometry calibrators with NIST-assigned ERF and concentration values for viral and EV analysis

Adam W. York,[1] Yu-Zhong Zhang,[1] Paul C. DeRose,[2] Linhua Tian,[2] Eric J. Welch[1] and Lili Wang[2]; [1]Thermo Fisher Scientific, Biosciences: Protein and Cell Analysis, 29851 Willow Creek Road, Eugene, OR, USA, 97402; [2]National Institute of Standards and Technology, Biomarker and Genome Analysis, 100 Bureau Drive, Gaithersburg, MD, USA, 20899

Abstract

Analysis of viruses and extracellular vesicles (EVs) by flow cytometry is a growing area of interest, which has been emphasized even more since the onset of the COVID-19 pandemic. Instrument hardware has and continues to be adapted for detection of nanoparticle-based samples. Currently, most nanoparticle detection problems by flow cytometry arise from poor instrument particle size resolution, inaccurate concentration measurements and/or inconsistent fluorescence intensity values due to improper instrument settings. To help address these detection issues, we collaborated with the National Institute of Standards and Technology (NIST) to develop the first ever fluorescence intensity and concentration nanoparticle standard kit, Invitrogen™ ViroCheck NanoParticle Reference Kit, to aid in the analysis of viruses and EVs by flow cytometry.

This first of its kind reference kit includes five fluorescent nanoparticle components: FITC-like (100nm), PE-like (100nm), APC-like (100nm) and Multicolor (200 & 500nm) nanoparticles all having NIST-assigned equivalent number of reference fluorophore (ERF) values. ERF values were determined when exciting each component by a UV, blue, yellow and/or red laser. In addition, both the 200 and 500nm components come supplied with NIST-confirmed particle concentration values. Herein, we will discuss and highlight the features and use of these new nanoparticle calibrators.

Introduction

Flow cytometry is typically employed to probe both the optical and fluorescent characteristics of cells in a high throughput manner.[1] Through the optimization of sample and instrument workflow protocols and pushing the boundaries of instrument hardware/detection the use of this powerful tool can now be extended beyond the analysis of cells and used in the direct detection and characterization of sub-micron particles (*e.g.* EVs and viruses).[2] Furthermore, as more efficient fluorescent labeling technologies and new detection equipment are developed the detection of sub 100nm viruses becomes feasible.[3] As the use of flow cytometry for investigating sub-micron biological particles expands, comparable sized calibrators are necessary to ensure proper instrument setup, normalization of the fluorescence scale and accurate day-to-day and lab-to-lab data comparisons. To address the needs of nano-sized calibrators for flow cytometry we have collaborated with NIST to develop nanoparticle standards for both fluorescence and particle concentration. Although further advances in nanoparticle calibrators will be needed and are expected by the research community, we hope that with the release of this kit we can start to lay a foundation for flow cytometry standards that can facilitate in the detection and analysis of sub-micron samples.

Materials and methods

ViroCheck NanoParticle Reference Kit Information

- Three single color 100nm nanoparticles with FITC-, PE- and APC-like fluorescence
- Two Multicolor fluorescent nanoparticles (200 & 500nm; broad ex/em)
- Concentration – 5 x10^6 particles/mL with NIST-confirmed values for 200 and 500nm components
- Volume – 500μL per component

Particle Sizing Methods

Particle sizing and distributions were measured by transmission electron microscopy (JEOL; JEM-200CX TEM) and dynamic light scattering (DLS; Malvern-Zetasizer Nano Series DLS detector with a laser operating at λ = 531 nm or 632.8 nm)

Flow Cytometry Methods

CytoFLEX™ S flow cytometer was used following manufacturer's recommended protocols. Violet (405nm) side scatter (SSC) served as a trigger for nanoparticle detection and discriminates from the noise. All buffers were 0.02μm filtered.

CytExpert™ software was used for data collection and analysis post acquisition.

Product features and highlights

ViroCheck NanoParticle Reference Kit provides nanoparticle fluorescence and concentration reference calibrators for the analysis of virus or EV samples via flow cytometry. Determining sample concentration and comparing fluorescence intensity data between instruments, laboratories and/or day-to day experiments are made possible.

- All components come with NIST-assigned ERF intensity values for 15 common filter set channels (**Table 1**)
- The 200 & 500nm Multicolor components are supplied with lot specific NIST-confirmed particle concentrations

Table 1. NIST ERF Value Channel Assignment for the ViroCheck NanoParticle Reference Kit.

Component	Excitation laser and Emission filter set				Measured concentration (beads/mL)
	UV (375 nm)	Blue (488 nm)	Yellow (561 nm)	Red (633 nm)	
100 nm FITC-like beads	–	530/30 574/26 610/20	–	–	N/A
100 nm PE-like beads	–	574/26	585/16 620/15	–	N/A
100 nm APC-like beads	–	–	–	660/20 670/14 720/30 780/60	N/A
200 nm Multicolor beads	405/30 450/45 525/40	530/30 574/26 610/20	585/16 610/20 675/30 695/40	660/20 670/14 720/30 780/60	Yes[1]
500 nm Multicolor beads	405/30 450/45 525/40	530/30 574/26 610/20	585/16 610/20 675/30 695/40	660/20 670/14 720/30 780/60	Yes[1]

[1] See component label for particle concentration.

Figure 1. Post-staining sizing statistics, representative TEM images and percent number distributions vs. hydrodynamic diameters for the 100 nm FITC-like (A; –), 200nm (B; –) and 500nm (C; –) Multicolor kit components.

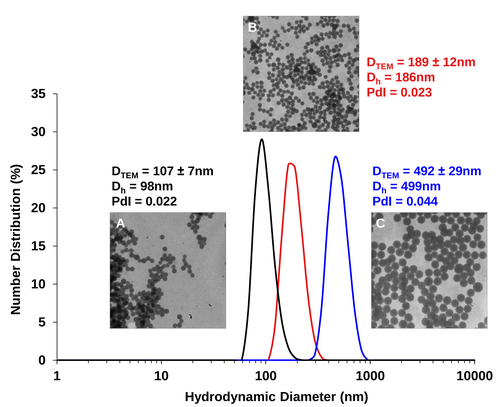


Figure 2. Flow cytometry data upon blue laser (488nm) excitation and 530/30 bandpass filter detection for 100nm FITC-like component and carboxy fluorescein-SE (CFSE) labeled extracellular vesicles (EVs). A) MFI vs. Violet SSC-H density plot and B) Count vs. MFI histogram for 100nm FITC-like component. C) CFSE labeled EVs MFI vs. Violet SSC-H contour plot. D) Example calculation for determining ERF value of the CFSE labeled EV sample.

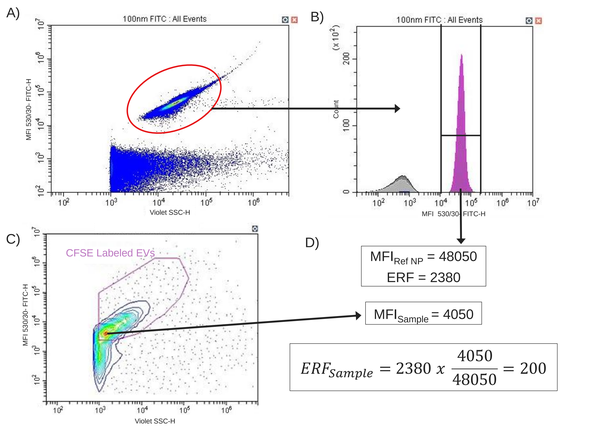
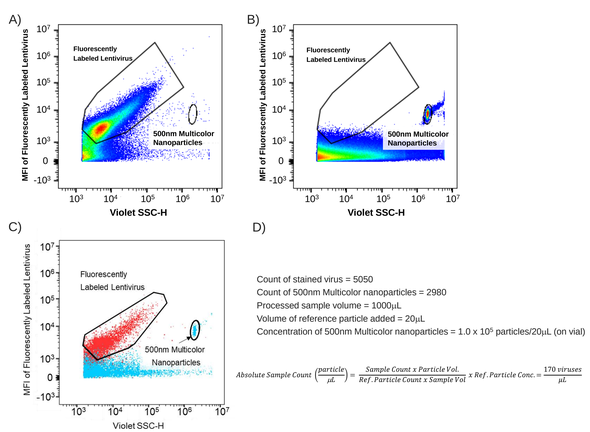


Figure 3. Example for determining number of virus particles per microliter using the ViroCheck NanoParticle Reference kit. MFI vs. Violet SSC-H density plots for A) fluorescently labeled lentivirus sample and B) the 500nm Multicolor component. C) Overlay dot plot of the fluorescently labeled lentivirus sample (red) and 500nm Multicolor component (blue). D) Example calculation for the concentration of lentivirus sample using count statistics.



Size distributions and particle concentrations of the dye-stained nanoparticles were measured by TEM, DLS, dry mass, fluorescence microscopy, particle tracking, microfluidic resistive pulse sensing, flow cytometry and virus counter. Data from TEM and DLS are shown in **Figure 1**. See Poster 145 for data on all other methods.

ERF values have been NIST-assigned to all nanoparticle components using NIST traceable fluorophores. An example of how to determine the ERF value of CFSE-stained EVs using the 100nm FITC-like kit component is presented in **Figure 2**.

In addition to ERF value assignment, the 200 and 500nm Multicolor kit components have NIST-measured and confirmed particle concentration values. This is the first nanoparticle concentration standard available that has been measured and confirmed by NIST. Knowing the concentration value of a standard can help researchers determine absolute particle concentration of their sub-micron samples. An example of determining the concentration of fluorescently labeled lentivirus using the 500nm Multicolor kit component and the count statistics is highlighted in **Figure 3**.

Conclusions

Through this collaboration we have developed a new fluorescent nanoparticle calibrator kit with NIST-assigned equivalent number of reference fluorophore (ERF) and nanoparticle concentration values for five separate nanoparticle components. To complete these assignments, several fluorescent, sizing and concentration measurement methods needed to be analyzed, developed and optimized (see Poster 145). Herein, we have highlighted the utility of the ViroCheck NanoParticle Reference Kit to aid in the analysis of virus and EV samples by flow cytometry. In summary, this kit will enable:

- Instrument setup and calibration of the fluorescence intensity scale for sub-micron samples
- Determination of sub-micron sample concentrations
- Accurate instrument-to-instrument, lab-to-lab, and day-to-day fluorescence intensity data comparisons.

References

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