

Overview of the principles of Luminex Calibration and the effect of altering RP1 target values on instrument reporter channel sensitivity.

Calibration of the Luminex is essential for optimal performance and day-to-day reproducibility of results. The Luminex should be calibrated each day after the start up procedure is complete and the optics have been warmed up. Also, the cytometer must be re-calibrated if the instrument temperature changes by more than 3° C during the course of the day.

Before calibrating, make sure that optics warm up is complete. The calibrators (CAL1 and CAL2 beads) are microspheres with stable fluorescent intensities in the RP1, CL1, and CL2 wavelength ranges. The calibration process uses these microspheres to adjust voltage settings for optimal and consistent microsphere classification and reporter readings over time and across different instruments. Current calibrated settings are automatically applied to any new session. The calibration procedure requires the bottle of CAL1 beads, the bottle of CAL2 beads, and sheath buffer water.

The CAL1 beads (Red cap) calibrate the Luminex doublet discriminator and classification channels (CL1 and CL2), while the CAL2 beads (Green cap) calibrate the reporter channel for reporter fluorescence detection.

The CAL2 calibration bottle supplied by Biorad lists two different RP1 target values:

- 1) Low RP1
- 2) High RP1

Biorad state that *"Bio-Plex phosphoprotein assays require calibration using the High RP1 target value, while Bio-Plex cytokine assays may be calibrated with either the Low or High RP1 target value. Other Luminex-type assays typically require calibration with the Low RP1 target value"*.

The Bioplex cytokine assay instruction manual (rev d) also contains the following guidelines , *"If you have prepared up the 2-32,000 pg/ml standards set, use the RP1 LOW target value for CAL2 calibration. If you have setup the 0.2-3,200 pg/ml standards set, use*

the RP1 HIGH target value for CAL2 calibration. Both the HIGH and LOW RP1 target values are listed on the CAL2 calibration bottle label."

"If you are running the 1.95 – 32,000 pg/ml cytokine standard curve (LOW RP1 Target value), do not change your Luminex settings. Calibrate with the Luminex CAL2 settings. Set gates according to Luminex procedure.

If you are running the 0.2 – 3,200 pg/ml cytokine standard curve (High RP1 target value), you will need to calibrate using HIGH RP1 (high PMT) calibration for CAL2. If you are using Luminex calibration beads, you will notice that the high RP1 value (high PMT) is not printed on the vial. The equation below provides the conversion factor to calculate the high RP1 (high PMT) value when using Luminex calibration beads.

Luminex RP1 x 4.55 = Bio-Plex high RP1 (high PMT)."

To better visualise the effect of altering the RP1 Target values on the sensitivity of the Luminex system we have performed the following test.

The instrument was first calibrated as normal and we collected samples of Low and High ACS QC beads * in triplicate. The mean MFI for the low and High beads were calculated.

Replicates of the identical ACS reagents were collected having calibrated the instrument to a range of different RP1 Target values as shown in the table below

RP1 Target Value	Resulting RP1 instrument Setting after Calibration	CL1/CL2/DD Voltage setting	Low Mean MFI	High Mean MFI
Normal (3885)	523	85/66/43	7	14407
X 4.55 (17676.75)	Would not calibrate			
/ 4.55 (853.85)	Would not calibrate			
+ 20% (4662)	540.7	85/66/43	9	17082
- 20% (3108)	511.6	85/66/43	5	11641

2000	480.10	85/66/43	2.3	7483.3
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*Applied Cytometry Systems QC "LOW" beads comprise Bead ID 54 with a "low" concentration of Phycoerythrin directly coupled to their surface. Applied Cytometry Systems QC "High" beads comprise Bead ID 54 with a higher concentration of Phycoerythrin than the "low" bead set directly coupled to their surface.

Note: Because calibration beads are highly concentrated, calibration via STarStation software is followed by four wash cycles using sheath Buffer.

Note: If you see a drastic change in a detector's voltage from one calibration to the next, it could indicate a problem with the instrument. A steadily increasing detector voltage may indicate that the laser is decreasing in intensity.

Note: You should never alter the target values for the CAL1 classification beads, since this could potentially influence the ability of the instrument to classify bead events.

Note: Performing a calibration with CAL2 alone does not reset the dCal value.

Conclusion

The effect of altering the CAL2 target value influences the sensitivity of the instrument.

In STarStation there is no direct way of altering the voltage of any of the detectors. Instead we can alter the sensitivity of rp1 detection for example via modification of the cal2 reporter target value.