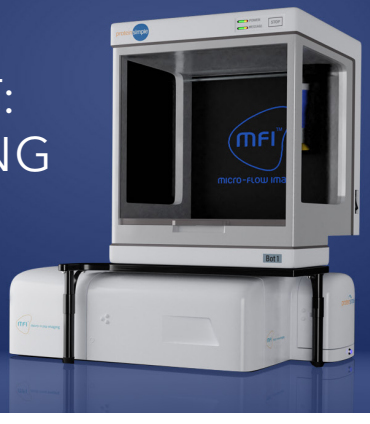
proteinsimple
a bio-techne brand

APPLICATION NOTE

DETERMINING RESIDUAL BEAD COUNT: APPLICATION OF MICRO-FLOW IMAGING TO CAR T-CELL MANUFACTURING



INTRODUCTION

Immunotherapy revs up the body's own natural defenses to fight cancer, veering away from traditional strategies that have, instead, focused on targeting the tumor and tumor cells. All of the enacting immunotherapeutic approaches incite hope, but especially those that uniquely engineer T cells to seek and destroy tumor cells. Chimeric antigen receptor (CAR) T-cell therapies, in particular, have produced booming immune responses and striking clinical outcomes with two such therapies already on the market and 800 clinical trials underway[1].

CAR T-cell therapy involves first isolating the patient's T cells and genetically modifying them to express a CAR on their surface capable of recognizing tumor-associated antigen(s). The engineered cells are then expanded ex vivo to an appropriate therapeutic dose and reinfused into the patient to stimulate an effective T-cell mediated anti-tumor immune response. From a manufacturing perspective, generating personalized batches of inherently complex CAR T-cell products poses real challenges for the biopharmaceutical industry. Regulatory agencies mandate that identity, purity, potency and safety attributes are closely monitored both in-process and for release. Of the process-related factors affecting product purity, residual beads during ex vivo expansion and activation of T cells pose safety and efficacy concerns with regard to triggering an unwanted endogenous immune response in vivo. Thus, compulsory limits are often set for residual bead counts to demonstrate product quality[2].

Residual bead count is typically determined manually by the naked eye and microscopy. But this approach is highly limited in its ability to accurately discern beads from cells and other potential in-process impurities, resulting in reporting uncertainties that risk regulatory approval. In this application note, you'll see how automation via image-based Micro-Flow Imaging™ (MFI) technology gets you the quantitative and morphological data you need to have confidence in distinguishing between beads, T cells or other potential contaminants.

HOW DOES MFI WORK AND WHY USE IT FOR EVALUATING RESIDUAL BEADS DURING CAR T-CELL MANUFACTURING?

The MFI 5000 platform series uses flow-imaging technology to detect, quantify and characterize subvisible particles in as little as 600 µL of solution (**FIGURE 1**). The MFI 5200, specifically, can scrutinize particles in the 1 µm to 70 µm size range, making it the choice platform for distinguishing between particles like beads and lymphocytes. By collecting data across 10 different morphological parameters, MFI and the accompanying View System Software (MVSS) suite and MFI Image Analysis enable accurate and precise discrimination of particle populations in a 21 CFR Part 11 compliant workflow. In this instance, MFI's powerful software and filtering tools let you quickly and accurately measure polystyrene bead residual counts and other subvisible in-process contaminants that may have made their way into the final CAR T-cell product.

The cell and gene therapy field is evolving fast, making variability and the lack of defined processes a key challenge for manufacturers. When it comes to the residual bead count parameter of product purity, manual microscopy-based methods that rely on the human eye for reporting are employed–this is not only labor-intensive but also tough to scale and highly prone to error. Instead, the regulatory compliant MFI 5200 system can be applied to analyze mixed populations containing lingering

polystyrene beads among a high concentration of T cells. By adopting MFI, you'll get automatically quantified bead counts (even in low numbers), morphological data and, notably, a streamlined, reliable process for subset composition analysis.

MATERIALS AND METHODS

PREPARATION OF T-CELL SAMPLES AND ACTIVATION BEADS

For individual analysis, Dynabeads® Human T-Activator CD3/CD28 (ThermoFisher, PN 11161D) activation and expansion beads ($4x10^7$ beads/mL in PBS) were resuspended in 10X dilution series from 1:$4x10^3$ to 1:$4x10^6$ in 5 mL of PBS. Human Jurkat T lymphocyte cell samples were resuspended in the 10X dilution series from 1:25 to 1:$2.5x10^5$ in 5 mL of Cell Wash Buffer (20 mM Bicine, 250 mM Sucrose and 0.1% Kathon CG at a pH 7.5). For analysis of mixed populations, Jurkat cells were resuspended in the cell wash buffer to a final dilution of 1:250. Then, this solution was used as a diluent to resuspend Dynabeads in a 10X dilution series from 1:$4x10^3$ to 1:$4x10^6$ in 5 mL of cell wash buffer diluent, maintaining a constant dilution of cells.

SAMPLE RUNNING AND DATA ANALYSIS USING MFI 5200

Each sample dilution was analyzed in triplicate on the MFI 5200 series equipped with a 100 µm SP3 flow cell. The volume dispensed was 0.9 mL; the volume analyzed was 0.6 mL.

The Dynabeads utilized herein are precoated with anti-CD3 and anti-CD28 antibodies and are commonly used for ex vivo T-cell research applications, as they consistently supply the necessary primary and co-stimulatory signals that T cells need for activation and expansion. However, their micromagnetic nature also means they require additional steps and technologies to enable their removal—especially important for CAR T-cell therapies set to be infused into patients. The reality is, residual beads may remain, and you need a reliable and industry-compliant systems approach to accurately assess particle populations that affect product purity and, therefore, your regulatory filing. To demonstrate the power of MFI in discriminating and quantifying Dynabeads from a mixed population of cells–even in low numbers–we'll walk you through proof-of-concept examples and then show you how you can use MFI Image Analysis Software to further customize your parameters of interest.

SETTING THE STAGE FOR SUCCESS: SIZE, COUNT, REPEAT

Let's establish some essential characteristics of the two types of particles we're working with here: Jurkat T cells versus Dynabeads. In **FIGURE 2**, we took a 0.9 mL sample of Jurkat T cells or Dynabeads and ran each through an MFI 5200 series to collect morphological data. An average equivalent circular diameter (ECD) of 8.29 ± 1.85 µm for the T cells (expected size is 11.5 µm) and a 4.66 ± 0.66 µm ECD for Dynabeads (advertised diameter is 4.5 µm) was

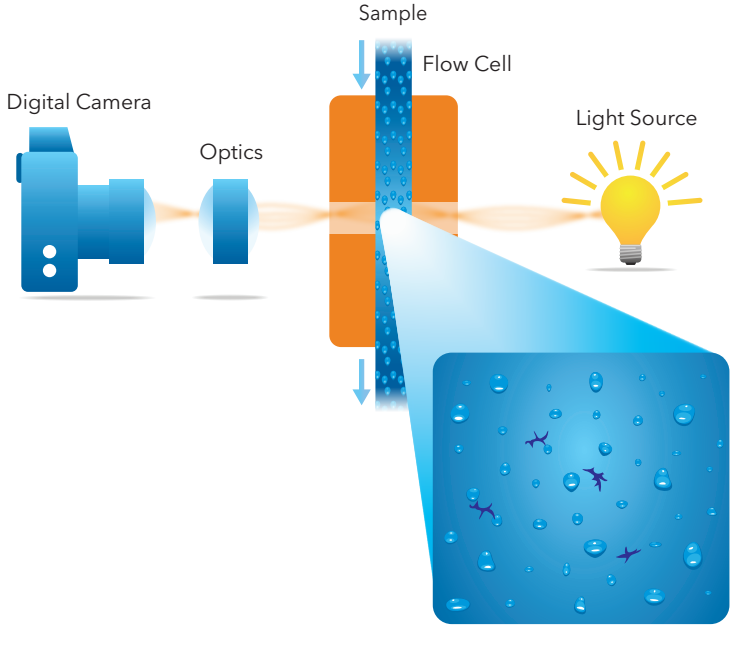


FIGURE 1. MFI technology principle. MFI uses a flow cell and digital optics to directly image, characterize and quantify subvisible particles in a liquid sample. The combination of digital microscopy and precise microfluidics gets you high-resolution images with 85% sampling efficiency. What's more, you'll get accurate counts and sizing information with full morphological detail for all subvisible particles in your sample.

identified. Overall, a clear difference is seen when looking at the optical and morphological properties of each population on MFI. But how sensitive and reproducible is MFI for the distinction and quantification of such particles?

How well a technique reproduces the same result is at the crux of comparative intra- and inter-lab analyses. Variations in results using the same sample in the same laboratory may result in indeterminate conclusions or, worse, false reporting. In addition to being frustrating for analysts, irreproducible results incur added cost to the development process and negatively impact overall operations. So, the power of MFI in minimizing inconsistencies and maintaining reproducibility is easy to appreciate! To evaluate the sensitivity and reproducibility of MFI, we created a ten-fold dilution series of Jurkat T cells (**FIGURE 3**) and Dynabeads (**FIGURE 4**) for analysis. The bar graph in **FIGURE 3, left** shows overall equal and highly reproducible cell counts between runs and across the Jurkat T-cell replicate samples analyzed—detecting as little as five cells per milliliter. Attesting to the reproducibility of the system is the linearity of assay detection data (**FIGURE 3, right**), which measures the dilution factor versus the observed cell count from **FIGURE 3**, **left**, where a robust coefficient of determination (R^2) was achieved (≥0.99). Similarly, for Dynabeads (**FIGURE 4**), reproducible particle counts were observed across the dilution spectrum, counting as low as 5–10 beads/mL (**FIGURE 4, left**) with a robust R^2 value (≥0.99) (**FIGURE 4, right**). Taken together, these data are proof-of-concept of MFI's ability to accurately assess particle and/or cell counts in a sample.

BEYOND SIZE AND COUNT: DISTINGUISHING BETWEEN CELLS AND BEADS IN THE SAME SAMPLE USING MFI IMAGE ANALYSIS SOFTWARE

After data acquisition, you can use MFI Image Analysis Software to intuitively design filters and distinguish between particles based on 10 different morphological parameters! In **FIGURE 5**, we demonstrate this capability for Jurkat T-cell and Dynabead samples. Dynabeads have a well-defined ECD of 4.66 ± 0.66 µm, whereas lymphocyte ECDs can range from about 6 µM to 15 µM depending on the type and activation status[3,4]. This size difference was used as a basis for building appropriate filters that would discriminate between the two population types, in combination with circularity and intensity standard deviation measurements (**FIGURE 5, top and middle panels**). These parameters are not exhaustive, and filters in MFI Image Analysis Software may be tailored for any particle type. For example, layers can be added or removed, and parameter limits can be tuned for each filter. A complete list of available customizable parameters is also displayed in **FIGURE 5, bottom panel**.

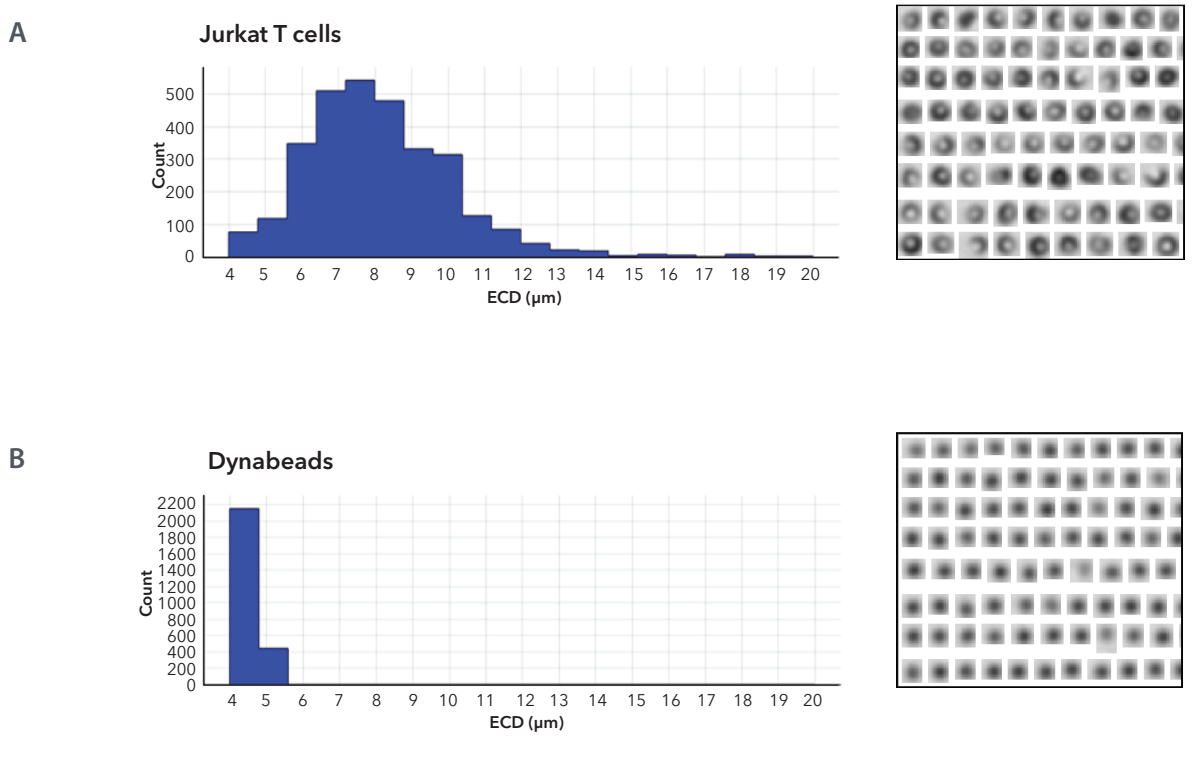


FIGURE 2. Particle size determination using MFI 5200. ECD measurements, which correlate to particle size, were automatically determined using MFI Image Analysis software for T cells (**A, left**) and Dynabeads (**B, left**). Each histogram shows the distribution of ECD in the two different particle types. Images of T cells (**A, right**) and Dynabeads (**B, right**) show distinct morphological features.

Previously, we analyzed Dynabeads and Jurkat T cells as individual samples. Now, in **FIGURE 6**, we demonstrate that MFI can parse the two populations, even from a mixed sample. The filter customization in **FIGURE 5**, which shows that circularity and intensity standard deviation are additional layers to the base layer of ECD, more precisely define each population in this sample based on their properties. Because ECD is the base property for these two filters, it is shown in **FIGURE 6** to represent the two populations of particles.

In a real-world example, Dynabeads are often used for cell selection and activation without the need for removal until harvest. As such, residual bead count checks are critical and commonly performed once the appropriate therapeutic CAR T cell dose is achieved, and you're ready for product lot release. To examine whether MFI could reproducibly count even small numbers of Dynabeads in the presence of a constant number of cells, we prepared a dilution series of Dynabeads while maintaining a constant dilution of Jurkat T cells (1:250). **FIGURE 7** represents these analyses, where MFI was able to reproducibly and accurately identify as few as 10 Dynabeads in a cell solution!

SAID AND DONE: CLEANING THE FLOW CELL

Subvisible particle detection and characterization via MFI is dependent on precise and accurate imaging of a solution as it passes through the flow cell. As such, this flow cell is treated with a hydrophobic silane coating to prevent particle adhesion, as proteins and particles can stick to its interfaces and surfaces. When working with biohazardous samples, a bleach solution is typically used to ensure all living materials and associated risks have been neutralized. To test the robustness of the flow cell silane coating, we tracked the percent of total particles that are either slow-moving or stuck after repeated cleanings (96 flushes at a rate of 6 mL/minute) with a 10% bleach solution (**FIGURE 8**). Then, the system was evaluated with suspensions of the National Institute of Standards and Technology Certified Particle Size Standard, 5 µm (ProteinSimple, PN 4004-001-001), which was analyzed after every other bleach flush. The total particle count and the percent of slow-moving and non-recovered particles were calculated (**FIGURE 8**). No increase in the percent stuck or slow-moving particles were observed over the course of our testing period, indicating that the silane coating remained intact.

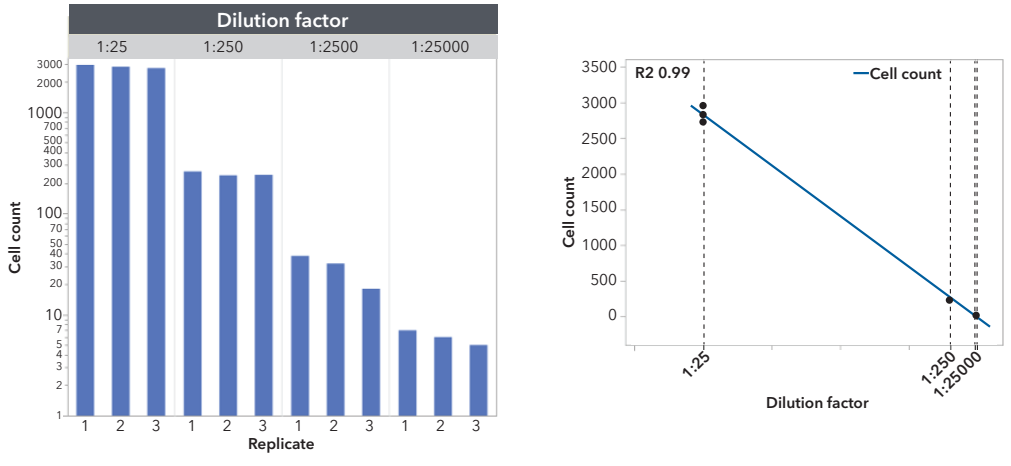


FIGURE 3. Quantitative reproducibility of MFI for the detection of Jurkat T cells. The observed cell count for the Jurkat T-cell dilution series analyzed in triplicate shows a linear decrease corresponding to the dilution factor that is reproducible with each replicate analyzed (**left**). The detection sensitivity was determined to be ~5 cells/mL. The linearity of this detection data (**right**) produced a robust R^2 value of ≥0.99

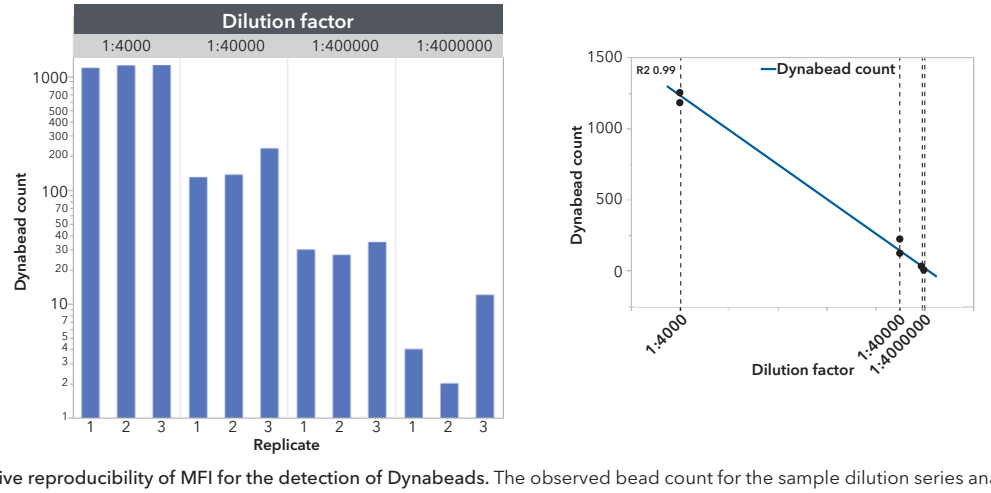


FIGURE 4. Quantitative reproducibility of MFI for the detection of Dynabeads. The observed bead count for the sample dilution series analyzed in triplicate shows a linear decrease corresponding to the dilution factor that is reproducible with each replicate analyzed (**left**). The detection sensitivity was determined to be 5–10 beads/mL. The linearity of this detection data (**right**) produced a robust R^2 value of ≥0.99.

Filters: T cells

Name	Property	Test	Level	Count	Concentration (#/ml)	Population (%)
All	Remove Stuck, Slow			5538	9070.13	98.84
Filter 1	ECD	>=	5.0	514	841.83	9.17
Filter 2	ECD	<=	15.0	514	841.83	9.17
Filter 3	Circularity	>	0.3	514	841.83	9.17
Filter 4	Intensity Std	>=	60.0	508	832.00	9.07
Filter 5	Intensity Std	<=	250.0	508	832.00	9.07

Filters: Dynabeads

Name	Property	Test	Level	Count	Concentration (#/ml)	Population (%)
All	Remove Stuck, Slow			5538	9070.13	98.84
Filter 1	ECD	>=	3.0	5199	8514.91	92.79
Filter 2	ECD	<=	5.0	4685	7673.09	83.62
Filter 3	Circularity	>	0.7	4680	7664.90	83.53
Filter 4	Intensity Std	>=	100.0	4662	7635.42	83.21
Filter 5	Intensity Std	<=	200.0	4662	7635.42	83.21

FILTERS AVAILABLE IN MFI IMAGE ANALYSIS SOFTWARE

- Equivalent circular diameter (ECD)
- Area
- Perimeter
- Circularity
- Aspect ratio
- Max Feret diameter
- X position
- Intensity mean
- Intensity standard deviation
- Intensity minimum
- Intensity maximum
- Time (%)
- Time (minutes)

FIGURE 5. Customizing filters using MFI Image Analysis Software. Presetting your own filters allows for the particle subpopulations present to be automatically filtered. Filter settings for Jurkat T cells (top panel); Dynabeads (middle panel) and a complete list of available filters (bottom panel) are shown. ECD, equivalent circular diameter; Circularity, the circumference of a circle with an equivalent area divided by the actual perimeter of the particle; Intensity Std, the standard deviation of the intensity of all pixels representing the particle.

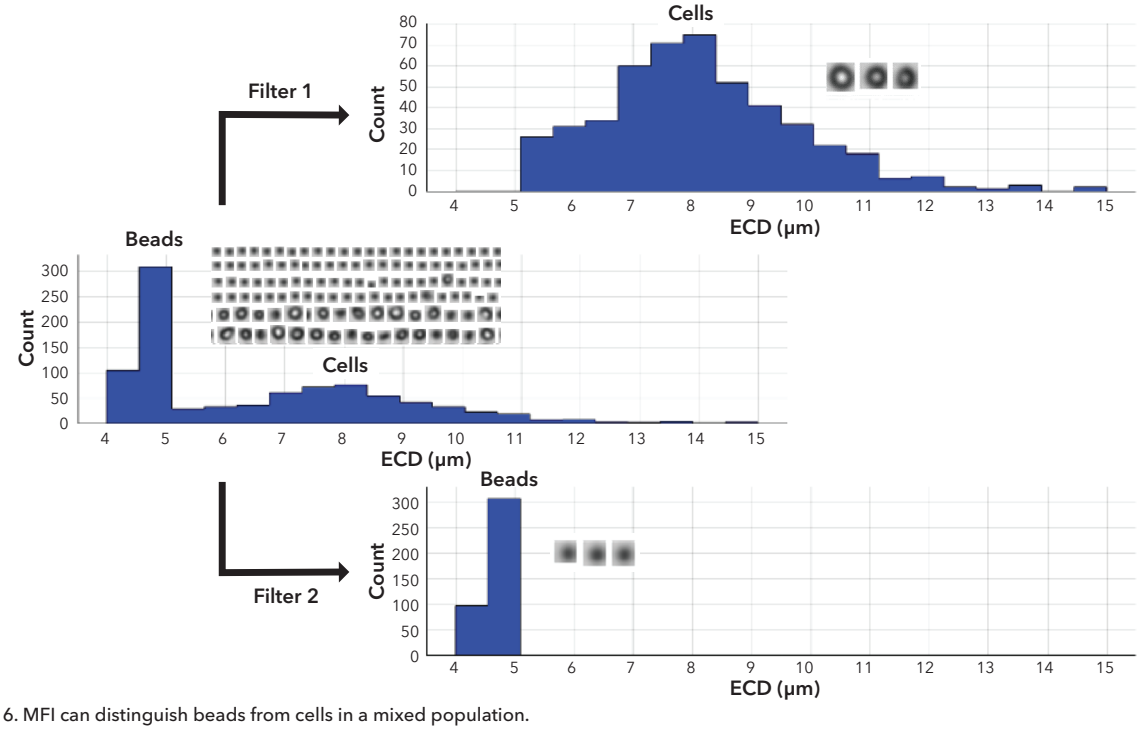


FIGURE 6. MFI can distinguish beads from cells in a mixed population.

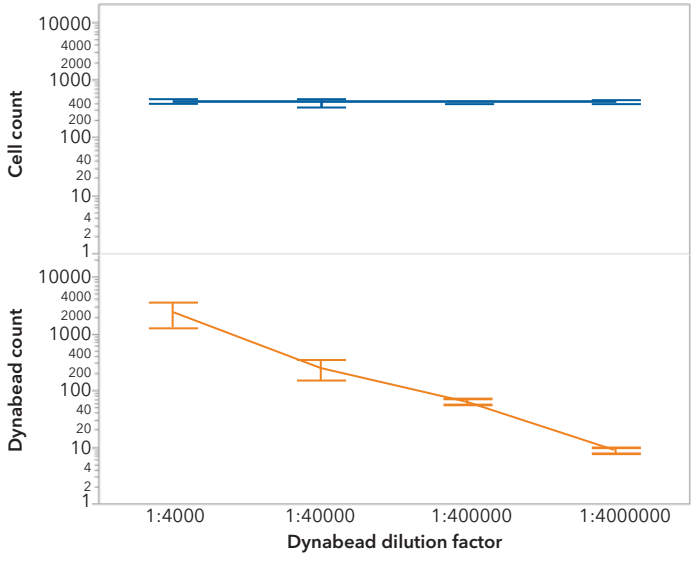


FIGURE 7. MFI can identify and count small numbers of Dynabeads in the presence of T cells. The counting of beads was unaffected by the presence of cells. A linear decrease in Dynabead count with dilution factor was observed despite the presence of T cells, and a reproducible number of Dynabeads was counted for each dilution.

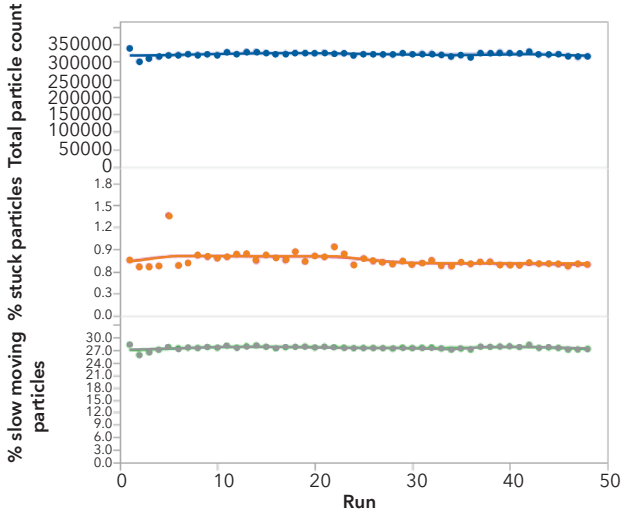


FIGURE 8. Particle count after MFI flow cell cleaning. The MFI flow cell was repeatedly cleaned with a 10% bleach solution, which did not affect the silane coating and all 48 subsequent runs tracking particle movement. The first data point corresponds to a run performed before bleach exposure, indicating no change in performance before and after bleach exposure.

CONCLUSION

Residual beads from in-process CAR T-cell manufacturing workflows may pose immunogenicity concerns and delay the release of your final product. Ensuring their removal is, therefore, an essential step to on-track regulatory approval. To confirm the product's purity, you'll need to accurately assess its composition for the presence of subvisible particles, including the reporting of residual bead count. Current approaches are not reliable or reproducible as they involve manual counting and rely on the human eye to discern between particle types and cells. But MFI is an image-based, automated system that gives you the advantage over compendial techniques for this type of analysis. By directly imaging particles and collecting morphological data, MFI enables the quantification and discrimination of T cells from activation beads in a mixed population. You'll also be able to detect even low numbers of beads within a large population of T cells, giving you more confidence in the vital decisions you make during development and manufacturing!

REFERENCES

1. The global pipeline of cell therapies for cancer, J.X. Yu, V.M. Hubbard-Lucey and J. Tang, *Nature Reviews Drug* Discovery, 2019, 18;821–822.

2. Standards and Best Practices for Cell, Gene and Tissue-based Therapies, R. Potts, U.S. Pharmacopeial Convention. Available online at cdn.ymaws.com/www.casss.org/resource/resmgr/cmc_no_am_jul_spkr_slds/2017_CMCS_PottsRebecca.pdf. Updated 18 July 2017. Accessed 8 January 2020.

3. Dynabeads® Human T-Activator CD3/CD28, ThermoFisher Instruction Manual. Available online at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/11131D_32D_61D.pdf. Accessed 13 January 2020.

4. The Histology Guide, Faculty of Biological Sciences, University of Leeds. Available online at https://www.histology.leeds.ac.uk/blood/blood_wbc.php. Accessed 13 January 2020.

Learn more | Request quote

WHERE SCIENCE
INTERSECTS INNOVATION™

bio-techne®

bio-techne.com

R&D SYSTEMS | NOVUS BIOLOGICALS | TOCRIS | protein simple | ACD | exosome dx

Global info@bio-techne.com bio-techne.com/find-us/distributors TEL +1 612 379 2956 North America TEL 800 343 7475
Europe | Middle East | Africa TEL +44 (0)1235 529449 China info.cn@bio-techne.com TEL +86 (21) 52380373

AN_MFI_CART-Cell_STRY0081621