Performance Qualification for MicroFlow Cytometers:

Understanding technical limitations to improve your research

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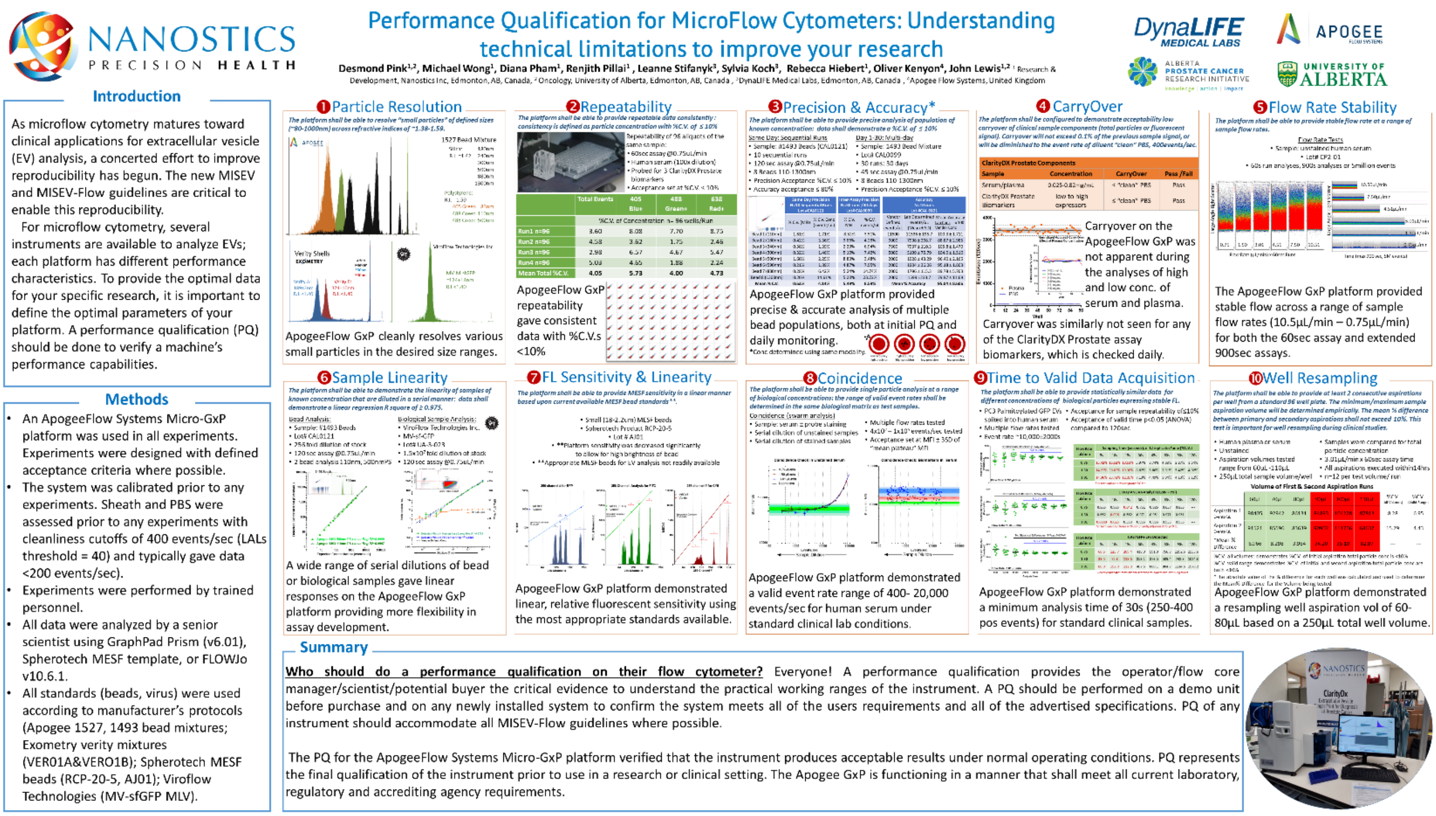
- Each section of the poster will be presented as a single page to aid the reader through the contents. This PDF will have more information compared to the stand alone poster pdf.



Introduction

- As microflow cytometry matures toward clinical applications for extracellular vesicle (EV) analysis, a concerted effort to improve reproducibility has begun.
- The new MISEV and MISEV-Flow guidelines are critical to enable this reproducibility.
- For microflow cytometry, several instruments are available to analyze EVs; each platform has different performance characteristics.
- To provide the optimal data for your specific research, it is important to define the optimal parameters of your platform.
- A performance qualification (PQ) is a collection of tests used to qualify equipment throughout the full range of the equipment capabilities as described in the machine specifications and based on user requirements.
- All key equipment used in the development of novel EV tests should be qualified using a similar approach as is required for your specific research. Acceptable performance on one instrument in one research area may not be sufficient for another.

Methods

- An ApogeeFlow Systems Micro-GxP platform was used in all experiments.
  - FCM Control v3.68; Histogram v255.0.0.279
  - 405nm (light scatter), 488nm , 638nm lasers tunable to 200mW
  - Autosampler – set for standard 96 well microplates
  - Sheath flow rate 0.75µL/min – 101µL/min
  - Event rate ≤20,000 events/sec for all experiments unless specified

- All experiments were designed with defined acceptance criteria where possible.
  - Experiments had defined pass/fail criteria based upon vender specifications and Nanostics-defined requirements
  - Where possible, statistical evaluation was used to analyze data.

- All experiments were performed on a calibrated platform.
  - The system (baseline PMT voltages) was calibrated daily using bead mixture ApoCal 1524, prior to any experiments being performed. Calibrations are based on preset specifications for optimal performance.

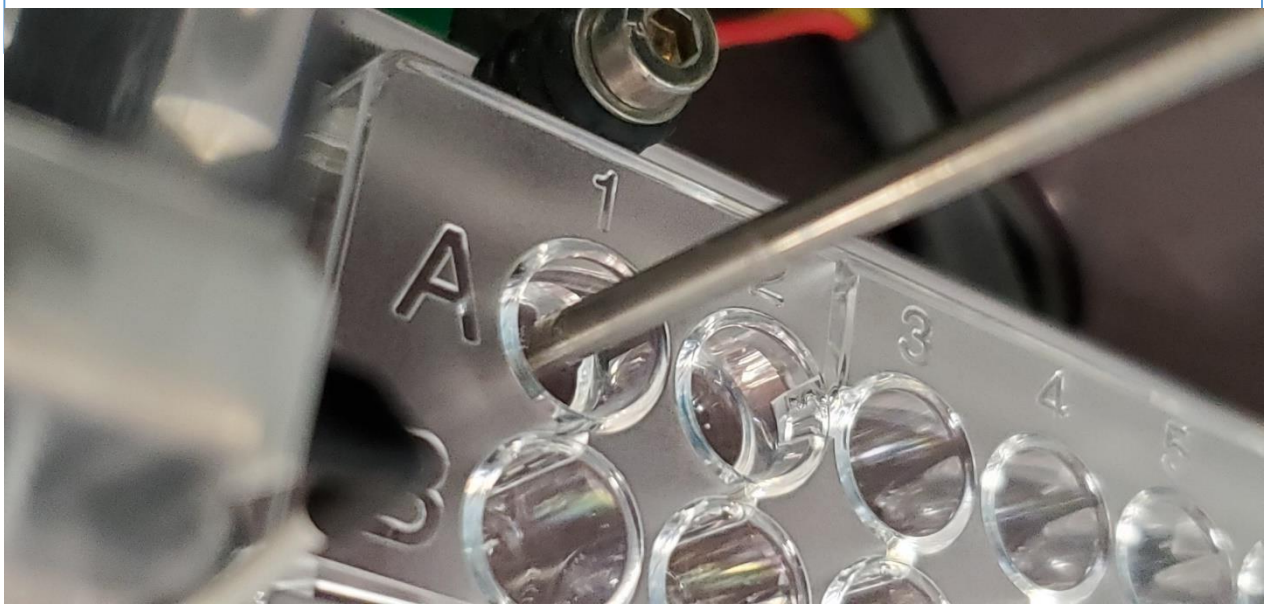
- All experiments were performed on a clean flow cytometer.
  - Sheath and PBS were assessed prior to any experiments with cleanliness cutoffs of 400 events/sec (LALs threshold = 40)
  - Daily readings typically demonstrate data <200 events/sec).

- Experiments were performed by trained personnel.
  - All operators were trained by Nanostics senior scientists for a minimum of 2 weeks.

Methods

- All data were analyzed by a senior scientist using various software:
  - GraphPad Prism (v6.01),
  - Spherotech MESF template,
  - FLOWJo v10.6.1.

- All standards (beads, virus) were used according to manufacturer's protocols:
  - Apogee 1524(LotCAL0139, exp 2024) , 1527 (Lot CAL0314,exp2021) , 1493 (LotCAL0099, CAL0121, exp2023,2024) bead mixtures;
  - Exometry verity mixtures (VER01A&VERO1B);
  - Spherotech MESF beads (RCP-20-5, AJ01);
  - Viroflow Technologies murine leukemia virus (MV-sfGFP MLV Lot S1005).



ApogeeFlow Systems GxP Cytometer Configuration for Validation Assays
General Set Up (specifics listed per assay page)

Platform	ApogeeFlow GxP	S/N 0139		
Parameter	Setting			
Sample Flow Rate	0.75 – 10.5 µL/min			
Pressure	150 units			
Acquisition time	5-900sec			
Sample Dilution	10-400x			
Volume/well	250µL			
Sample volume	10 uL			
Antibody/spike volume	varies uL			
Diluent volume	varies uL			
Channel	Laser Power (mW)	PMT	Gain	Threshold
405nm	0-200 (110)			
488nm	0-200 (80)			
561nm	N/A			
638nm	0-200 (80)			
405-SALS		362	1.0	40
405-LALS		320	1.0 (0.5 MESF expt)	40
405-Blue		405	1.0 (0.1 MESF expt)	
405-Green		573	1.0	
488-Green		405	1.0 (0.1 MESF expt)	
488-Orange		368	1.0	
488-Red		390	1.0	
561-Orange		---	---	
561-Red		---	---	
638-Red		628	1.0 (0.1 MESF expt)	
638-Far Red		---	---	
Beads	Product number	Lot number	Expiration	
Calibration	Apogee 1524	☐NA	☐NA	
Monitor	Apogee 1493	☐NA	☐NA	
Monitor	Apogee 1527	☐NA	☐NA	
Reference	Exometry Rosetta	☐NA	☐NA	
Reference	Exometry Verity A	☐NA	☐NA	
Reference	Exometry Verity B	☐NA	☐NA	
Reference	ViroFlow MV-sfGFP	☐NA	☐NA	
Apogee Protocol*	location			*new protocols only
ApoVirus	C:\			

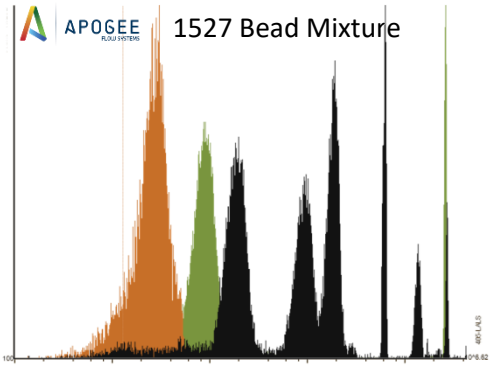
Particle Resolution

1

The first parameter to be tested for EV analyses should be …
Can the system resolve small particles from noise?
Under normal operating conditions?

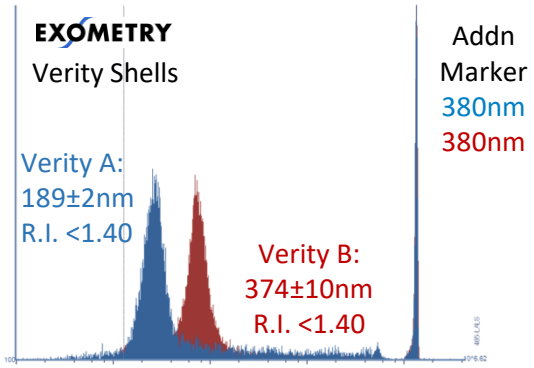
Apogee 1527 and Exometry Vero1A, 1B beads were analyzed under standard operating conditions. Analysis of MV-M-sfGFP was performed under increased 405nm power.

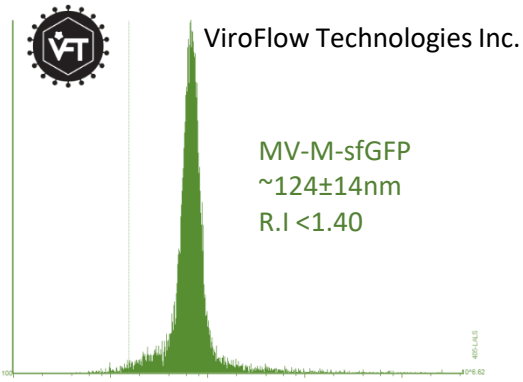
Acceptance Criteria: The platform shall be able to resolve "small particles" of defined sizes (~80-1000nm) across refractive indices of ~1.38-1.59.



Silica: R.I. =1.42
180nm
240nm
300nm
590nm
880nm
1300nm

Polystyrene: R.I. =1.59
405 Green 83nm
488 Green 110nm
488 Green 500nm





ApogeeFlow GxP cleanly resolves various small particles in the desired size ranges. Resolution of discrete particle populations 20nm difference is possible (e.g. 100-120nm Si not shown).

Repeatability

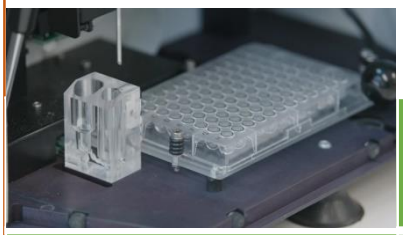
2

Any flow cytometer needs to provide repeatable data. For Nanostics, this means blood derived EVs in plasma or serum, hence repeatability should be demonstrated in this sample type.

Acceptance criteria: The platform shall be able to provide repeatable data consistently: consistency is defined as particle concentration with %C.V. of ≤ 10%

Repeatability of 96 aliquots of the same sample:

- 60sec assay @0.75uL/min
- Human serum (100x dilution)
- Probed for 3 ClarityDX Prostate biomarkers
- Acceptance: Variability shall be set at %C.V. ≤ 10% (= Pass)



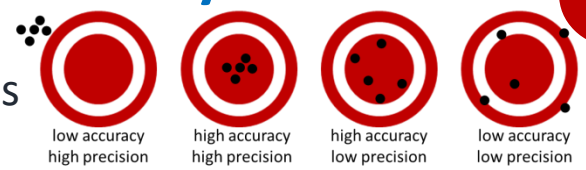
	Total Events	405 Blu+	488 Green+	638 Red+
	%C.V. of Concentration n= 96 wells/Run			
Run1 n=96	3.60	8.08	7.70	8.75
Run2 n=96	4.58	3.62	1.75	2.46
Run3 n=96	2.98	6.57	4.67	5.47
Run4 n=96	5.03	4.65	1.88	2.24
Mean Total %C.V.	**4.05**	**5.73**	**4.00**	**4.73**

ApogeeFlow GxP gave highly repeatable data with %C.V.s <10%.

Precision & Accuracy*

3

Any flow cytometer needs to provide precise analysis of samples of known compilation.



Accuracy is more difficult to measure due to biological variability and sample degradation. Here flow cytometry was also used to define concentration.

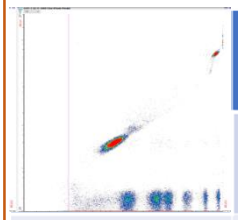
Acceptance criteria: The platform shall be able to provide precise analysis of population of known concentration: data shall demonstrate a %C.V. of ≤ 10% to have a PASS.

Same Day: Sequential Runs

- Sample: #1493 Beads (CAL0121
- 10 sequential runs
- 120 sec assay @0.75uL/min
- 8 Beads 110-1300nm
- Precision Acceptance %C.V. ≤ 10%
- Accuracy Acceptance ≤80%

Day 1-30: Multi-day

- Sample: 1493 Bead Mixture
- Lot# CAL0099
- 30 runs: 30 days
- 45 sec assay @0.75uL/min
- 8 Beads 110-1300nm
- Acceptance set at %C.V. ≤ 10%



	Same Day Precision N=10 Sequential Runs Lot #CAL0121		Inter-Assay Precision N=30 runs /30 days Lot# CAL0099		Accuracy N=10 runs Lot #CAL 0121		
	% C.V. (MFI)	% C.V. Conc (Events/uL)	% C.V. MFI	% C.V. events/uL	Vendor Defined events/uL	Lab Determined events/uL (Mean ± SD)	Mean Accuracy Lab Conc x 100 Vendor Conc
Bead 1 (110nm)	1.61%	1.71%	8.52%	3.51%	11500	11536 ± 196.7	100.3 ± 1.711
Bead 2 (180nm)	0.42%	3.36%	3.86%	4.28%	9000	7998 ± 268.7	88.87 ± 2.985
Bead 3 (240nm)	0.38%	1.39%	2.29%	6.94%	7500	7937 ± 110.3	105.8 ± 1.470
Bead 4 (300nm)	0.32%	1.46%	5.10%	7.42%	5000	5199 ± 75.79	104.0 ± 1.516
Bead 5 (500nm)	1.60%	2.25%	8.83%	3.48%	2000	1928 ± 43.29	96.40 ± 2.165
Bead 6 (590nm)	0.21%	1.89%	4.87%	7.95%	2000	1904 ± 36.06	95.18 ± 1.803
Bead 7 (880nm)	0.28%	6.42%	5.24%	14.74%	2000	1796 ± 115.3	89.78 ± 5.763
Bead 8 (1300nm)	0.20%	14.61%	5.23%	23.72%	2000	1599 ±233.7	79.97 ± 11.69
Mean % C.V.	**0.63%**	**4.14%**	**5.49%**	**8.59%**	**Mean % Accuracy**		**95.04 ± 8.606**

The ApogeeFlow GxP platform provided precise & accurate analysis of multiple bead populations, both at initial PQ and daily monitoring.

*Conc determined using same analysis modality.

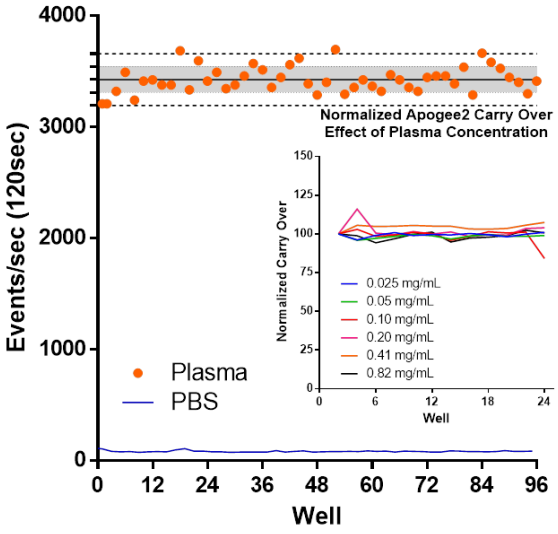
Sample CarryOver

4

Sticky fluorochromes, sample constituents and fluidics problems can cause samples to stick to flow cells and tubing. If any sample signal is not cleaned between runs, the validity of the subsequent run data is compromised.

Acceptance criteria: The platform shall be configured to demonstrate acceptably low carryover of clinical sample components (total particles or fluorescent signal). Acceptance: Carryover shall not exceed 0.1% of the previous sample signal, or will be diminished to the event rate of diluent "clean" PBS, 400events/sec.

ClarityDX Prostate Components			
Sample	**Concentration**	**CarryOver**	**Pass /Fail**
Serum/plasma	0.025-0.82mg/mL	≤ "clean" PBS	Pass
ClarityDX Prostate Biomarkers	low to high expressors	≤ "clean" PBS	Pass



Carryover on the ApogeeFlow GxP was not apparent during the analyses of high and low concentrations of serum or plasma.

Carryover was similarly not seen for any of the ClarityDX Prostate assay biomarkers, this carryover is also checked daily.

Flow Rate Stability

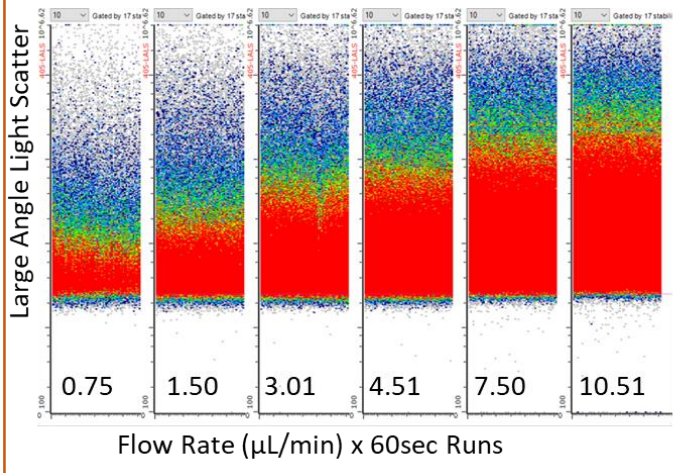
5

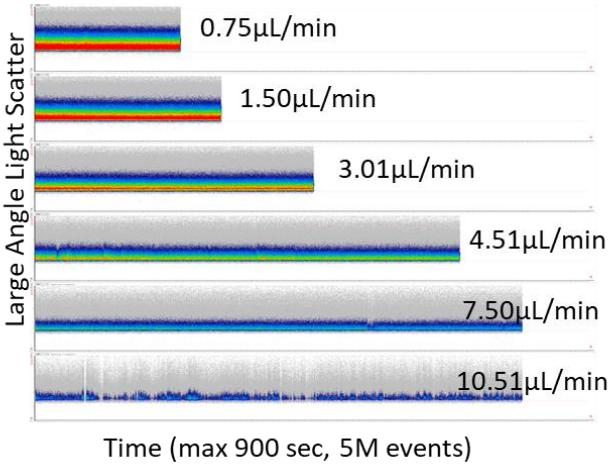
The presentation of sample at a steady, even flow rate into sheath flow is necessary for accurate analysis of sample composition. Unsteady flow may indicate fluidics problems but in practice is defined only by visual analysis.

<u>Acceptance criteria:</u> The platform shall be able to provide stable sample flow rate at a range of rates for the duration of standard analysis requirements. Flow stability is monitored as a running average of sample concentration x time. A deviation of >10% from the average is considered unacceptable. *A sample must have ≥75% of all particles within the average event rate.*

<u>Flow Rate Tests</u>

- Sample: unstained human serum
- Lot# CP2-01
- 400µL sample injection
- Event rate ≤20,000 events/sec
- 60s run analyses, 900s analyses or 5million events





The ApogeeFlow GxP platform provided stable flow across a range of sample flow rates (10.5µL/min – 0.75µL/min) for both the 60sec assay and extended 900sec assays.

Sample Linearity

Sample dilution for ClarityDX Prostate has been standardized. However, some samples may fall outside the range of acceptable event rate – in this case, an additional dilution may be necessary. Quantification of sample components (e.g. concentration) should be linear with respect to dilution.

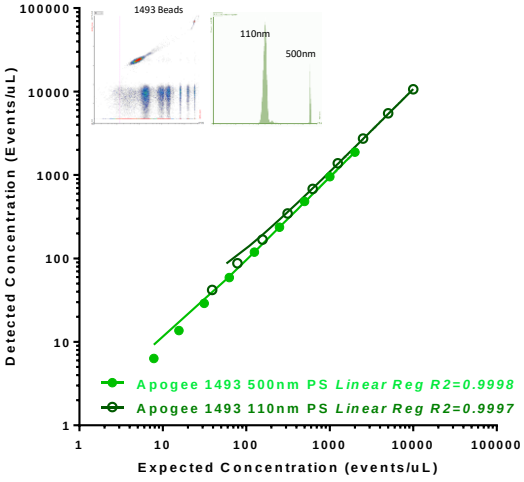
Acceptance criteria: The platform shall demonstrate a linear analysis of samples of known concentration that are diluted in a serial manner: data shall demonstrate a linear regression R square of ≥ 0.975.

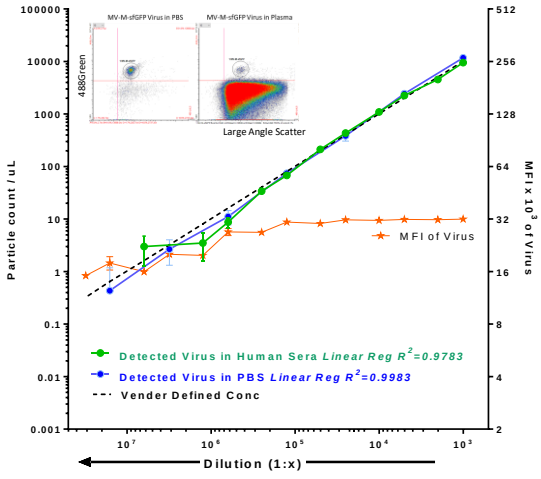
Bead Analysis:

- Sample: #1493 Beads
- Lot# CAL0121
- 256 fold dilution of stock
- 120 sec assay @0.75uL/min
- 2 bead analysis 110nm, 500nmPS

Biological Sample Analysis:

- ViroFlow Technologies Inc.
- MV-sf-GFP
- Lot# UA-3-023
- $1.5x10^7$ fold dilution of stock
- 120 sec assay @0.75uL/min





A wide range of serial dilutions of bead or biological samples gave linear responses on the ApogeeFlow GxP platform providing more flexibility in assay development.

FL Sensitivity & Linearity

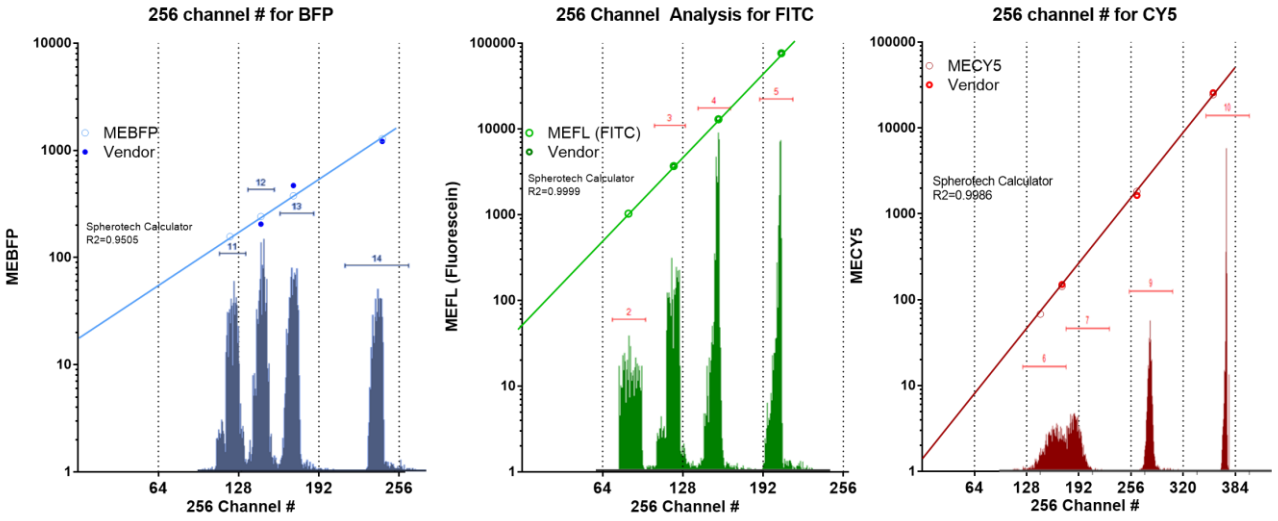
7

MESF (Molecules of Equivalent Soluble Fluorochrome) analysis enables the standardization of fluorescence intensity units for applications in quantitative fluorescence cytometry. Unfortunately, most current MESF beads are too large and have too many attached fluorochromes to reflect the fluorescent intensity of stained biological EVs or small particles.

*<u>Acceptance Criteria:</u> The platform shall be able to provide MESF sensitivity in a linear manner based upon current available MESF bead standards**.*

- Small (18-2.2um) MESF beads
- Spherotech Product RCP-20-5
- Lot # AJ01

- **Platform sensitivity (GAIN,PMT) was decreased to allow for high brightness of bead
- **Appropriate MESF beads for EV analysis not readily available



ApogeeFlow GxP platform demonstrated linear, relative fluorescence sensitivity using the most appropriate standards available.

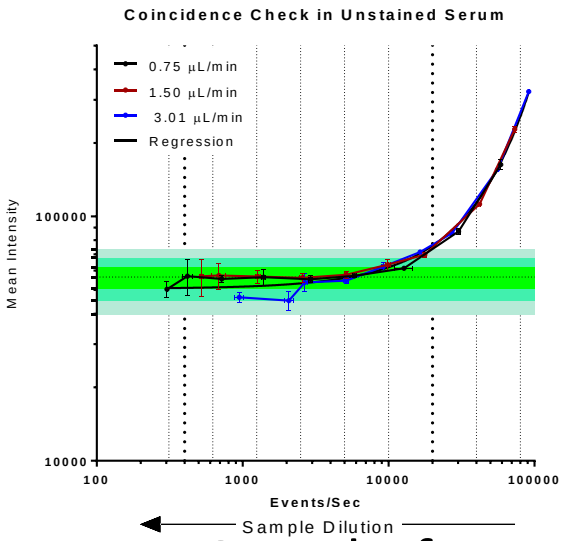
Coincidence

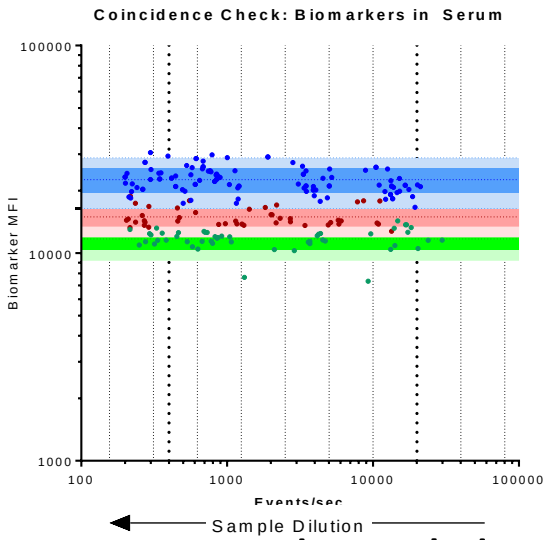
For accurate sample composition, only single particles must be evaluated at a time. Coincidence (swarm) is the simultaneous analysis of multiple particles in the laser beam. This occurs when sample concentration is too high. To define the optimal sample range, a broad sample dilution is performed and the single particle range defined by the "plateau" of fluorescent intensity against event rate.

<u>Acceptance criteria:</u> The platform shall be able to provide single particle analysis at a range of biological concentrations: the range of valid event rates shall be determined in the same biological matrix as test samples.

<u>Coincidence (swarm analysis)</u>

- Sample: serum ± probe staining
- Serial dilution of unstained samples
- Serial dilution of stained samples
- Multiple flow rates tested
- $4x10^2$ – $1x10^5$ events/sec tested
- Acceptance set at MFI ± 3SD of "mean plateau" MFI





Apogee GxP platform demonstrated a valid event rate range of 400 - 20,000 events/sec for human serum under standard clinical lab conditions.

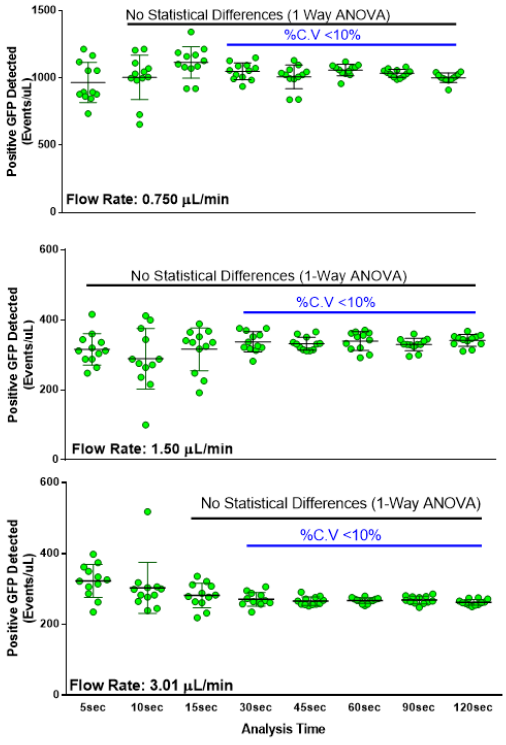
Time to Valid Data Acquisition

9

Unlike conventional flow cytometry, the concentration of the component of interest in liquid biopsies may be extremely low (<<<1% of total sample concentration). While the detection of 100,000s particles can occur in seconds, the actual time needed to accurately detect the particles of interest needs to be defined.

<u>Acceptance criteria:</u> The platform shall be able to provide statistically similar data for different concentrations of biological particles expressing stable fluorescence.

- PC3 Palmitoylated GFP EVs spiked into human serum at high–low concentrations
- Multiple flow rates tested
- Event rate ~10,000±2000s
- Acceptance for sample repeatability of ≤10%
- Acceptance of valid time p<0.05 (ANOVA) compared to 120sec



Flow Rate µL/min	Sampling Time (seconds), n=12 replicates/time (%C.V.)							
	5s	10s	15s	30s	45s	60s	90s	120s
0.75	15.42%	16.52%	10.53%	5.97%	8.74%	4.03%	2.87%	3.54%
1.50	14.22%	30.02%	19.38%	8.62%	5.60%	7.91%	5.42%	4.92%
3.01	14.36%	23.72%	12.35%	7.13%	4.49%	3.04%	4.13%	3.12%

Red text indicates unacceptable %C.V.s

Flow Rate µL/min	1 Way ANOVA Analysis (120s = ctrl)							
	5s	10s	15s	30s	45s	60s	90s	120s
0.75	0.865	0.985	0.042	0.751	0.985	0.657	0.865	---
1.50	0.662	0.036	0.662	0.971	0.951	0.971	0.951	---
3.01	0.0003	0.025	0.593	0.955	0.955	0.955	0.955	---

Red text indicates times with significant different data, p<0.05.

Flow Rate µL/min	Total GFP+ EVs Detected							
	5s	10s	15s	30s	45s	60s	90s	120s
0.75	60.5	125.7	209.4	418.9	591.0	756.8	1192.5	1553.6
1.50	39.5	72.3	118.6	253.1	373.5	509.4	742.3	1024.3
3.01	80.9	152.0	212.0	407.5	600.1	804.7	1214.5	1578.2

Red text indicates total events detected at inadequate sampling times.

ApogeeFlow GxP platform demonstrated a minimum analysis time of 30s (250-400 positive events) for standard clinical samples

Well Resampling Volume

10

A failed aspiration may require the resampling of a standard assay well. In order to sample multiple times, the maximum volume that can be aspirated twice must be empirically determined.

Acceptance criteria: The platform shall be able to provide at least 2 consecutive aspirations per well from a standard 96 well plate. The minimum/maximum sample aspiration volume will be determined empirically. The mean % difference between primary and secondary aspirations shall not exceed 10%. This test is important for well resampling during clinical studies.

- Human plasma or serum
- Unstained
- Aspiration volumes tested: 60µL-110µL
- 250µL total sample volume/well
- Samples were compared for total particle concentration
- 3.01µL x 60sec assay time
- All aspirations executed within14hrs
- n=12 per test volume/ run

Volume of First & Second Aspiration Runs

	60µL	70µL	80µL	90µL	100µL	110µL	%C.V. (All Volumes)	%C.V. (Valid Range)
Aspiration 1 Events/uL	98405	92942	86131	84893	104228	87813	8.28	6.95
Aspiration 2 Events/uL	91521	85596	83639	90902	111706	68637	15.79	4.43
*Mean % Difference	6.966	8.298	3.964	34.20	35.19	82.97	---	---

%C.V. all volumes: demonstrates %C.V. of initial aspiration total particle conc is <10%
%C.V. valid range demonstrates %C.V. of initial and second aspiration total particle conc are both <10%
*The absolute value of the % difference for each well was calculated and used to determine the Mean% Difference for the Volume being tested.

Apogee GxP platform demonstrated a resampling well aspiration volume of 60-80µL based on a 250µL total well volume.

Summary

Who should do a performance qualification on their flow cytometer? Everyone!

- A performance qualification provides the operator/flow core manager/scientist/potential buyer the critical evidence to understand the practical working capabilities of the instrument.

When and why should a PQ be executed?

- A PQ should be performed on a demo unit before purchase and on any newly installed system to confirm the system meets all of the users requirements and all of the advertised specifications.
- A partial PQ should be performed if significant service or relocation of the instrument occurs.
- A PQ of any instrument should accommodate all MISEV-Flow guidelines where possible.

How should a PQ be performed?

- A PQ should be executed by trained personnel using standard operating conditions expected to reflect normal usage.
- Similar types of samples, or reasonable surrogates, should utilized, for all testing. Bead mixtures should not be used for all experiments.

Summary

ApogeeFlow Micro-GxP

- The performance qualification for the ApogeeFlow Micro-GxP platform verified that the instrument produces acceptable results under normal operating conditions.
- Performance qualification represents the final qualification of the instrument prior to use in a research or clinical setting.
- The ApogeeFlow Micro-GxP functions in a manner that shall meet all current laboratory, regulatory and accrediting agency requirements.
- A summary of the test data are presented below:

Test	Data Summary	Summary
1. Particle resolution	~120nm biological particle	Pass
2. Repeatability	Mean %C.V 4.05%	Pass
3. Precision & accuracy	Precision %C.V=4.14% Accuracy ~95%	Pass
4. Carry over	< 0.1%	Pass
5. Flow rate stability	Stable flow 0.75-10.5µL/min	Pass
6. Sample linearity	Linear with R^2 >0.975	Pass
7. Sensitivity & linearity	MESF bead analysis linear with R^2 >0.950	Pass
8. Coincidence	Valid range 400-20,000 events/sec	Pass
9. Time to valid data acquisition	Minimum time is 30sec or ~250-400 events For low-high concentration targets	Pass
10. Well resampling volume	Aspirations of 60-90µL permit 2 sample runs (20uL) total sample volume	Pass

Contact Us

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