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TECHNICAL APPENDIX: ADDITIONAL GUIDELINES FOR ABSOLUTE COUNTING TECHNIQUES: HOW TO IMPROVE PRECISION AND ACCURACY

A. Pipetting Technique

1. The primary sample pipetting step plays the major role in influencing measurement precision and accuracy.
2. Reverse pipetting is always mandatory. Press the pipette plunger at second stop, insert tip into the sample, draw sample (an excess sample is aspirated), then dispense at the first stop (the tip contains the excess sample, which must not be distributed).
3. Never use the first sample taken for dispensing (dry tip dispensing). Draw sample at the second pipette stop, then do two to three gentle dispense/draw cycles at the first stop, keeping the pipette tip within the sample. You are ready to dispense at the first stop (wet tip dispensing).
4. Before dispensing, wipe away the extra sample that sticks to the outer wall of pipette tip using gauze or tissue. Avoid touching the terminal tip hole to prevent inadvertent sample absorption due to capillarity.
5. Before dispensing, place the pipette tip at the dry round bottom of the test tube. Do not touch the tube wall

with the tip side. This applies only with the primary sample dispensing (see point B.3).

6. DISPENSE at the first plunger stop.

7. If repeated sample dispensing with the same tip is made by manual pipetting, do not lift the thumb after each dispensing. Keep the plunger pressed steadily at the first stop until another take/dispensing cycle is done (points A.3–A.6).

8. The same rules apply to the drawing and dispensing of FlowCount microbeads and for other small-aliquot reagents in volumetric counting techniques.

B. Pipette Calibration

Always use the above described pipetting technique.

1. Blood is much more viscous than water, and its specific gravity is estimated around 1.08 g/mL.

2. The pipette (either manual or electronic) must be calibrated using a precision weighing scale. First, adjust the volume using distilled water (1 μ L water = 1 mg) in a repeated series of reverse dispensings. Second, calibrate for blood dispensing keeping the 1.08 correction factor for specific gravity (i.e., 100 μ L blood = 108 mg).

3. Pipette calibration must take into account the decision about how to deal with residual droplets that may remain at the pipette tip end. This mostly applies to the dispensing of secondary reagents such as counting beads.

4. Residual sample droplets are sensitive to thumb energy: if the dispensing action is quick, droplets do not tend to form and vice versa.

5. Another calibration decision may also derive from the dispensing habit, namely, if the secondary reagent is injected in air from above the existing sample or if the tube wall is touched just above the cell suspension surface. In either case, the residual droplet has a different influence and must be taken into account accordingly.

6. The size of the pipette tip terminal bore influences the residual droplet formation. Narrow bores ensure less droplet formation. Recalibrate the dispensing procedure when new lots or different brands of pipette tips are used.

7. When FlowCount microbeads are added to lysed blood (i.e., when the sample is already present in the tube), the points from B.3 to B.5 are particularly critical.

C. General Rules for Counting With Microbeads

Whatever microbead type and brand are used, the following rules must be applied to ensure best assay performance. The above-described pipetting technique and calibration procedures must be applied.

1. Beads are mixed with samples so that the approximate final concentration of 1,000 beads per microliter of original sample is usually obtained.

2. Sample cell concentration must be not higher than 30,000 cells per microliter and the total cell/bead event ratio should not be >10 . In case sample dilution is required, always use PBS supplemented with 1% BSA to ensure an adequate protein content.

3. At least 1,000 bead events must be included in the sample list mode file (best 2–3,000). The relevant cell events must not exceed more than four to five times the bead events to ensure proper statistic robustness.

4. When the total analyzed sample amount is the measurement endpoint (e.g., in leukoreduced blood products analysis), the number of acquired microbeads is a rough indicator of how much sample has been processed.

5. Ensure proper instrument setup and sample injection pressure to acquire at rates not higher than 3,000–4,000 events per second. This helps to minimize event loss for coincidence and to reduce bead peak CV.

6. Ensure that highly intense microbead fluorescence signals are as much as possible on scale. This helps to avoid prolonged photomultiplier tube (PMT) blinding and event loss due to PMT oversaturation (increased PMT dead time).

7. Ensure that sheath fluid tank is over 50% full. This seems to increase flow stability.

8. Avoid bubble formation in any instance. Bubbles capture beads by capillarity and remove them from the suspension.

9. Vortexing can be applied with care, provided proteins are present in the resuspension medium. Vortexing start must be gradual, and the mechanical mixing must not exceed 5 s at maximum 50% of tube height. Mixing samples by inversion after tube capping is also applicable.

10. During very lengthy sample runs (i.e., more than 8–10 min), beads tend either to sediment or to float, thus causing a change in the proportion of cells and beads simultaneously aspirated. An additional mixing is therefore warranted if the run lasts more than 8–10 min.

D. Additional Usage Tips for Beckman-Coulter FlowCount Beads

The following guidelines may be in some contrast with official manufacturer's instructions.

1. Always keep the bead bottle in upright position and tightly stopped.

2. FlowCount beads completely float at the suspension medium surface if the bottle is kept unperturbed for more than 12 h.

3. The first bottle opening must be preceded by a very thorough mixing and the aluminum foil seal must be completely removed after the first mixing.

4. Mark the date of the first opening and use the bottle preferably within 1 month.

5. The bottle must be vortexed once a day, in the morning, then gently mixed by inversion just before any usage during the same day.

6. The same pipetting precautions described above must be applied to the drawing and dispensing FlowCount beads.

7. FlowCount beads must be dispensed using exactly the same pipette and tip type used for the primary sample.

8. After adding FlowCount to lysed sample, mix by gentle vortexing (as described above) or by inversion after tube capping.

9. Analyze immediately after mixing or keep the samples in melting ice in the dark until analysis.

10. Instrument setup can include FSC as the primary threshold and trigger, provided low-volume debris and electrical noise are appropriately gated out during acquisition.

11. Take into account only bead singlets. Include time as an additional parameter to better define bead singlets and to monitor possible fluidic perturbations in the acquisition process. Try to keep bead singlet CV for FSC and fluorescence within 3%.

12. FlowCount beads generate end-scale fluorescence signals when used on Becton Dickinson instruments set up for routine IF analysis. Ensure that the bead events are fully on scale in at least one fluorescence channel.

13. FlowCount beads are not excited by He-Ne red lasers in instruments equipped with dual-laser configuration.

E. Additional Usage Tips for Becton Dickinson TruCount Beads

The following guidelines may be in some contrast with official manufacturer's instructions.

1. Always keep the TruCount tube bag carefully airtight (best with adhesive tape) and use an opened bag within 1 month.

2. Discard tubes where bead bolus is fragmented or if bead dust is evident.

3. Discard the entire sample if the bead bolus does not dissolve quickly and completely.

4. Ensure maximum pipetting precision when using TruCount beads. All the measurement precisions rely on this step and it is not compensated by the dual-step pipetting (i.e., sample and bead pipetting) as with FlowCount.

5. TruCount beads must be used with primary threshold and trigger on a fluorescence channel, because they are too small to be acquired when FSC is used as a threshold parameter.

6. A fluorescence marker must be used to define at least the relevant cell population to be analyzed.

7. TruCount beads are some 10 times less brilliant than FlowCount beads. This implies that end-scale bead events are usually not generated, but that some interference between cell and bead signals can occur.

8. All bead events, including doublets and multiple aggregates, must be taken into account for absolute count calculation. This is best accomplished with a dual-fluorescence display and a upperright corner window including end-scale events.

9. TruCount beads tend to sediment with time.

10. TruCount beads are excited also by the He-Ne red laser.

F. Additional Tips for Volumetric Counting Systems

1. Maximum dispensing precision and accuracy must be applied in any step. However, the primary sample dispensing still has the major influence on overall accuracy.

2. If a limited amount of final sample is aspirated by volumetric instruments, verify that the relative proportion between primary sample and lysing is set to ensure the acquisition of the minimum positive cell events or the minimum primary sample volume.

3. Avoid inadvertent final sample dilution by spillage of sheath fluid drops from the sample injection port.