AUTOCOMPENSATION

Prepared By: Kacee Floyd, MSc, BS, MB(ASCP)^{cm} Date: 10/11/2021
Document Control Number: MOL.20.010.00

Approved By:

Scott Arnold, MD Date: 10/29/21

Geoffrey Hahm, MD Date: 10-29-21

Jason Meyer, MD Date: 10/27/21

Ersie Pouagare, MD Date: 10/28/21

Date:

Adopted: Date: 11/1/2021

Reviewed:		Date:	
Reviewed:		Date:	
Reviewed:		Date:	
Reviewed:		Date:	
Reviewed:		Date:	

Retired: Date:

Kettering Health (KH) Organization-Wide Policy

KH adopts this policy for any of the following facilities that offer these services. The facilities are Kettering Health Main Campus, Kettering Health Miamisburg, Kettering Health Dayton/Kettering Health Washington Township, Kettering Health Greene Memorial, Soin Medical Center, Kettering Health Hamilton, Kettering Health Troy, all hospital off-sites, and KH Support Services.

POLICY

BD™ CompBeads are polystyrene microparticles which are approximately 3.0-3.4 µm in diameter and are used to optimize fluorescence compensation settings for multicolor flow cytometric analyses. The set of anti-mouse Ig, κ microparticles includes two populations. One population is a negative control, which has no binding capacity. The second population can bind any mouse κ light chain-bearing immunoglobulin. When the beads are mixed together with a fluorochrome-conjugated mouse antibody, they will provide distinct positive and negative (or background fluorescence) stained populations which can be used to set compensation levels in a manual process or with the autocompensation protocols in the instrument software. BD FACSDIVA™ software includes a compensation setup feature that can automatically calculate spectral overlap values for an experiment, which can help to save time and eliminate inaccuracies introduced with manual compensation. Since the compensation adjustments are made using the fluorochrome-labeled antibodies that are utilized in the experiments, the combination of CompBeads with the compensation setup in DIVA allows the laboratory to accurately establish the spectral overlap corrections for any combination of fluorochrome-labeled antibodies.

To determine compensation values, spectral overlap values are measure for all fluorophores (via single –color control samples) in every detector. An array of spectral overlap values is inverted by matrix algebra to create useable, and traceable, compensation values. These compensation values are what can be used by the flow cytometer to subtract out the contributions of non-primary colors overlapping into a particular detector. This allows the fluorescence of interest to be seen without spillover from the other fluorophores.

SPECIMEN:

No specimen is utilized for this procedure, only control material. Verification was based on comparison of beads to multiple specimen types (data on file).

SUPPLIES AND EQUIPMENT:

1. Corning™ Falcon™ Round-Bottom 12 X 75 mm (5mL) polystyrene tubes. Fisher HealthCare Catalog #14-959-6 (Corning 352052). Or appropriate equivalent brand.
2. Assorted-size sterile, filtered pipet tips for adjustable pipets.
3. Canto Carousels
4. Multiple adjustable pipettors (10 µl, 20 µl, 100 µl, 200 µl, 1000 µl)
5. Vortex-Genie mixer
6. BD Canto II Flow Cytometer
7. BD Lyse Wash Assistant (LWA)
8. Dade Immufuge™

REAGENTS:

All antibodies have the storage requirement of 2-8°C, and each individual lot will have the expiration date printed on the label. In addition, bottles should be kept in the dark.

1. BD Pharmingen™ Stain Buffer (BSA), 500 mL. BD Catalog # 554657. Stored at 2-8°C.
2. Gibco™ Dulbecco's Phosphate Buffered Saline (DPBS) without Calcium and Magnesium, 1X concentration, 6 x 1L case. Fisher HealthCare Catalog # 14-190-235 (Gibco 14190235). Stored at 15-25°C (room temperature).
3. BD Anti-Mouse Ig, κ/Negative Control Compensation Particles Set. BD Catalog #552843. Stored at 2-8°C.
4. Cytometer Setup & Tracking (CS&T) Beads Kit (FACSDiva v7+), 150 tests. BD Catalog # 655051. Stored at 2-8°C.
5. BD FACSFlow™ Sheath Fluid, 20 L. BD Catalog # 342003. Stored at 15-25°C (room temperature).
6. BD™ Stabilizing Fixative 3X Concentrate, 90 mL. BD Catalog # 338036. Stored at 15-25°C (room temperature).
    a. Diluted to 1X for working concentration (see preparation in 32 00 01 Immunophenotyping procedure)
7. CD19 FITC ASR Clone SJ25C1. BD Catalog # 340719
8. CD5 PE ASR Clone L17F12. BD Catalog # 340697
9. CD8 PerCP-Cy5.5 ASR Clone SK1. BD Catalog # 341049
10. CD13 PE-Cy7 ASR Clone L138. BD Catalog # 338432
11. CD2 PE-Cy7 ASR Clone L303.1. BD Catalog # 335804
12. CD5 PE-Cy7 ASR Clone L17F12. BD Catalog # 348800
13. CD33 PE-Cy7 ASR Clone P67.6. BD Catalog # 333949
14. CD10 PE-Cy7 ASR Clone HI10A. BD Catalog # 341102
15. CD56 PE-Cy7 ASR Clone NCAM16.2. BD Catalog # 335809
16. CD25 PE-Cy7 ASR Clone 2A3. BD Catalog # 335807
17. CD200 PE-Cy7 ASR Clone MRC OX-104. BD Catalog # 655735
18. CD34 APC ASR Clone 8G12. BD Catalog # 340667
19. CD20 APC-H7 ASR Clone L27. BD Catalog # 641405
20. CD3 APC-H7 ASR Clone SK7. BD Catalog # 641406
21. CD14 APC-H7 ASR Clone MφP9. BD Catalog # 643077
22. CD4 BV421 ASR Clone SK3. BD Catalog # 659476
23. CD45 V500-C ASR Clone 2D1. BD Catalog # 647450

All reagents and antibodies have expiration dates on the individual components that must be observed.

PROCEDURE:

INSTRUMENT START-UP:

1. Prior to warming up the Canto II cytometer, empty the waste container and condensation trap.
2. Power on the instrument and allow to warm up for at least fifteen (15) minutes.
3. Power on the accompanying computer, if not already on. The computer user should be BDAdmin (Administrator) and the login password is BDIS#1.
4. Launch the BD FACSDiva software from the desktop icon.
5. Once the software indicates that the cytometer is connected and warmed-up, perform "Fluidics Start-up".
6. Perform daily cytometer QC by using the Cytometer Setup and Tracking (CS&T) module in the software menu.

a. Note: This will display a separate software screen.
b. Set up CS&T tube by pipetting 350 µl of sheath fluid into a 5 mL tube.
c. Gently vortex (quick tap) the CS&T beads, and place one drop into the tube.
d. Perform a quick vortex, and then place the tube in position 1 of a Canto Carousel for acquisition.
e. Use the "Check Performance" option to acquire, after verifying that the lot is accurate.
f. Check ΔPMTV values and P/F columns and/or values for outliers prior to moving forward. If any value is out of range, CS&T may need to be repeated to confirm value. See user guide for values out of range.
    i. Note: The software will use internal ranges to yield a Pass or Fail result for the run. This should be used as the initial data check. If any ΔPMTV is trending ±10 or a PMTV value is >2SD on the trend plot, repeat testing should be performed. Any continuing outlying trends should prompt a call to BD Technical Support for further guidance.
    ii. The CS&T software will calculate optimal voltages for the instrument with this daily run data and individual tube data – yielding consistency in testing.

7. Once CS&T has passed successfully, close out the module and return to FACSDiva proper.
8. If needed, perform baseline testing on CS&T prior to a daily performance check (every 6 months / lot change).
    a. Add 1 mL of sheath fluid to a 5 mL tube.
    b. Gently vortex (quick tap) the CS&T beads, and place three drops into the tube.
    c. Perform a quick vortex, and then place the tube in position 1 of a Canto Carousel for acquisition.
    d. Use the "Define Baseline" option to acquire, after verifying that the lot is accurate. New bead lot files can be found on BD Biosciences website.
    e. Once the Baseline passes, a daily tube will need to be tested prior to returning to the FACSDIVA software.
    f. If values are out of range, repeat set-up with a new bottle of beads. If values are still out of range – consult user guide or contact BD Technical Support.

COMPENSATION TUBE PREPARATION:

1. Set up a tube rack that contains eighteen (18) of the 5 mL tubes. Label tubes according to Table 1 below.
2. Remove compensation bead set, stain buffer, and antibodies from the refrigerator and take to the biosafety cabinet (BSC).
3. Using an adjustable micropipettor, add 100 µl of stain buffer with BSA to each compensation tube.
4. Carefully vortex the compensation beads with quick bursts as well as manual inversion / hand-rolling to mix the beads well.
    a. The white cap bead bottle contains a nil population of beads – to act as a guide to population density and SSC/FSC on the cytometer. The blue cap bead bottle should be used for all tubes that require staining.

5. Using the squeezer drop top on the bead bottles, carefully add one drop of the appropriate bead solution to each of the compensation tubes. The white cap should only be used for the "Unstained" tube, while the blue cap solution should be utilized for all tubes to be stained.
    a. If beads should happen to drop to the side of the tube instead of down into the stain buffer, either tap down the drop, pipette it down, or flush with the stain buffer from the bottom of the tube.
    b. The "Unstained" tube is now finished and can be put aside.
6. For all the tubes with antibodies – carefully add the matching antibody and volume to the appropriately labeled tube. See Table 1 for reference if needed.
7. Perform a quick vortex to each tube to mix well.

Tube Name	Bead drop	Antibody	Volume of Antibody
Unstained	1 from white cap	No antibody	----
FITC generic (CD19)	1 from blue cap	CD19 FITC	20 µl
PE generic (CD5)	1 from blue cap	CD5 PE	20 µl
PerCP-Cy5.5 generic (CD8)	1 from blue cap	CD8 PerCP-Cy5.5	20 µl
PE-Cy7 generic (CD13)	1 from blue cap	CD13 PE-Cy7	5 µl
PE-Cy7 specific CD2	1 from blue cap	CD2 PE-Cy7	5 µl
PE-Cy7 specific CD5	1 from blue cap	CD5 PE-Cy7	5 µl
PE-Cy7 specific CD33	1 from blue cap	CD33 PE-Cy7	5 µl
PE-Cy7 specific CD10	1 from blue cap	CD10 PE-Cy7	5 µl
PE-Cy7 specific CD56	1 from blue cap	CD56 PE-Cy7	5 µl
PE-Cy7 specific CD25	1 from blue cap	CD25 PE-Cy7	5 µl
PE-Cy7 specific CD200	1 from blue cap	CD200 PE-Cy7	5 µl
APC generic (CD34)	1 from blue cap	CD34 APC	5 µl
APC-H7 generic (CD20)	1 from blue cap	CD20 APC-H7	5 µl
APC-H7 specific CD3	1 from blue cap	CD3 APC-H7	5 µl
APC-H7 specific CD14	1 from blue cap	CD14 APC-H7	5 µl
BV421 generic (CD4)	1 from blue cap	CD4 BV421	5 µl
V500 generic (CD45)	1 from blue cap	CD45 V500	5 µl

Table 1: Compensation tube layout and volumes

8. Incubate the tubes in the dark at room temperature (15-25°C) for 15 minutes.

9. Remove the tubes from the dark and add 2 mL of stain buffer to each tube.
10. Centrifuge at 600g for 3.0 minutes (Immufuge-high speed)
11. Decant the fluid away from the bead pellet into a waste flask.
    a. Manually pipetting can be done as well to remove the fluid, as long as the pellet is not disturbed.
12. Re-suspend the pellet in 500 µl of 1X Stabilizing Fixative
    a. Can re-suspend in 500 µl of stain buffer if acquiring promptly
13. Vortex the tubes thoroughly in quick bursts
14. Proceed to acquisition.

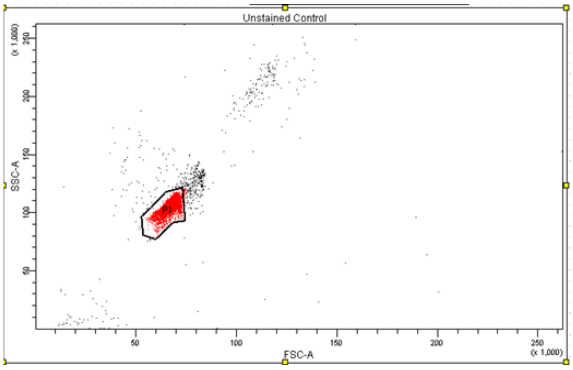
PLEASE NOTE: An automated process is available on the BD Lyse Wash Assistant that will allow for steps 8-13 to be done as a hands-off process. The "Comp-Inc-Wash-Fix" process is available. A manual program can be used as well if the staining incubation is done off-board.

EXPERIMENT SET-UP AND ACQUISITION:

1. Under the Compensation folder with monthly sub-folder, create a new experiment.
2. From the 8C tab of experiments, select the *8C Compensation_BV421* experiment.
    a. This template includes all the tubes in order for testing.
3. Right click on the "Application Settings" heading and go to "Apply".
4. Apply the *8C APPLICATION SETTINGS BV421.*
    a. If prompted to "Set all to zero" for values for FSC scaling, click "Yes".
5. Load the prepared compensation tubes onto a Canto carousel in the order matched to what is listed in the experiment.
6. Load the carousel onto the Canto – noting which number it is.
7. Go to the FACSDiva menu for "Carousel" and choose the "Carousel Setup".
8. Apply the matching carousel number to the tubes.
9. On the right-hand screen, toggle the first button so that the Global worksheet (green tab) is on top.
10. On the acquisition pane, click "Run Carousel".

AUTO-COMPENSATION / OPTIMIZATION:

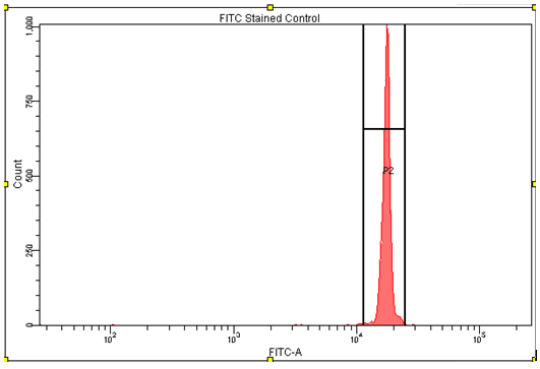
1. Once acquisition has finished, toggle the worksheet from Global to Normal (green tab to white tabs).
2. Narrow in the P1 gate around the low FSC/SSC population, capturing the majority of events around the dense center. Example shown below.
    a. Do not over-narrow, as this can result in event loss.

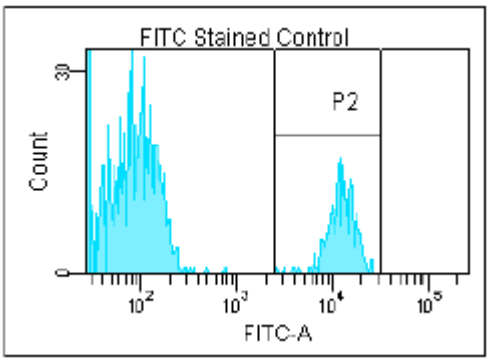


P1 Gate Settings

3. Once the P1 gate is narrowed, right click the actual gate border and choose the command that will apply the gate to all the other tubes (Apply to All Compensation Controls).
4. The gate will then copy to all the other tubes and should capture a matching population on each. If a tube's population of interest falls outside this gate, re-acquire. If the new acquisition is still out of range, the tube will need to be set up again.
5. Proceed tab by tab to narrow the gate around the positive population on the histogram per fluorophore. See examples below.
    a. The gate should be brought in around positive peak without maintaining a lot of debris around the base – closer to the red peak P2 than the light blue P2 for the shown examples.
    b. Do not over-narrow, as this can result in event loss.

P2 Histogram Settings





6. After all the P2 gates have been adjusted, go to the "Experiment" menu and scroll down to "Compensation Set-up"
7. Click "Calculate Compensation"
    a. If the auto-compensation has passed via the comparison algorithms in FACSDiva, the software will show a successful calculation and allow for the option to "Link and Save".
    b. If a tube in the auto-compensation fails, the software will prompt review.
        i. Review the gate and if it is in the proper place, then it may be possible then that individual tube will need to be re-run. Some peaks are too wide, or some populations have dual expression due to partial staining – resulting in a dual "horn-like" peak. Both of these circumstances demonstrate failed compensation examples.
        ii. If repeat tubes fail, review of antibody quality controls should occur. If multiple tubes fail, the bead mixture should be reviewed for well mixing as well as the mixing with the antibodies.
8. As compensation is performed on beads, but the experiments are set up for cellular material, some manual adjustments to the compensation must be made.
9. Go to the "Cytometer" menu and select "Catalogs"
10. Sort the files to select the matching compensation that just passed calculation, then click "Edit".
11. Reduce the FSC by a value of 200 to accommodate cells (e.g. 462 for beads →262 for cells).
    a. Note: This is the only regular voltage change utilized for daily experiments. If data looks skewed, the value may need to be manually adjusted so populations are rounded and discrete. Negative and positive populations should be well separated and distinguishable.
12. Change the threshold from 10,000 for beads to 30,000 for cells.
13. Save the corrected settings.
    a. If a prompt comes up about settings already being linked and should changes be saved – respond with "OK" then close the pop-up.

APPLYING COMPENSATION:

1. Once the daily compensation has been run and adjusted for cells, it is able to be utilized for patient testing.
2. Under the appropriate month folder, create a new experiment using the "Experiment" menu in the top bar.
3. Select the choice experiment that needs to be utilized (e.g. 8-color basic or titration)
4. Rename the experiment with the date (and type if needed)
5. Right click on the experiment name and select "Application settings" then choose "Link Setup"
6. Choose the newest compensation that was acceptable and click "Link"
7. New specimens can now be added to the experiment and will run with the compensation data filter that was attached

CRITICAL POINTS:

The auto-compensation process performed by FACSDiva serves as a quality control for instrument operation and experiment optimization for multi-color immunophenotyping.

- If the color compensation fails repeatedly upon re-acquisition and re-setup – contact BD technical service for additional trouble shooting.
- Beads re-suspended in stain buffer should be acquired within 30 minutes of the process being finished.
- If one particular parameter shows shifting beyond values seen in the spectral overlap for previous runs, investigate the antibody lots and expiration dates.
- If multiple parameters show shift on the same color laser, note which ones are affected and contact BD technical service for laser adjustment/cleaning.
- Make sure beads are well mixed to facilitate the proper amount of bead to antibody ratio.

REFERENCES:

1. BD FACSCanto II Instructions for Use BD642239 Rev A June 2007.
2. BD FACSDiva Software Quick Start Guide BD338574 Rev A September 2004.
3. BD™ CompBeads Technical Data Sheet, BD552843 Rev 5 – 2017.