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Title: Instrument Optimization for BD FACSCanto Instruments - Creating Application Settings for White Blood Cells using Lyse/Wash, or Lyse/No Wash methods

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OUTLINE

Instrument optimization is an often underestimated source of low resolution and high variability. It is important to optimize voltages for each PMT to determine and maximize the dynamic range available for positivity. An optimal dynamic range provides the best resolution for dim staining, while maintaining maximum range for very bright staining. The process described below, using objective values obtained from CS&T, should be followed to create an objective, optimized setup prior to assay validation. Once determined, CS&T Application Settings can be used to maintain optimized settings, while target particles can be used to standardize multiple instruments and reset optimization after a service visit.

PROCEDURE

Cloud Resources: See more information about this procedure and standardization using in the links to the BD Standardization Webinar & Technical Bulletin.

1. http://www1.bd.com/videos/bdb/webinars/facsdiva_pt1_9-2010/
2. http://www.bing.com/search?q=BD+CST+standardization&src=IESearchBox&FORM=IENTTR&conversationid

1. ***Select the laboratory's desired CS&T Configuration; perform a CS&T Baseline and Performance Check***
    a. BD Default configurations include one or more fluorochrome labels for each PMT, as well as a defined Window Extension (WE=7 for Canto A/Canto II and WE=3 for Canto 10 Color.). Some labs choose to create a customized configuration with optimized PMT labels and Window Extension. (See BD Window Extension Technical Bulletin). A WE=3 is recommended for the Canto.
    b. See CS&T Application Guide to create a custom configuration. Briefly, a BD Default configuration can be copied and pasted in the configuration view, renamed, and edited as desired.
    c. Click "Set Configuration" on the configuration to be used.
    d. Run a CS&T Baseline and Performance Check on the selected Configuration. (Baseline values will be used below.)

2. ***Prepare Cells***
    a. Typically, clinical labs use lyse-washed peripheral blood to create optimized settings.

b. Cells with the lowest autofluorescence should be used to optimize low-end resolution (typically lymphocytes,) and the brightest staining cells should be used to determine maximum voltages.

c. Cells should be lysed and washed according to the normal laboratory procedure. Examples include FACSLyse procedure or Pharmlyse/Ammonium Chloride Bulk Lyse Procedure. (See BD Document for bulk whole blood PharmLyse or Ammonium Chloride lysing procedure.)

d. In some cases, different optimized settings may be desired for largely varied sample types (e.g. RBC or Platelets vs. WBC.)

Tip: Prepare 2-3 tubes of unstained cells for procedures below.

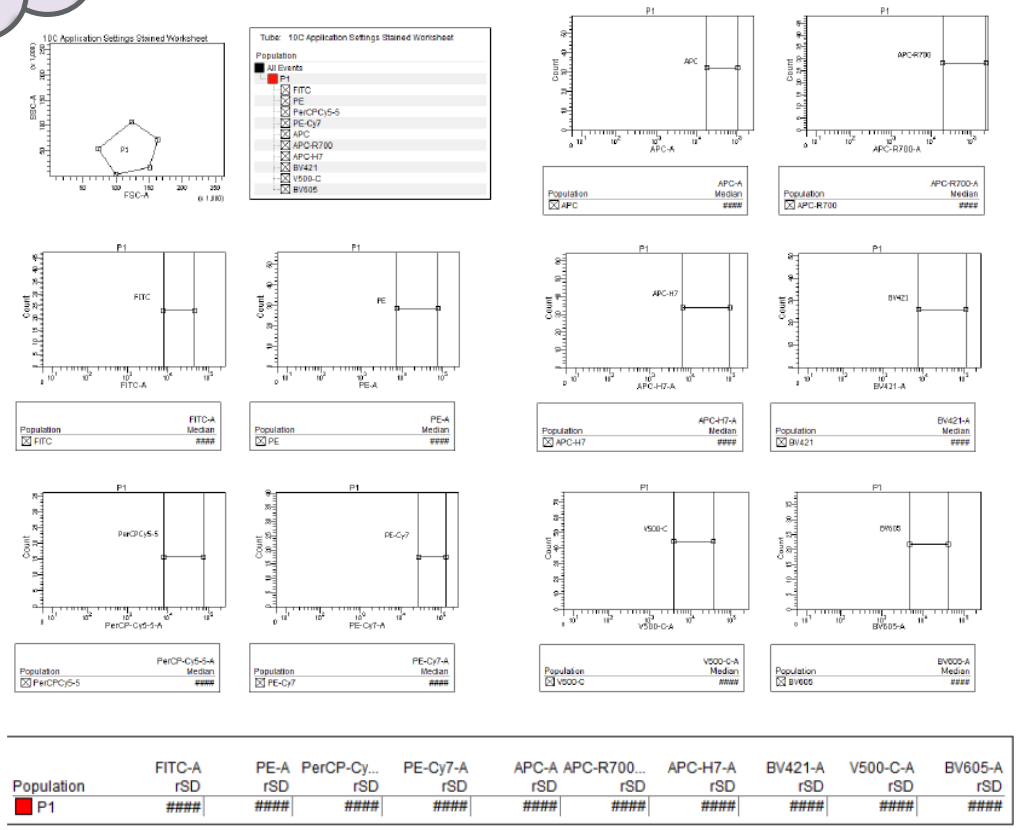
3. *Create an Experiment in Diva*

Note: Figure 1 is a template. A similar template can be obtained from a BD Application Specialist. The same template can be used for the unstained and stained samples (see Section 5) provided that the template includes the SD_{EN} (standard deviation of electronic noise) statistics. See tech note link for further explanation of SD_{EN} and FACSDiva.

Cloud Resources: Statistics Tech Note https://www.bdbiosciences.com/documents/Robust_Statistics_in_BDFACSDiva.pdf

a. Creating a new experiment and select the parameter labels to be used (Figure 1). Select FSC/SSC height and/or width if doublet discrimination is desired. Right click on the settings and Apply Current CS&T Settings.

b. Create a Global Worksheet with FSC vs. SSC plot.



Population	FITC-A rSD	PE-A rSD	PerCP-Cy... rSD	PE-Cy7-A rSD	APC-A rSD	APC-R700... rSD	APC-H7-A rSD	BV421-A rSD	V500-C-A rSD	BV605-A rSD
P1	####	####	####	####	####	####	####	####	####	####

Figure 1. Diva Experiment Template for Creating Application Setting.

Cloud Resources: See Video on this procedure http://www1.bd.com/videos/bdb/webinars/facsdiva_pt1_9-2010/

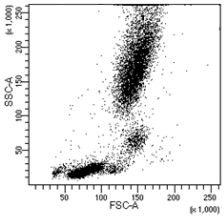
4. *Adjust FSC/SSC Voltages*

a. Acquire and adjust FSC and SSC voltages to place cells on scale between 50 and 100 on the FSC axis scale, just below 50 on the SSC axis (if linear scale,) and a FSC threshold to eliminate most of the debris (Figure 2).

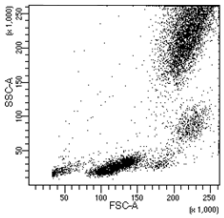
b. With optimized FSC, threshold is usually set to 30,000.

c. If linear SSC is used, it usually requires only small adjustments, if any.

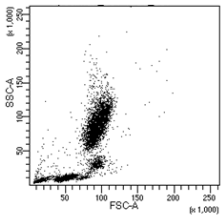
Tip: Set acquisition rate to low. This will allow ample time for adjustments.



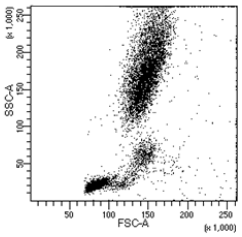
FSC/SSC and Threshold just right



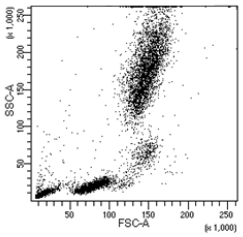
FSC/SSC both too high



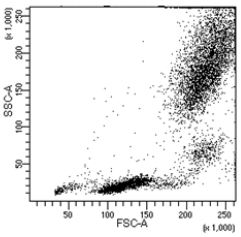
FSC/SSC both too low



Threshold too high



Threshold too low



Only FSC too high

Figure 2. FCS and SSC voltages optimally adjusted, too low and too high.

5. *Adjust PMT Voltages*

Note: An importable template can be obtained from a BD Application Specialist. The Experiment and Worksheet as shown Figure 1 can be used provided that the template includes the SDEN statistics. Create a statistics views for SDEN in each PMT.

a. Optimize low-end resolution.

i. From the baseline report, record the SDEN of each fluorescent channel.

Tip: For automatic calculations, enter into an excel worksheet (See Appendix) Extra channels can be left blank for <10 colors

ii. Calculate 2.5 x SDEN for each PMT. A value that is equal to, or greater than SDen×2.5 will be the Application Settings Target Values for each detector (Figure 3).

iii. Create a gate around cells of interest in the FSC/SSC plot created above (typically lymphocytes.) Create a statistics view and show the rSD values for each fluorochrome/PMT (Figure 3).

iv. Collect 5000 unstained cells prepared as indicated above. (Events must be RECORDED to obtain rSD values.)

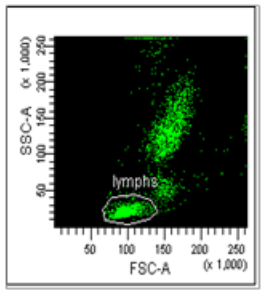
v. Compare rSD values to target SDEN values.

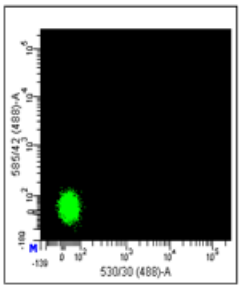
vi. Click "next" in the acquisition dashboard to obtain a new blank tube. Adjust voltages up or down to meet the targets.

vii. Repeat this procedure until all parameters are equal to or slightly above the SDEN targets.

Note: Although beyond the scope of this document, target values can be created with target particles to standardize additional instruments and/or easily recreate application settings in the future. See standardization bulletin, and refer to BD Application Specialist for advice regarding appropriate target particles. (Note: templates can be obtained from a BD Application Specialist.)

Target Values	
Detectors	rSDEN/ 2.5 x rSDEN
530/30 (488)-A	16.2/41
585/42 (488)-A	18.5/46
670 LP (488)-A	19/48
780/60 (488)-A	19/48
660/20 (633)-A	14.5/36
780/60 (633)-A	15/38
450/50 (405)-A	14.5/36
510/50 (405)-A	16/40





Population	530/30 (488)-A rSD	585/42 (488)-A rSD	670LP (488)-A rSD	780/60 (488)-A rSD	660/20 (633)-A rSD	780/60 (633)-A rSD	450/50 (405)-A rSD	510/50 (405)-A rSD
Lymphs	40	47	48	49	37	38	37	40

Figure 3. Adjusting the PMT Voltages. PMT voltages were adjusted as described in Section 5a using the SDEN based on the instrument's CS&T baseline values.

b. Optimize maximum signal.

Note: templates can be obtained from a BD Application Specialist.

i. From the Baseline Report, record the maximum linearity for each detector (MS Excel Worksheet above can be used.)

ii. Multiply each value by 0.5 to allow room for variation in individual patient staining. These values are the maximum median values for stained cells.

iii. Stain cells for the brightest signal to be detected in each detector. If unknown, CD8 can be used in every channel. However, this may unnecessarily reduce low end resolution.

iv. Create histograms for each parameter (based on a FSC/SSC gate.)
v. Create interval gates with median channel statistics for each parameter.
vi. Run cells prepared in iii and verify/adjust bright positive signal to be below the values determined in ii above (Figure 4).
    a) Adjust P1 gate over the appropriate population if needed-lymphocytes, granulocytes, monocytes, ect.
    b) Adjust histogram gates around the bright peaks
    c) Ensure that the Medians of all bright peaks fall below the maximum value determined in step 3.
    d) If any populations are above the maximum median, lower the voltage in that channel until the median meets the criteria
vii. If BD Comp Beads will be used for compensation, lower threshold to allow beads to be detected, or return FSC voltage to CS&T voltage. Repeat step vi above for stained Comp Beads. (typically less room for variability is required here.)
viii. Select FSC and/or SSC height and/or width if double discrimination is desired.
ix. Save settings as Application Settings. Use these Application Settings to obtain the optimal dynamic range (low end resolution to high end maximum) for all staining of similar sample type. (see Figure 2 above.).

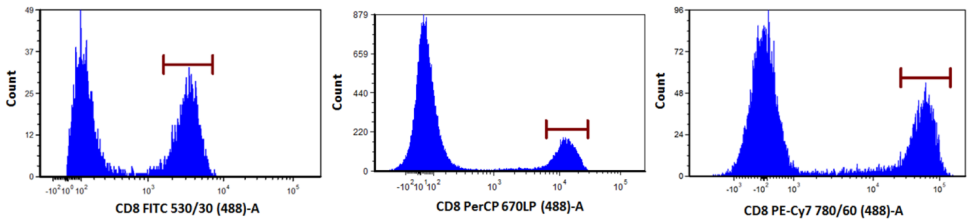


Figure 4. Maximum Signal Verification. Whole blood was stained with CD8 using a lyse/wash method to verify that the maximum signal was within the linear range as described in Section 5b.

Cloud Resources:

BD Compensation:
https://www.bdbiosciences.com/documents/BD_FACSDiva_setup_system.pdf

BD Technical Bulletin
http://static.bdbiosciences.com/documents/Compensation_Multicolor_T

7) Perform Compensation

a) Create compensation control experiment.
    i) Using the laboratory's panels, create a list of single-stained compensation controls. Ensure you have label-specific controls for all tandem reagents. For example, if your set-up contains CD4 APC-H7 and CD8 APC-H7, you will create a control for each marker (CD4 & CD8). Furthermore, the stained cells or bead must have only one fluorophore on it, so that measured

Tip: For easy re-creation save as an Experiment Template

spectral overlap values are due only to that single fluorophore (see BD compensation technical note; link above in tips).

ii) Create an experiment with parameters and labels desired for lab. Select FSC/SSC height and or width if doublet discrimination is desired. Choose SSC log or linear based on lab preference.

iii) Create a population gate in FSC vs. SSC plot.

iv) Apply Application Settings created in step 5 above.

v) Create compensation controls using list created in 7a. above. (See Diva Manual for detailed instructions on creating compensation controls) (Note: see b3. below. Either check or un-check "use separate unstained control" when creating compensation controls.)

Tip: For easy re-creation save the compensation specimen as a Panel Template

b) Prepare compensation controls.

i) Stain cells or BD Comp Beads with antibodies per the list created in 7ai above. (BD Comp Beads are highly recommended, but the laboratory must assess suitability.)

(1) Note that BD Comp Beads are species-specific, and can only be used with antibodies bearing an anti-kappa chain. (See product inserts.)

(2) Use the same staining protocol to be used for samples when using cells as controls. If using comp beads, follow SOP provided in package insert.

Tip: Create a Global Worksheet to acquire using the loader

(3) Unstained cells or beads with similar autofluorescence must be used as negative controls.

(4) Appropriate negative control particles can added to each positively stained tube (see 3 above,) or if all compensation tubes use the same particles, one negative particle tube can be used as control for all positive tubes.

c) Acquire and record compensation controls in compensation panel.

d) Review FSC/SSC gate and interval gates; adjust as necessary. (See Diva Manual.)

e) Review the compensation matrix to verify results are within reason and that no mistakes have been made.

f) Compensation is automatically calculated for each tube based on the single-stained control, name and save compensation.

Cloud Resources: Product inserts here: http://www.bdbiosciences.com/us/solrSearch?text=comp+beads&x=0&y=0

Tip: If BD comp beads were used to create compensation, edits FSC and SSC values for cells in the Compensation Settings Catalog

REFERENCES

1. RA Hoffman: Standardization, calibration and control in flow cytometry. In: JP Robinson, Z Darzynkiewicz, P Dean, L Dressler, H Tanke, P Rabinovich, C Stewart and L Wheeless, eds. Current Protocols in Cytometry. John Wiley & Sons, Inc., New York. (2005) 1.3.1- 1.3.21.
2. RA Hoffman and JCS Wood: Characterization of Flow Cytometer Instrument Sensitivity. Current Protocols in Cytometry. John Wiley & Sons. 1.20.1- 1.20.18 (2007)
3. Becton Dickinson Technical Note Standardizing Application Setup Across Multiple Flow Cytometers Using BD FACSDiva™ Version 6 Software. Ellen Meinelt, Mervi Reunanen, Mark Edinger, Maria Jaimes, Alan Stall, Dennis Sasaki, Joe Trotter
4. Package Insert from BD Cytometry Setup and Tracking Beads (CS&T), Becton Dickinson Biosciences, 2350 Qume Drive, San Jose, CA 95131-1807. June 2007.
5. Package Insert from BD CompBeads Anti-Mouse Ig, k/Negative Control (FBS), Becton Dickinson Immunocytometry Systems, 2350 Qume Drive, San Jose, CA 95131-1807. 552843 Rev 2.

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