Using BD FACSDiva™ CST To

Evaluate Cytometer Performance,

Create Custom Assay Settings

and

Implement Cross-Instrument and Cross-Site Standardization of Assays

PART 2

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Agenda: Designing and Performing a Multicolor Assay Across Sites

Part 2

- **Review Key Points from PART 1**
- **Choosing reagents**
- **Insuring equivalent fluorescence intensities (MFI) across Multiple instrument**
    - **Insuring consistent MFI over time (Multi-year studies)**
    - **Using Application settings**
- **Optimizing for multiple cytometers-**
    - **Accounting for different instrument performance**
    - **Test assay by "detuning" an instrument**
- **A "real-world" example**
    - **The NIH ICS Assay Quality Assurance Project**

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BD CS&T: Qr and Br – Relative Q and B

- **Qr** is *photoelectrons per fluorescence unit* and indicates how bright a reagent will appear on the sample when measured in a specific detector.
  - It is a function of
    - The instrument [laser power and alignment; optical design]
    - The reagent [quantum yield of the fluorochrome]

- **Br** is *measured optical background*, which helps indicate how easily (dim) signals may be resolved from unstained cells in that detector by providing a practical estimate of competing optical background.

- Qr and Br are independent variables, but both affect sensitivity.

- The relative detector sensitivity for a specific fluorochrome is proportional to Qr and Br:

$$\text{Sensitivity}_{\text{relative}} \propto \sqrt{\frac{\text{Qr}}{\text{Br}}}$$

Qr: Anti-CD10 PE Example (BD FACSCanto™)

The laser and detectors were attenuated by ND filters over a 30-fold range to illustrate the effects of decreasing detector sensitivity on population resolution.

CS&T standardized the settings to place the positive at the same location.

Qr	Br
0.2274	277
0.0867	251
0.0379	255
0.0135	254
0.0071	266
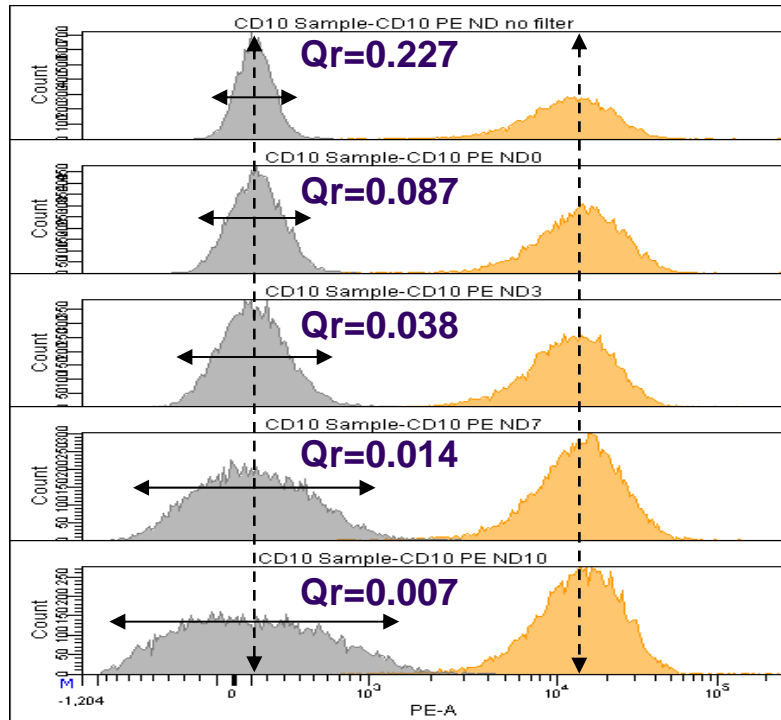
Trans-mission	SI Corrected
100%	83
35.5%	55
17.8%	35
7.1%	20
3.5%	14

Factors to Consider for an Optimal Gain Setup

- **Things to consider in *optimizing* the cytometer setup for the immunofluorescence application**

  1. *Electronic Noise* **can affect resolution sensitivity**
     - ✓ A good *minimal* application PMT voltage would place the dimmest cells (unstained) where electronic noise is no more than 10% to 20% of the total variance.

  2. **Dynamic range assessment for each fluorescence parameter**
     a) Are the brightest populations within the *linear range* of the detector?
        - Leave room for ~ 2-fold increase in expression levels and ensure the cells are in the linear range of the detector.
     b) Are the compensation controls within the linear range of the detector?
        - If positive cells are out side of the linear range compensation may be inaccurate
     c) Are the negatives (in a stained sample) too high?
        - This is a matter of taste

  3. **An optimal cytometer gain setting is one for which both conditions are met.**

An Alternative Approach to Settings Gains

- One of the criteria for setting gain is to ensure that electronic noise does not impact low end sensitivity
  - The goal is have the dimmest cells (unstained) where electronic noise is no more than 10% to 20% of the total variance.
- CST uses Dim particle MFI which is normalized to dim cell brightness to set the MFI gain
  - While this is a good general approach (for a single instrument) it does not take into account differences in cells or assay conditions
    - Cellular autofluorescence
    - Autofluorescence due to fixation
    - Intrinsic variance (standard deviation) of the negative cells
- **An alternative approach is for a given assay to measure the rSD of the negative cells at different gain (PMTV) settings.**
  - **A good rule of thumb is to set the gain so that the rSD of the negative cell is greater than 2.5 times the SD of the electronic noise**

$$rSD_{Neg\,Cells} > 2.5 \times SD_{EN}$$

  - **Adjusted Gain settings can then be applied through the use of Application Settings (explained in detail in the next Part)**

Setting Gains for Use Across Multiple Instruments

- **When determining gain settings for assays to work across multiple instruments, the gains need to be set according to the limitations of the poorest performing instrument.**

- Example*:

Inst. No.	SDen	Qr	Br	Sens	Upper End of Linearity
1	26	0.015	976	3.9	230,000
2	17	0.042	92	21.4	200,000
3	23	0.01	298	5.8	180,000
4	22	0.01	613	4.0	200,000
5	9	0.007	2322	1.7	230,000
6	20	0.018	2768	2.6	190,000

- Instrument 3 has the lowest upper end of linearity: 180,000.
    - The gain should be low enough so the brightest population in the assays is lower than 180,000 on any instrument (150,000 would give some room for variability).
- Instrument 1 has the highest electronic noise: 26.
    - The gain should be high enough so the SD of negative cells is >2.5 x 26 = 65.
        - This is critical only if you are measuring dim events in this channel.
- If both conditions can't be met, then you must choose which is more important for this channel: identification of bright populations or resolution of dim populations.

 * This variation in instruments is outside that expected for properly maintained instruments.

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Designing Multicolor Experiments for Use Across Multiple Instruments

2. Taking into Account Differences in the Performance of Fluorochromes
(Relative Stain Index)

Designing Multicolor Experiments

- **Determine which and how many markers are required for:**
  - **Identifying the sub-population(s) of interest**
    - **eg, CD8 T cells , NKT cells**
  - **Measuring the biological functionality**
    - **eg, level of activation, cytokines, phosphorylation**

- **Which markers should go on which fluorochromes?**
  - **Which instrument(s), number of lasers; number of fluorescence parameters**
    - **BD FACSCanto II (3 lasers; 8 parameters)**
    - **BD LSRFortessa™ cell analyzer (5 lasers; 18 parameters)**
  - **Goal of panel design**
    - **Cleanly identify the subpopulations**
    - **Maximize the resolution in experimental parameters for the sub-populations of interest**

Which Fluorochrome for which Specificity?

1. **For most experiments the parameters are either "Classifying" or "Experimental".**
   - **Classifying: eg, CD4, CD8- define subpopulations**
     - **Bright (CD4) vs dim (CD25)**
     - **Population of interest vs exclusion "Dump" channels**
   - **Experimental: Unknown, dim, or changing expression levels**

2. **In general, experimental parameters need the best low-level resolution (especially if the expression level is unknown).**
   - **Use fluorochromes with brightest intensity (Stain Index).**
   - **Use fluorochromes with minimal spillover from other channels.**
     - **Total spillover into a given channel is a function of the sum of the % spillover x intensity (MFI) from all other channels.**

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Factors in Selecting Reagents

- **Assay Specific**
  - **The Stain Index of the reagents**
    - **Intensity of the fluorochrome**
    - **Density of the specificity on the cell**
    - **Autofluorescence of the cell in the channel**
      - **Lower in red channels**

  - **Spillover of other fluorochromes into the channel**
    - **Spillover increases spread, reduces resolution**
    - **Adding colors generally adds background and usually complicates population resolution: "spread" increases**
      - **APC ⇐PE, PerCP-Cy™5.5, Alexa Fluor® 700, APC-Cy™7, AmCyan**
      - **PE ⇐FITC, PE-Texas Red®, PE-Cy7, AmCyan**

- **Instrument Specific**
  - **Qr, Br, SDen of the channel / fluorochrome**

Know the Stain Index of Your Reagents

Fluorochrome	Stain Index				
		Reagent			
	CD19	CD4	CD8	CD20	CD45
FITC	18	51	182	218	350
PE	154	338	1131	782	
PerCP	26	57	208	204	292
PerCP-Cy5.5	77	177	754	242	325
PE-Cy7	223	448	1494	1060	
APC	174	377	1193	729	440
APC-Cy7	27	31	132	118	209
APC-H7	19	35	103	118	174
Horizon V450	47			277	382
AmCyan	24	57	34		252
Horizon V500	24	61	21	83	136

- **The Stain Index of a reagent is a function of:**

  – Relative Fluorescence Intensity of the fluorochrome used

  – Density of the specificity on the cell

  – Autofluorescence of the cell in the channel
    - Lower in red channels

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Designing Multicolor Experiments for Use Across Multiple Instruments

3. Ensuring Equivalent Fluorescence Intensities
   a. Standardizing a Cytometer

BD

Setting Up Your Instrument

- **Only two things need to be done to set up a single instrument for a given type of assay.**

    1. **Set the gain [PMT voltage], to achieve reproducible fluorescence intensity (MFI)**

    2. **Correct for background from fluorescence spillover [compensation], which is instrument dependent.**

- **For comparing results of assays to be run on multiple instruments, it is important that the MFIs are consistent and reproducible between the instruments.**

Reproducibility of MFI Gain Settings Using CS&T Beads

- **CS&T setup was repeated 15 times side-by-side on three BD FACSCanto II cytometers.**
- **The accuracy is achieved due to the low CV of the CS&T bright bead and the direct measurement of MFI.**
- **The accuracy of the target MFI is typically <2-3%.**

Legend: FITC, PE, PerCP-Cy5.5, PE-Cy7, APC, APC-Cy7, PE-Texas Red®, Alexa Fluor® 700, Horizon V450, Horizon V500
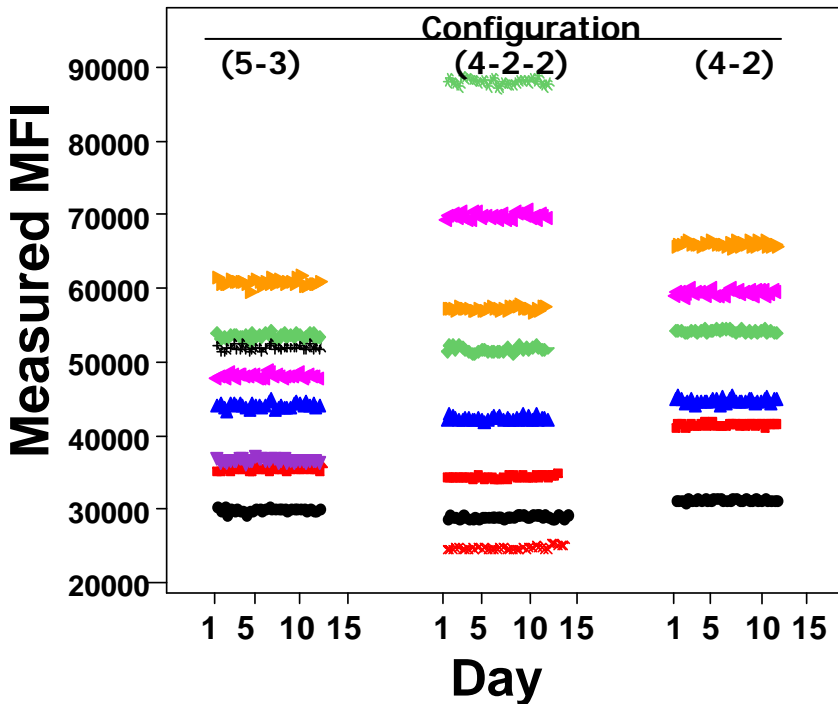
	Within-day CV%		Between-day CV%	
	PMTV	MFI	PMTV	MFI
FSC	*0.1*	*0.4*	*0.6*	*0.5*
SSC	*0.1*	*0.7*	*0.3*	*0.9*
FITC	*0.1*	*0.5*	*0.1*	*0.6*
PE	*0.0*	*0.3*	*0.1*	*0.5*
PerCP-Cy5.5	*0.1*	*0.5*	*0.2*	*0.5*
PE-Cy7	*0.1*	*0.7*	*0.2*	*0.7*
APC	*0.1*	*0.5*	*0.1*	*0.5*
APC-Cy7	*0.1*	*0.5*	*0.2*	*0.6*
V450	*0.1*	*0.4*	*0.2*	*0.6*
V500	*0.1*	*0.3*	*0.1*	*0.1*

Using CS&T Application Settings to Standardize Assay Fluorescence: Cross-Site, Cross-Instrument

- **The CS&T system is designed to set fluorescence gain to optimize low-end sensitivity for each instrument.**
  - Thus, different instruments can report different MFIs for the same input fluorescence intensity.

- **One of the most under-utilized features of BD FACSDiva 6 / CS&T software is the ability for users to create their own application settings for each Assay type.**
  - MFIs can be set by the user, saved, and reproducibly reused.

- **Using BD FACSDiva 6/ CS&T's Application Setting functionality, it is possible to standardize multiple instruments (different platforms and different sites) to give equivalent fluorescence.**
  - This can be done for different instrument platforms using BD FACSDiva 6 / CS&T.

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CS&T Saves Your Assay Specific MFI Targets

- **Run a CS&T Performance Check to standardize the instrument.**

- **Adjust the PMT voltages so that you have the fluorescence intensities (MFIs) that are appropriate for your assay.**

  - **Select "Application Settings-Save" (Right-click on Cytometer Settings)**
    - **BD FACSDiva 6 / CS&T software remembers the target MFI values.**

  - **These settings can then be applied to future experiments.**

  - **Gives reproducible data**
    - **Experiment to experiment or**
    - **Instrument to instrument**
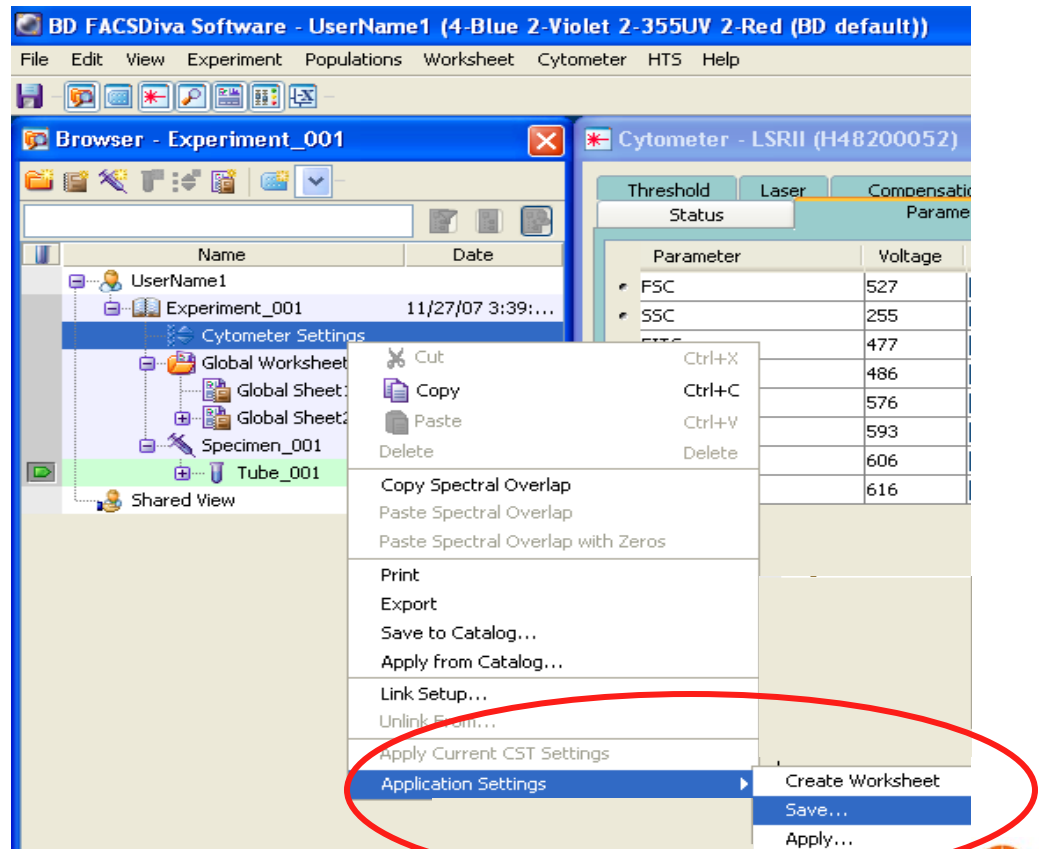
Maintaining Consistent Fluorescence Measurements Over Time- Application Settings

Configurations, Baselines,
Bead Lots & MFI Target Values

Part 2

Application Settings, Baselines and MFI Target Values

- **Application Settings are ratios relative to the CS&T MFI Target Values used at the time the Application Setting was created**
  - Thus, the Application Setting is linked to the Baseline / Bead Lot / MFI Target Values used at time of creation

- **Applying Application Settings to an experiment that is setup using a different Baseline with different MFI targets will result in inconsistent fluorescence settings**

- **If you create a new Baseline (with new or old beads) which results in new MFI Target Values you must re-create all of the associated Application Settings**

BD
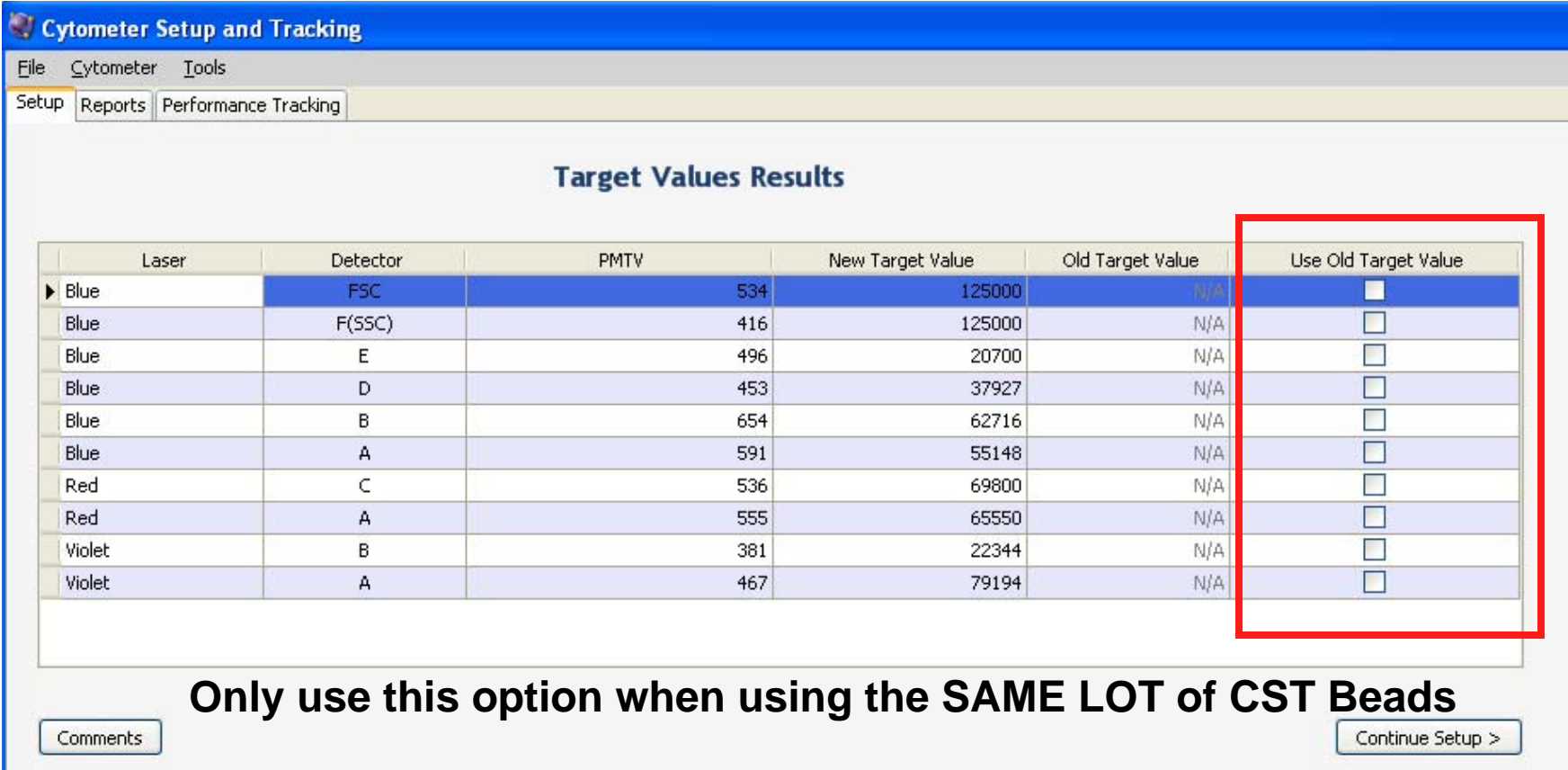
How to Maintain Consistent MFI Targets Over Time

- **Every time you run a new Baseline, CST will create new MFI Target values depending upon the performance of the instrument.**
  - **It is trying to optimize for that instrument.**
  - **Differences may be smaller or larger depending upon conditions.**
    - **Mainly affected by changes in SDen.**

- **However, if the primary objective is to have consistent fluorescence MFIs over long time frames (years), you want to insure that MFI Target Values are maintained when you perform a new Baseline.**
  - **Increases in SDen could affect data.**
  - **Make sure original Gains/PMTVs are sufficiently high to allow for changes in SDen**

- **How to do this when:**

  A. Using the same lot of beads as the previous Baseline.

  B. Using a different lot of CS&T beads.

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How to Maintain Consistent MFI Targets Over Time

A. Using the same lot of CS&T Beads

- **When performing the Baseline the last dialog allows you to choose between the new calculated Target Values and the Old Target Values**
  - **Select "Use Old Target Value"**

Cytometer Setup and Tracking

File   Cytometer   Tools

Setup | Reports | Performance Tracking

Target Values Results

Laser	Detector	PMTV	New Target Value	Old Target Value	Use Old Target Value
▶ Blue	FSC	534	125000	N/A	☐
Blue	F(SSC)	416	125000	N/A	☐
Blue	E	496	20700	N/A	☐
Blue	D	453	37927	N/A	☐
Blue	B	654	62716	N/A	☐
Blue	A	591	55148	N/A	☐
Red	C	536	69800	N/A	☐
Red	A	555	65550	N/A	☐
Violet	B	381	22344	N/A	☐
Violet	A	467	79194	N/A	☐

Only use this option when using the SAME LOT of CST Beads

Comments   Continue Setup >

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How to Maintain Consistent MFI Targets Over Time

B. Using a different lot of CS&T Beads

1) Use "Reset Target Values" (See Part 1)

 ➢ Restricted to resetting to one original lot.

 ➢ Only good for the lifetime (expiration) of the original lot

2) Manually adjust PMTVs during the "Define Baseline" procedure

 ➢ Requires more operator involvement.

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How to Maintain Consistent MFI Targets Over Time
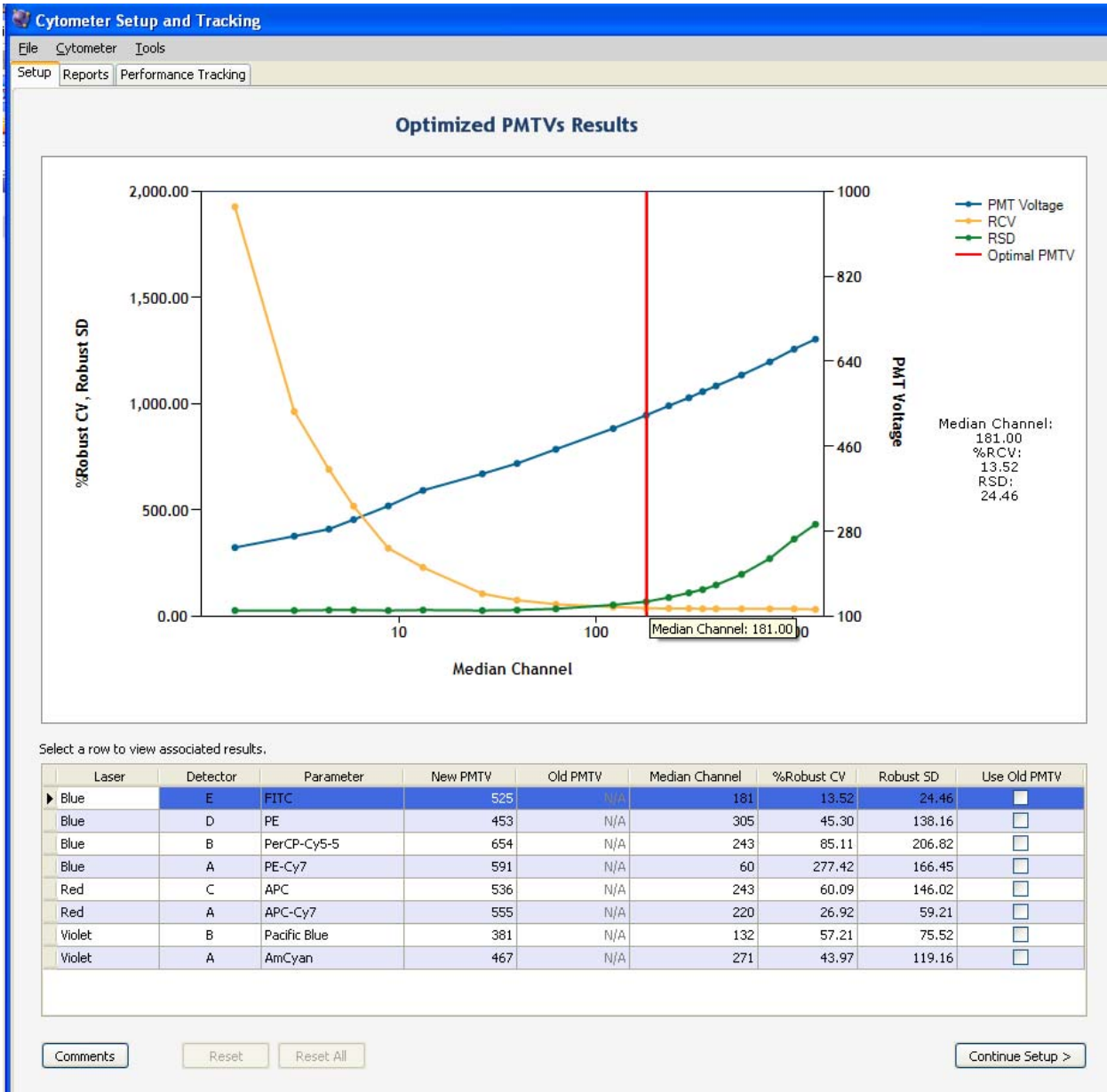
- Dialog appears after data collection
- Shows the the suggested PMTVs that CST has determined to minimize effect of SDen
- Users can override these values and specify the PMTVs to be used to generate the MFI Target Values

Adjusting PMTV During Baseline Procedure

How to Maintain Consistent MFI Targets Over Time

B. Using a different lot of CS&T Beads

1) Run "Check Performance" with current / old lot of CS&T beads

2) Record the PMT voltages set.
   i. These are the PMTV that meet the current MFI Target values
   ii. The goal is to get MFI Target values for the new lot of beads that will give the same PMTV (on that day)

3) Run "Define Baseline" with new lot of CS&T beads

4) When "Optimized PMTVs Results" dialog appears manually adjust PMTVs for each parameter to be equal to the values recorded for the current lot (Step 2)

5) Press "Continue Setup"
   1) New MFI Target Values will then be determined at these PMTVs
   2) MFI Target Values for the old and new Baselines may be different but both will set the instrument to the same gains / PMTVs insuring consistent MFI from biological samples over time

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Designing Multicolor Experiments for Use Across Multiple Instruments

3. Ensuring Equivalent Fluorescence Intensities Across Instruments

    a. Using Application Settings

Using Application Settings for BD™ CBA Flex Set Assays

- **Application Settings enable reproducible MFI values, experiment to experiment.**
  - **Accurate, direct comparison of data over time, across sites**

Day 0:
Application Settings for 30-plex BD Cytometric Bead Array (CBA) created using control CBA beads and the SOV for the red channels determined

Subsequent days:
Setup was done using standard CS&T setup. No other controls.

Application settings applied to the experiment, and SOVs from Day 0 experiment copied.

This is data post compensation.

Day 0   Day 70

BD
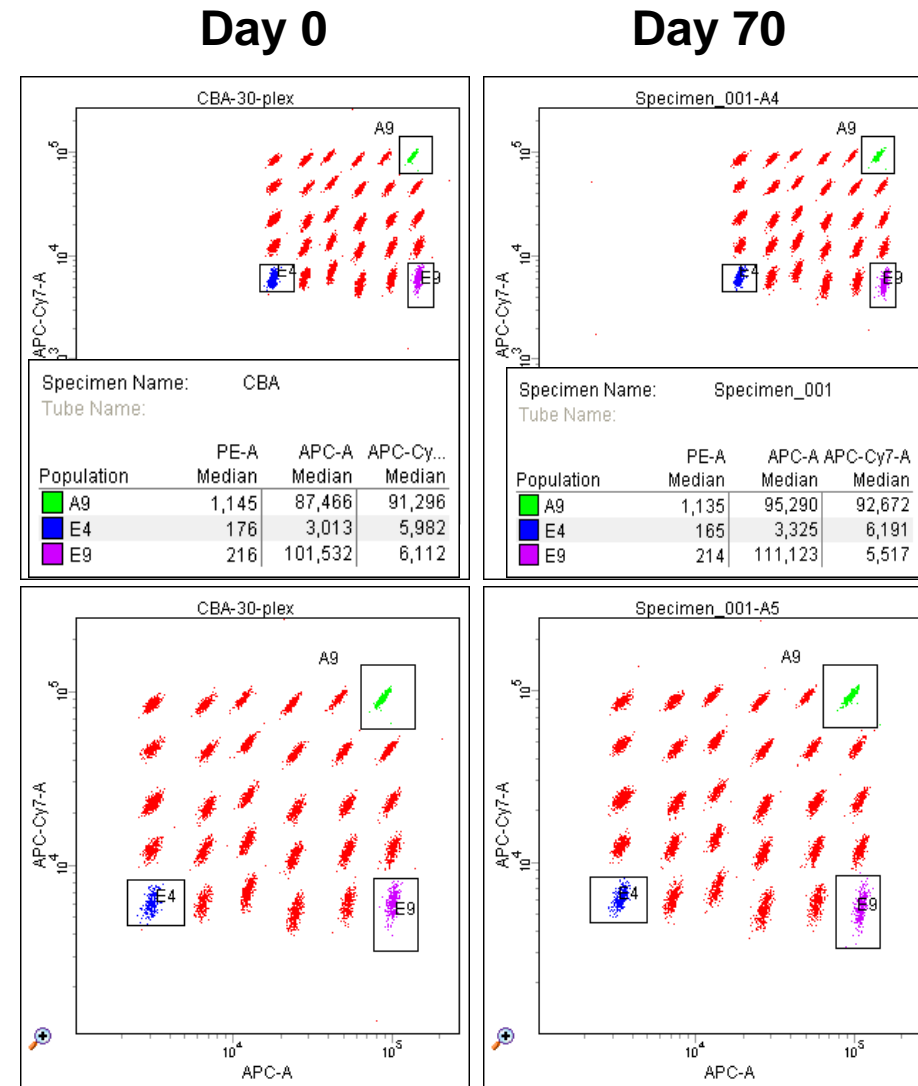
Transferring an Assay

BD FACS Calibur

- **CD4 Alexa Fluor® 488**
- **CD8 PE**
- **CD3 PerCP**
- **CD20 APC**

- Dual laser flow cytometer
  - Blue laser: **488 nm**
  - Red laser: **633 nm**
- Four-color detection
  - **FITC**   -**PerCP**
  - **PE**   -**APC**
- Analog signal processing using **peak** height mode, 1,024-channel resolution

BD FACSCanto II

- Three laser flow cytometer
  - Blue laser: **488 nm**
  - Red laser: **633 nm**
  - Violet laser: **405 nm**
- Eight-color detection
  - **FITC**   - **PE**   - **PerCP**
  - **PE-Cy7**   - **APC**   - **APC-H7**
  - **Horizon V450** - **Horizon V500**
- Digital signal processing using **area** signal processing, 2^{18} channel resolution

Does it Work? Same Sample on Different Instrument Platforms

- Using **BD CellQuest™** software
  - Analyze the CS&T bead data using linear values (1 – 10,000).
  - Record the **median** channel values of the CS&T bright bead from each detector.
  - Convert: **Calibur MFI x (2^{18} / 10,000)**
  - Example: (Calibur MFI PE = **1,370**) x (2^{18} / 10,000) = PE target for Diva/Canto = **35,914**
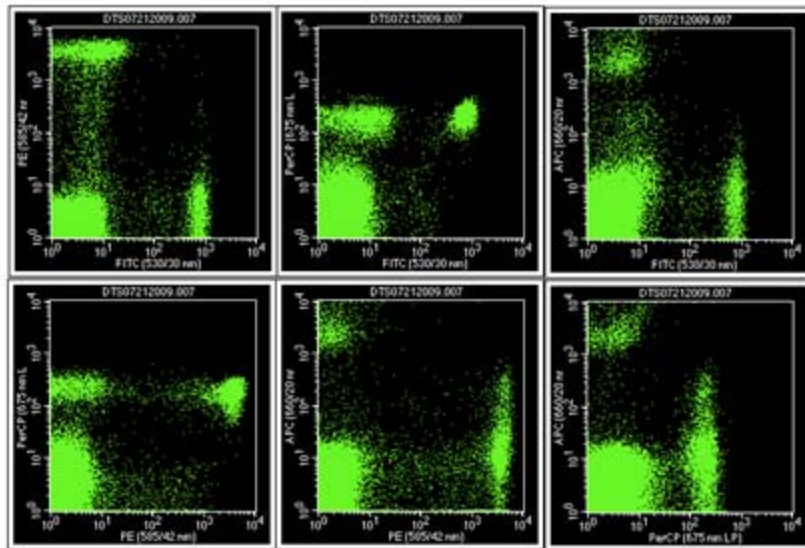
BD FACSCalibur – BD CellQuest Pro   BD FACSCanto II – BD FACSDiva 6
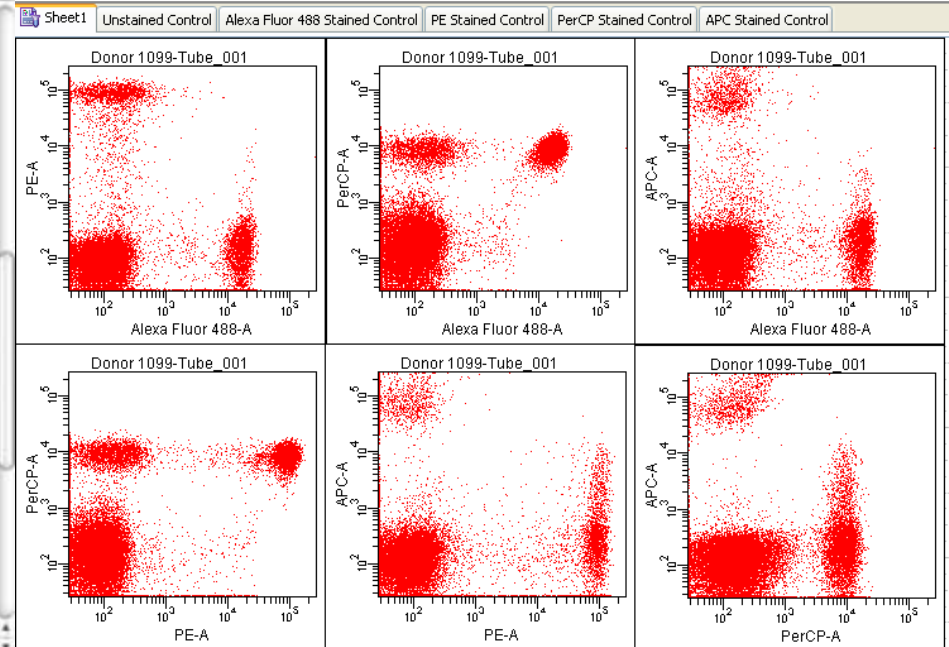
Transferring Assays Among Digital Instruments

1. **On the primary instrument, adjust gains (PMTV) as required for the assay.**

   - **Save Application Settings (Assay 1)**

2. **Using fluorescence controls (CS&T, BD™ CompBeads, stained cells) determine the MFI target values.**

   - **When transferring between instruments with equivalent laser and filter combinations, you can just *use CS&T beads.***
   - **When transferring between instruments with different laser and / or filter combinations (ie, PE off a blue 488-nm laser vs a yellow-green 658-nm laser) *use fluorochrome-matched controls such as BD CompBeads.***

3. **Using the same controls, on all other instruments adjust gains (PMTV) to meet MFI targets.**

   - **On each instrument, save Application Settings (Assay 1).**

One Assay, Run on Four Platforms

BD FACSCanto II
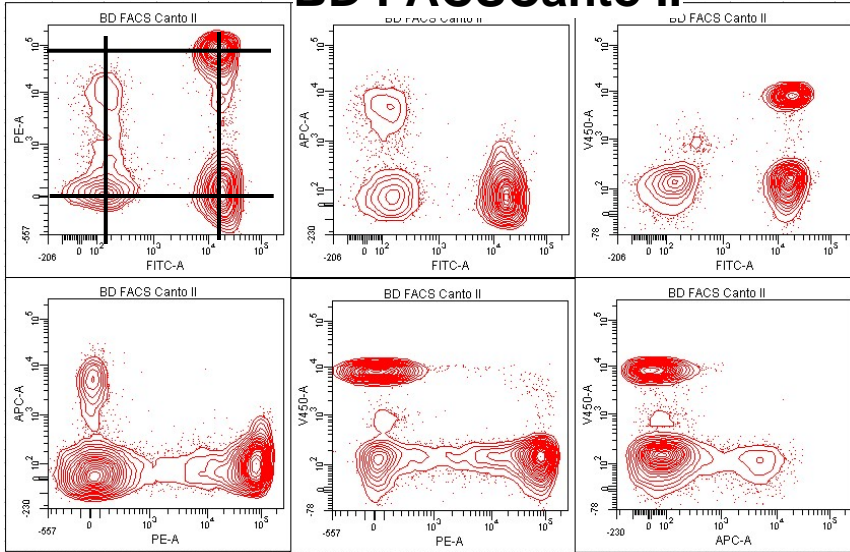
BD™ LSR II
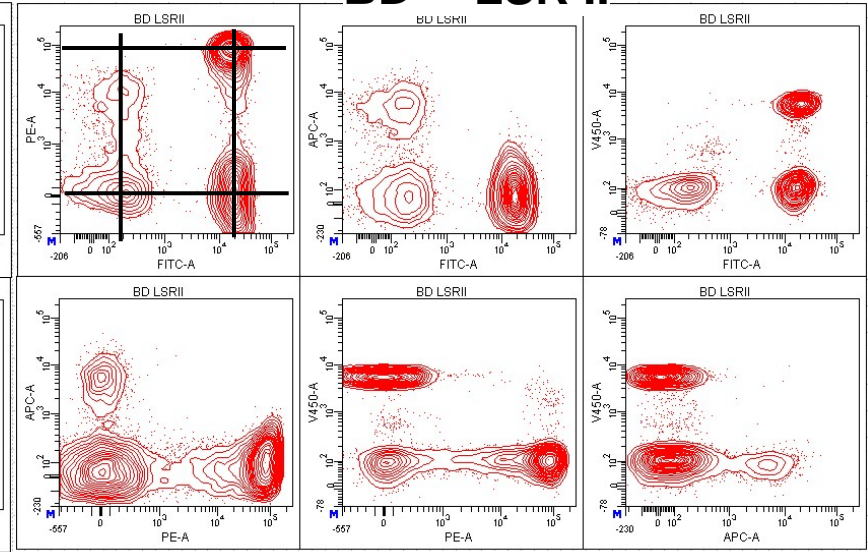
BD FACSAria™ III
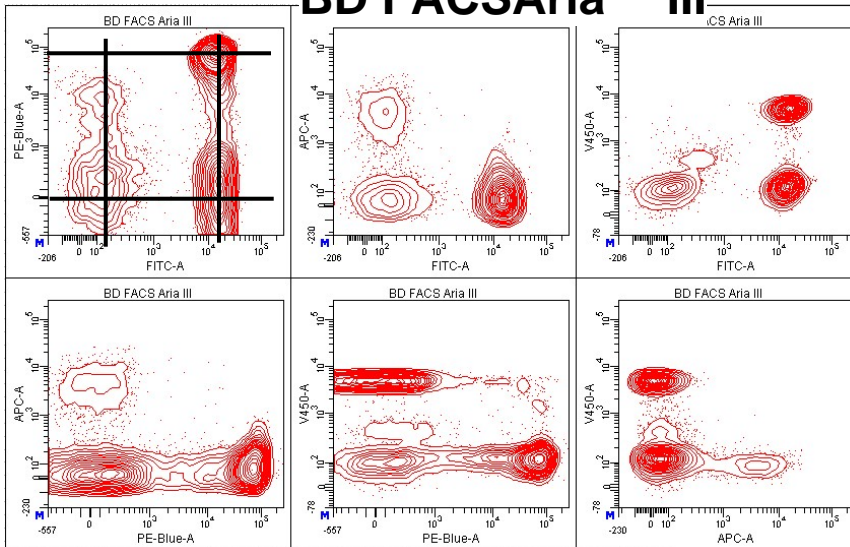
BD LSRFortessa
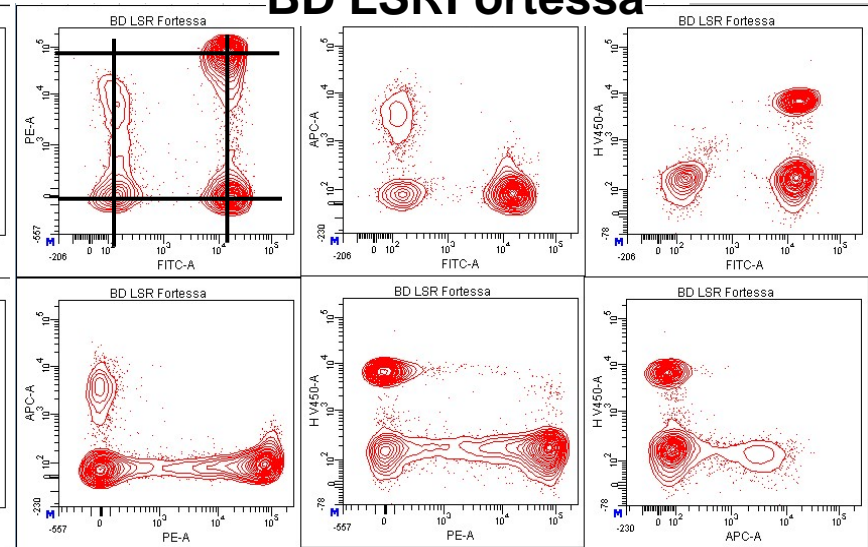
Designing a Multicolor Experiment for Use Across Multiple Instruments

4. Optimizing Assays for Multiple Cytometers
   -Taking into Account Variance in Instrument Performance

BD

Optimizing an Assay for Multiple Instruments: Principles

- **When working across multiple instruments the goal is to ensure the assay will perform acceptably on the poorest performing instrument.**

- **Examine a given assay's performance on multiple instruments and platforms (BD FACSCanto, BD LSR II, BD LSRFortessa, etc).**
  - **QC every instrument for critical performance parameters:**
    Q_r, B_r, SDen, Linearity, Sensitivity
  - **Establish major contributors for degrading assay sensitivity.**

- **Correlate assay sensitivity requirements with instrument performance by detuning instrument performance.**
  - **Establish minimum instrument performance for assay quality assurance.**

Different Instruments Have Different Resolution Sensitivities

BD LSR II
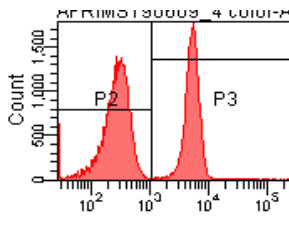PE: 50 mW Blue; APC: 18 mW Red
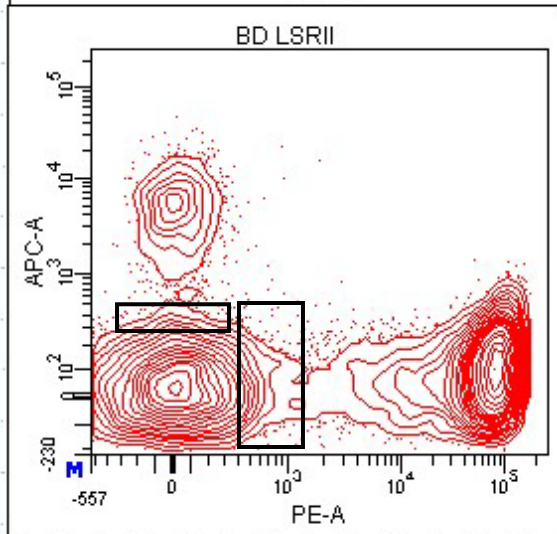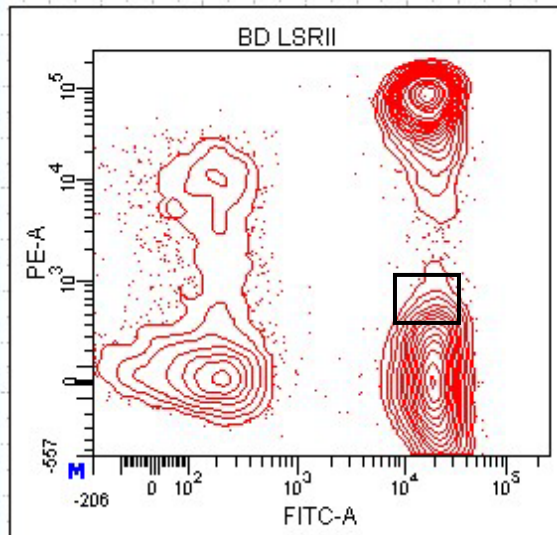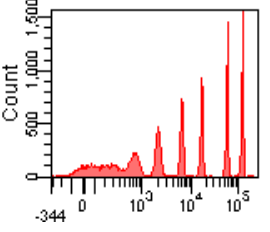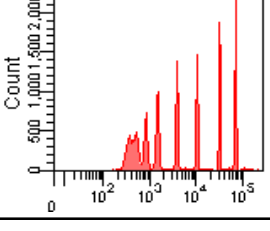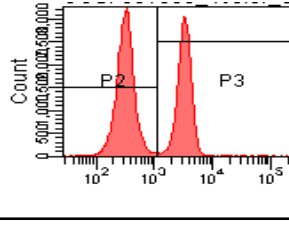
BD LSRFortessa
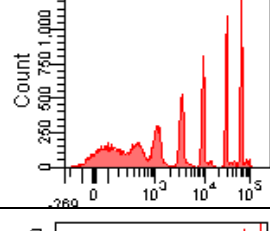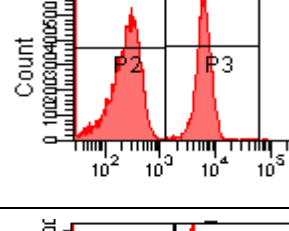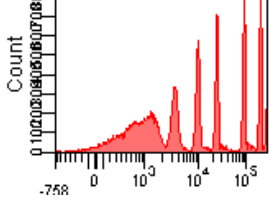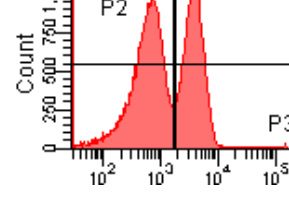PE: 50 mW Y/G; APC: 50 mW Red
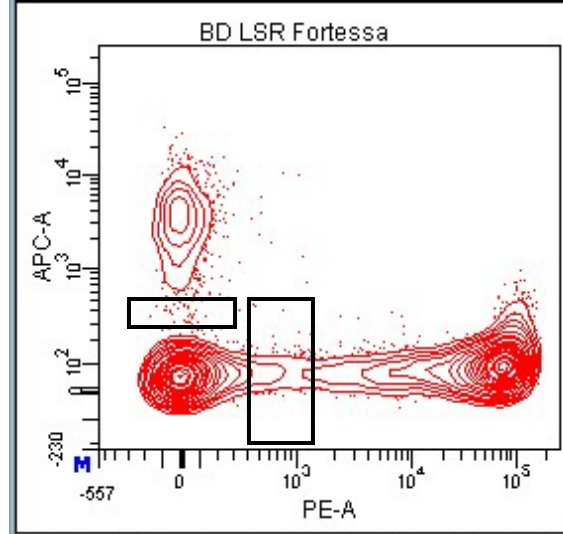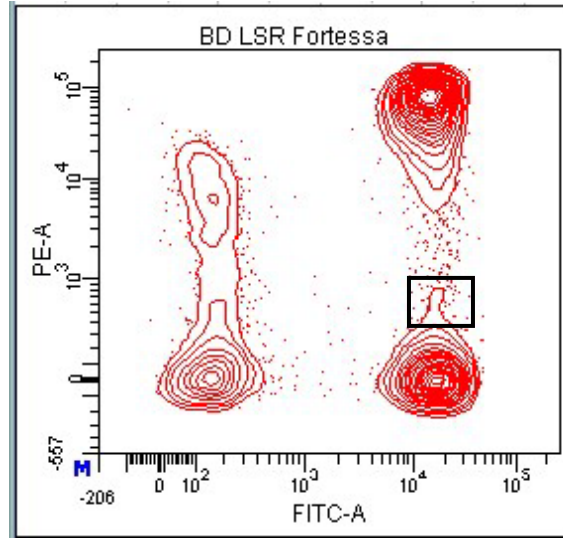
If an assay requires the identification of a dim population, the brightness required to resolve that population is limited by the instrument with the lowest resolution.

In this case, for the PE channel it is the BD LSR II that controls the minimum separation required.

Variation in Instrument Performance*

| Lab	SDen	Qr	Br	SI Comp beads	8 peak beads	Lymphocytes (CD4 FITC)
8	26	0.015	976	55.6		
9	17	0.042	92	88.2		
11	22	0.010	613	66.0		
12	9	0.007	2322	13.7		

* This variation in instruments is outside that expected for properly maintained instruments.

Designing a Multicolor Experiment for Use Across Multiple Instruments

5. Optimizing Assays for Multiple Cytometers

    -Testing Assay Performance Limitations by "Detuning" an Instrument

Effect of Detuning on Individual Fluorochromes / Channels

- **Degradation of instrument performance impacts low resolution in some channels more than others.**
- **Running an assay on a detuned instrument (ND filters / free dye) lets you estimate how much "room" you have for each parameter in your assay.**
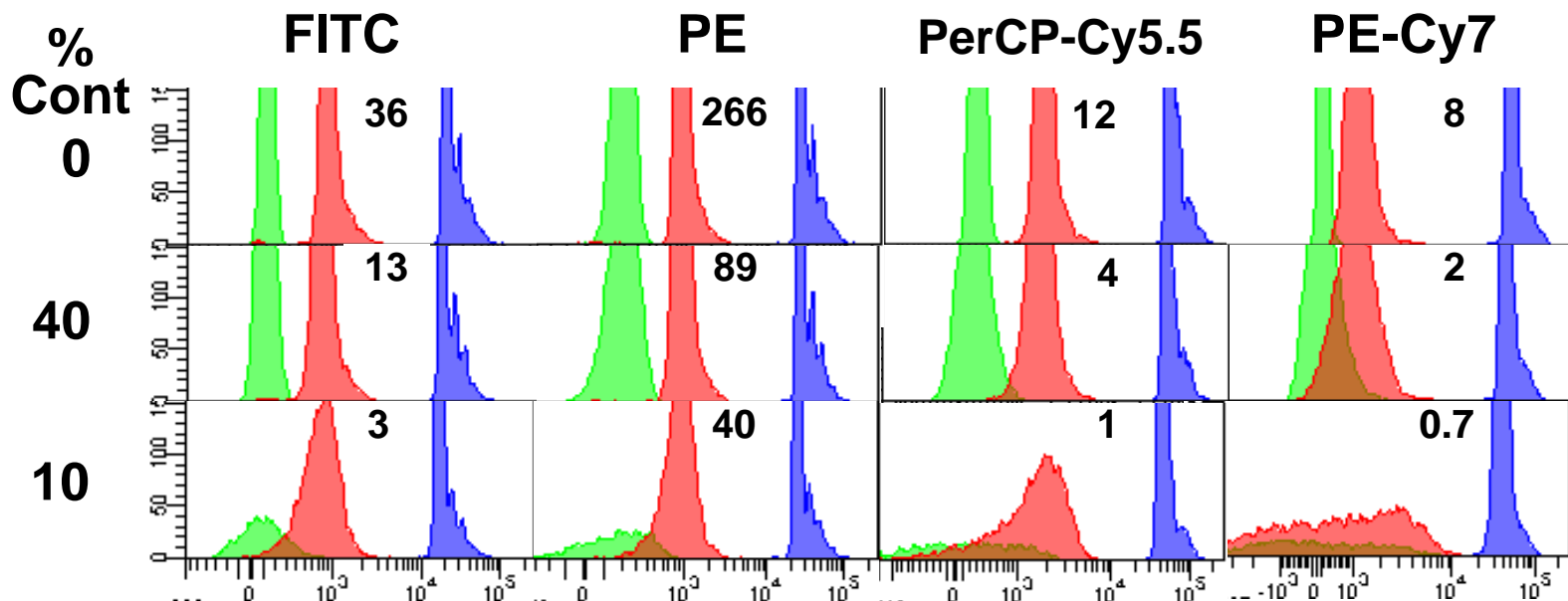

- **FITC is duller (lower SI), but with higher Qr is more resistant to changes in instrument performance (alignment and/or laser power).**
- **PE-Cy7 has a high SI because of low autofluorescence, but the low Qr means resolution of dim populations is significantly affected by changes in performance**
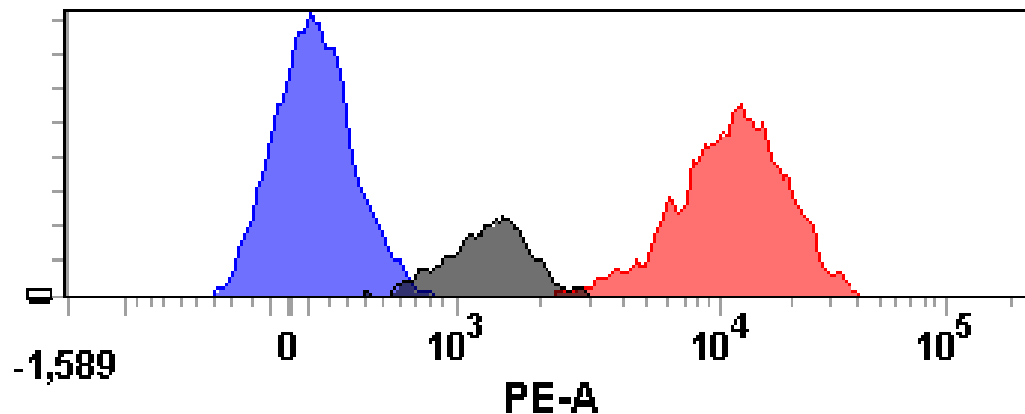
Anti-CD10 PE Staining Assay: Detuned

- CD10 staining good in all samples. Potential issue is failure to separate Eosinophils population in the FITC vs PE dot plot due to high autofluorescence in the detuned instrument.
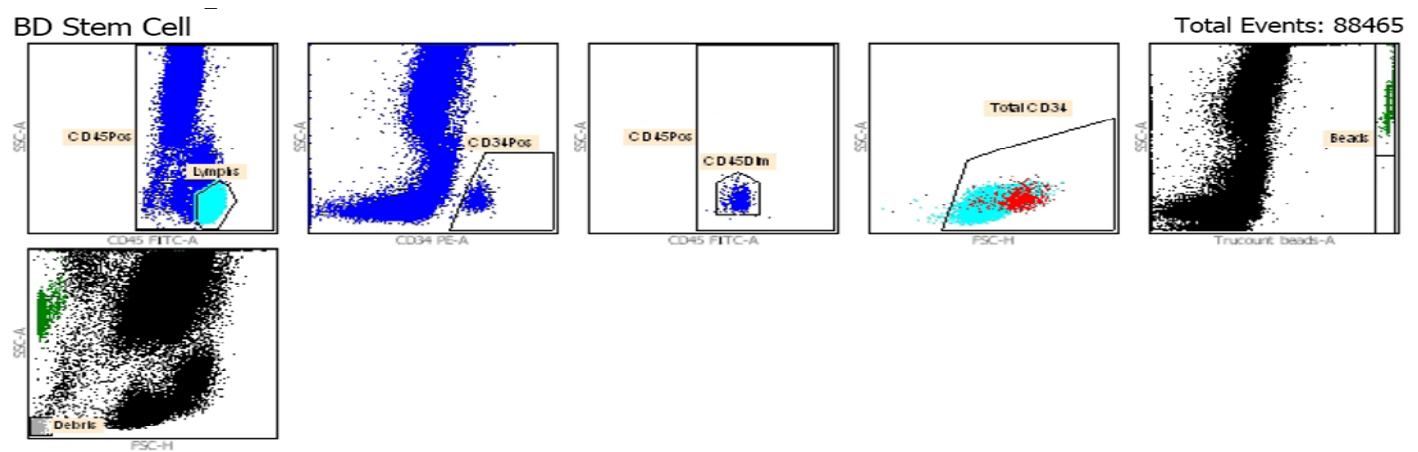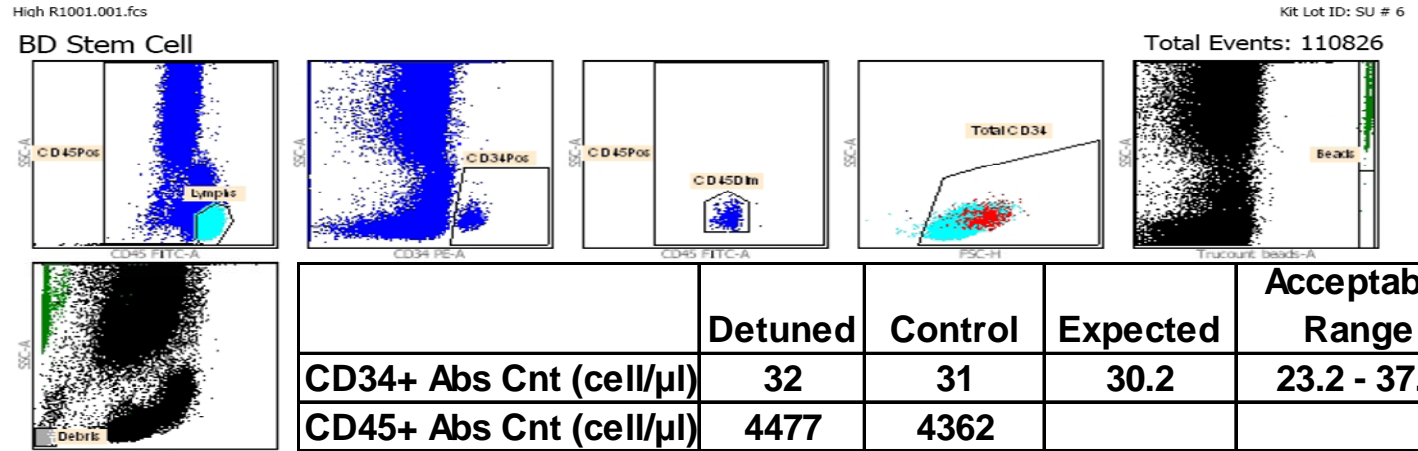

Normal Instrument
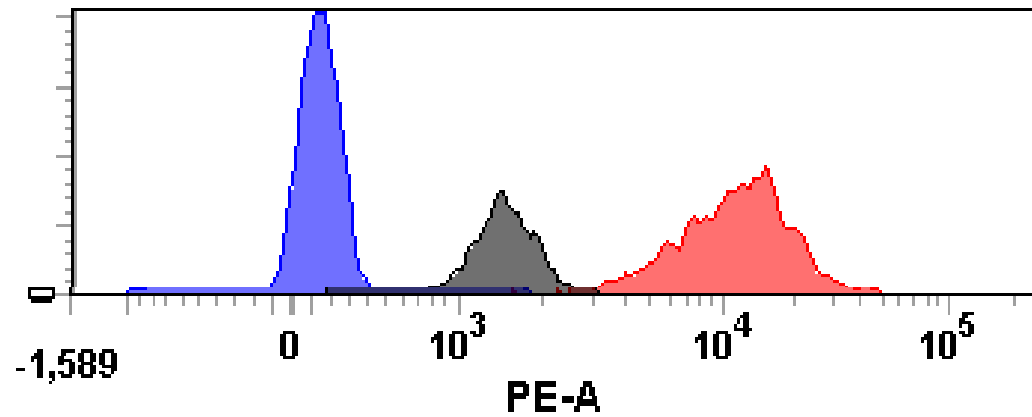
Detuned instrument (18% of Control)

CD34+ Cell analysis: Detuned vs Control Instrument

Control Instrument

Detuned Instrument (18% of Control)

	Detuned	Control	Expected	Acceptable Range
CD34+ Abs Cnt (cell/µl)	32	31	30.2	23.2 - 37.2
CD45+ Abs Cnt (cell/µl)	4477	4362		
Bead Events	8248	8460		
CD34+ as % CD45	0.75	0.64	0.723	0.56 - 0.89

Detuned and control give comparable results.

Summary: Steps to Creating a Cross-Site Multicolor Assay

1. Collect CS&T performance data on all instruments to be used in a study.
2. Choose which fluorochrome will be used with which reagent (CD).
   a) Use information on SI, antigen density, Qr, Br.
3. Set fluorescence intensity (gains) on the primary instrument.
   a) For each channel, identify worst linear range and SDen among instruments.
   b) Run stained cells to ensure all positive and negative populations are appropriately on scale.
4. Transfer gain settings to all cytometers in the study.
   a) Use common CS&T and or fluorochrome-specific controls.
   b) Use Application Settings to store and reproduce common gain settings across all instruments.
5. Confirm performance of the assay.
   a) Run the assay on instruments with the lowest performance characteristics.
   b) Run the assay on a detuned instrument.
      • Mimic the worst case scenario.

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Designing a Multicolor Experiment for Use Across Multiple Instruments

A Real-World Example:
The NIH Intracellular Staining (ICS) Assay
Quality Assurance Project
Maria Jaimes

NIH ICS Assay Quality Assurance Project

- **Fourteen labs world wide participated**
  - **BD FACSCanto and BD LSR II instruments with various instrument configurations.**

- **Common instrument QC bead**
  - **BD CS&T and Spherotech 8-peak beads**
    - **Monitoring Qr, Br, SDen, Linearity, Sensitivity**

Common lyophilized reagents
  - **BD CompBeads for compensation**
  - **BD reagents and protocol**
  - **Pre-stained control cells**
    - **Samples were analyzed for % positive for a number of intracellular cytokines.**

- **Goal: to correlate the instrument performance parameters with assay performance and establish minimum instrument performance for a given assay.**

Observation of Project Results

- **Initial poor assay performance is caused by**
  - **Instruments with low Qr and/or high Br**
    - **Correlates with the single color stain index**
  - **Differences in instrument setup**
  - **Instruments with high spillover**
    - **High spillover index might be due to**
      - **Poor filter selection**
      - **Gain settings**

- **Instrument performance, stable reagents, and setup are the keys to success for assay quality assurance.**
  - **Need stable fluorochrome-specific particles for cytometer standardization**

Instrument Performance and Assay Results Summary

Lab	Laser power	Filter	SDen	Qr	Br	SI Comp beads	8 peak beads	Lymphocytes BRIGHT MARKER (CD4 FITC)
8	20	530/30	26	0.015	976	55.6		
9	20	530/30	17	0.042	92	88.2		
10	20	530/30	23	0.010	298	80.5		
11	20	515/20	22	0.010	613	66.0		
12	20	525/50	9	0.007	2322	13.7		
13	90	530/30	20	0.018	2768	16.2		

* This variation in instruments is outside that expected for properly maintained instruments.

Improving Cross-Site Assay Reproducibility Using Standardized Instrument Setup

Inter-laboratory variability of cytokine responses across seven rounds of the Intracellular Staining Quality Assurance Program (ICS QAP) using a 4-color cocktail

SDs were calculated for all measurements (%positive/cytokine/antigen) done in a given round (each dot is the SD for a given response).
The horizontal lines represent the mean.

Majors improvements in data reproducibility after the first round were realized by optimizing instrument performance (PMs) and standardizing the assay setup

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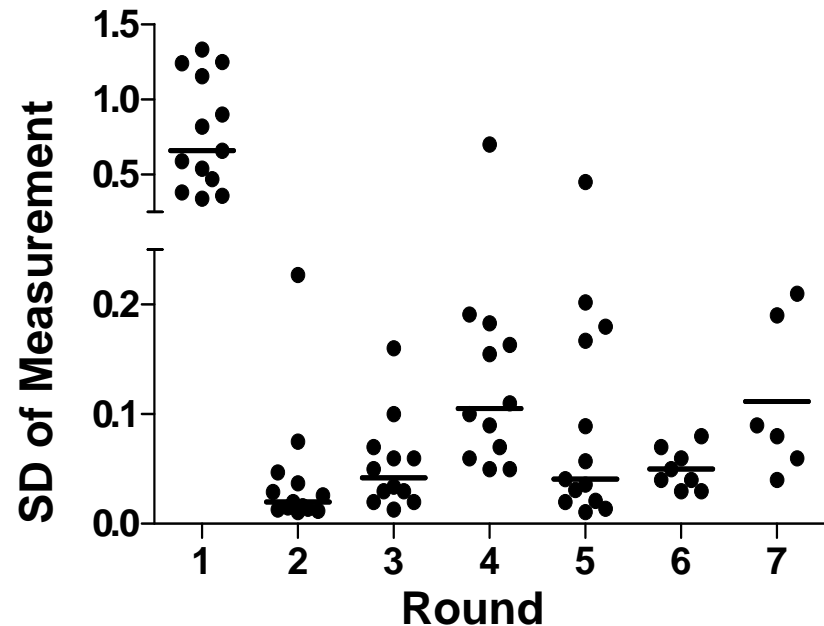
QUESTIONS?

Questions can also be emailed to researchapplications@bd.com

Acknowledgments

- **Ming Yan**

- **Maria Jaimes**

- **Dennis Sasaki**

- **Bob Hoffman**

- **Joe Trotter**

- **Neha Pathak**

- **Mark Edinger**

- **Pat Collins**

- **Yang Zeng**

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