Protocol: Experiment Setup (BD Analog Instruments)

Steps:

1. Select either the FACS Comp or manual method from the chart below, and follow the steps for voltage setting, then compensation. Use the instructions for "First-Time Run of a New Panel" when running a new combination of antibodies or an existing combination of antibodies on a new cell type. Use the instructions for "Subsequent Runs of the Same Panel" when repeating experiments with the same antibodies and cells.

2. Run experiment samples, followed by appropriate shutdown procedures for the instrument.

Notes:

1. FACSComp, while simple and highly automated, offers only two types of voltage settings (LW or LNW). In some cases, neither of these will be ideal for a particular reagent panel. Additionally, compensation can be slightly less accurate than with manual setup.

2. Compensation should be calculated for each day's experiment. Importing previous settings (including compensation) is only acceptable if there is to be NO change in PMT voltages, and if NO tandem dyes are used in the experiment (these can vary experiment-to-experiment in their compensation requirement).

3. CompBeads are generally preferable to single-stained cells as compensation controls, as long as the antibody efficiently binds the appropriate bead. Calibrite beads are also acceptable in most cases.

4. PerCP and PerCP-Cy5.5 have different compensation requirements, and are best not combined in the same experiment.

Option	**First-Time Run of a New Panel**		**Subsequent Runs of the Same Panel**	
	Voltage Setting	**Compensation**	**Voltage Setting**	**Compensation**
FACS Comp	1. Open FACSComp software, choose 3- or 4-color LNW or LW setup and appropriate FL3 color (PerCP or PerCP-Cy5.5). 2. Load appropriate beads as prompted by the software. 3. Opening CellQuest Pro after successful completion of FACSComp will automatically import the voltage and compensation settings thus established.			
Manual	1. Run unstained cells and place peak in the middle of the first decade for each color. 2. Run fully stained cells and decrease the voltage for any color in which events are off-scale. 3. Run mid-range beads and record medians in each color. These will be your target channels.	1. Run single-stained CompBeads for each color. 2. Acquire with compensation off, and calculate compensation in FlowJo prior to analysis, using the compensation calculation tool.	1. Run mid-range beads, and adjust voltages (if needed) to place medians within 10% of the target channel for each color.	1. Run single-stained CompBeads for each color. 2. Acquire with compensation off, and calculate compensation in FlowJo prior to analysis, using the compensation calculation tool.

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Reagent Recommendations:

Reagent	**Catalog Number**	**Instructions for Use**
BD Calibrite beads	340486 (unlabeled, FITC, PE, PerCP beads) 340487 (APC beads) 345036 (PerCP-Cy5.5 beads)	Use 1 drop + 350 μL FACS Flow buffer.
BD Mid-range beads	556298 (Sphero rainbow particles, mid-range FL1 fluorescence)	Use 1 drop + 350 μL FACS Flow buffer.
BD CompBeads	552843 (anti-mouse κ) 552844 (anti-rat κ) 552845 (anti-rat/hamster κ)	Use 1 drop per sample. Negative CompBeads can be included in each tube and/or run as a separate negative control (the latter is preferable).