Cell Therapy Clinical Manufacturing

Novel Detection Method for Residual Beads in CAR T-Cell Manufacturing Using Imaging Flow Cytometry

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Introduction

Gene-modified cell therapies, such as Chimeric Antigen Receptor T-cell (CAR-T) therapy, have revolutionized cancer treatment and show potential for treating genetic disorders and autoimmune diseases. The manufacturing process involves isolating, activating, and expanding human T cells, for which CD3/CD28 Dynabeads™ offer a streamlined solution. These beads have been used in over 200 clinical trials; however, they are non-biodegradable making their removal from the final product crucial. Newer systems like detachable beads aid in this removal, however, both systems require quantification of residual beads as contaminants, with the FDA requiring fewer than 100 beads per 3 million cells in the final product. Consequently, a precise and accurate assay for detecting and quantifying residual beads is essential for product release testing. Current methods are either labor-intensive, have low throughput, or require specialized equipment. This study introduces a novel imaging flow cytometry-based assay designed to improve quality control and reliability in product release testing.

Attune™ CytPix™ for Residual Bead Detection

This assay leveraged the Attune™ CytPix™ flow cytometer, which combines traditional cytometry with brightfield imaging, making it ideal for detecting rare events like residual beads in cell products. The CytPix™ analysis software offers 26 image processing parameters which can be used for post-hoc analysis.

This novel assay offers a reliable alternative to existing residual bead detection methods and can be implemented on a platform commonly available in cell therapy manufacturing facilities.

Materials and Methods

T-Cell Isolation and Culture

Cells for Assay Establishment and Development: Apheresis material, collected from healthy donors (AllCells), was processed using the CliniMACS™ Prodigy™ and CD4/CD8 microbeads (Miltenyi), and cryopreserved. This ensured a T-cell population without existing Dynabeads. Vials were thawed and washed in dPBS or basal medium for use in assay.

CAR-T Drug Product: The CAR-T Drug Product followed a 10-day process where on day 0 T-cells were isolated using CD3/CD28 Dynabeads using the CTS™ DynaCellect™ Magnetic Separation System. On day 2, the isolated T-cells were debeaded, modified, and further expanded for 8 more days. On Day 10 the cells were cryopreserved, and samples were collected for testing.

Assay Establishment

Over 300,000 flow events and images were captured, and the resulting images were processed using the instrument software for object and pixel attributes. Twenty-six image analysis parameters were explored in various combinations to determine the optimal gating and analysis strategy for distinguishing cells and beads. A combination of 5 parameters was identified for clear separation between cells, beads, and cell-bound beads.

Assay Development

The assays performance was evaluated following ICH Q2(R2) guidelines, across 3 users and 3 runs. To determine Limit of Detection (LOD) beads were serially diluted from 800 to 12.5 beads/mL to in basal medium to determine the lowest concentration the Attune could detect beads from the background. To determine Lower Limit of Quantification (LLOQ) a mixture of known bead concentrations, ranging from 800 to 20 beads per million cells was used. After LOD and LLOQ curves were defined, blind tests were performed by different users to test bias and imprecision of assay. The assay was then transferred to a partner site for testing and validation.

Assay Deployment

To assess residual beads, samples were run using the Attune™ Cytpix™ Flow Cytometer. Image capture settings were adjusted to ensure captured objects were in focus and centered on the screen. A minimum of 360,000 images per sample were collected, evaluated using defined gating strategy, and compiled for statistical analysis.

Data Analysis

Cell counts, flow- and image-based methods were used to collect results at (NucleoCounter® NC-3000™, Attune™ CytPix™). GraphPad Prism and Biorender were used to prepare figures. Data are represented as mean +/- SEM.

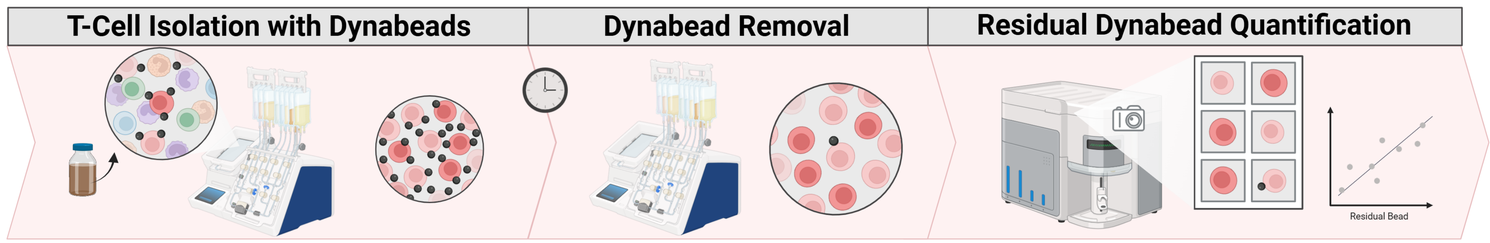


Figure 1. Dynabead Workflow for Cell Therapy Applications and Residual Bead Detection. CTS CD3/CD28 Dynabeads can be used to isolate and activate T-cells simultaneously from apheresis product. The CTS™ DynaCellect™ Magnetic Separation System can be leveraged to process apheresis material on a closed, semi-automated platform for both cell isolation and bead removal. Once the Dynabeads are removed from product or at the end of manufacturing process, samples can be analyzed for the residual beads. The residual bead assay leverages the Attune™ Cytpix™ flow cytometer, where images are captured and analyzed for the number of residual beads remaining in the sample.

Results

Establish Residual Dynabead Detection Using Attune™ Cytpix™ Flow Cytometer

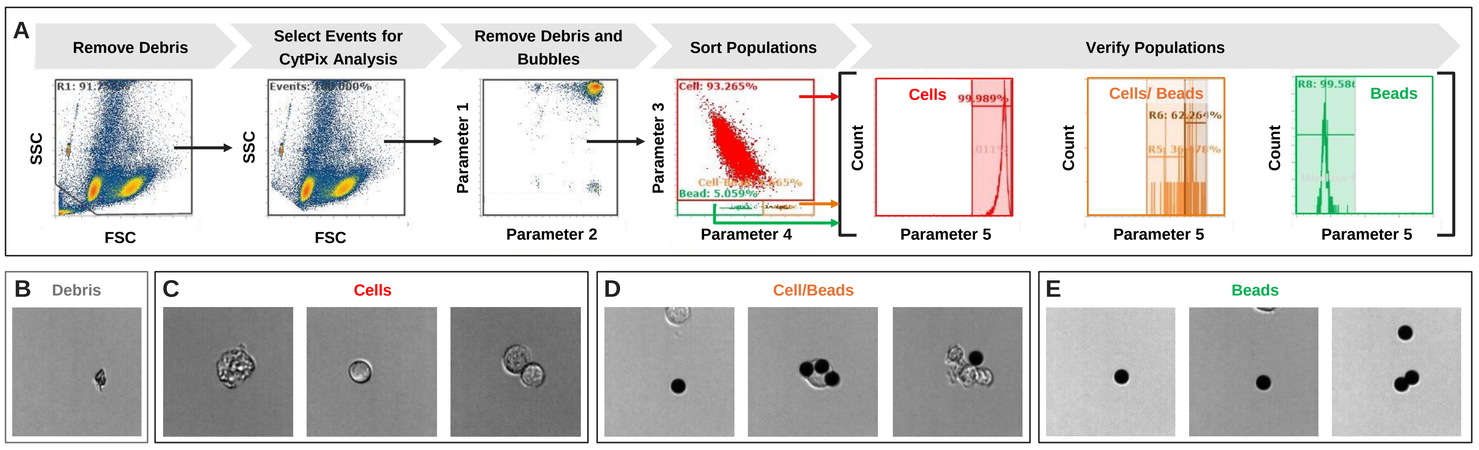
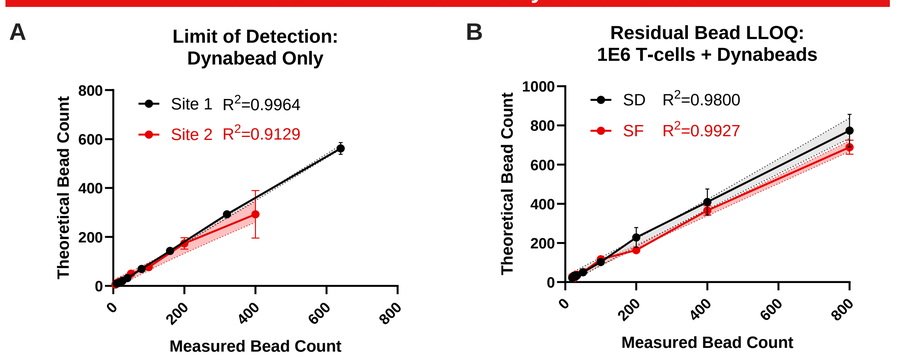


Figure 2. Residual Dynabead Detection Gating Scheme. The Attune™ Cytpix™ flow cytometer software offers 26 image processing parameters for post-hoc analysis. Different variations of the 26 parameters were tested for residual bead detection until a combination of 5 parameters were determined to provide optimal separation of cells, beads and cell-bound beads, and would be suitable for residual bead detection. (A) The gating strategy removes debris and bubbles utilizing forward scatter (FSC) and side scatter (SSC) to determine size and complexity, respectively, as well as Parameter 1 and Parameter 2. This removes events that should not be analyzed downstream and limits noise within the sample. After debris removal, samples were sorted into 3 main populations: Cells, Beads, and Cells/Bead Mixture utilizing Parameter 3 and Parameter 4. These populations were then verified utilizing Parameter 5. Any events that fall outside of the Parameter 5 gate can be manually re-classified. At this stage, the beads captured within the “Beads” and “Cell/Beads” gate are visually confirmed and counted. (B-E) Representative images of debris, cells and cell aggregates, cells and beads (bound and unbound), and cells (single and aggregated), respectively.

Evaluate Residual Bead Assay Performance



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Assay Performance (LLOQ) – Site 1								
Theoretical Beads	20	30	33	50	100	200	400	800
Measured Beads	21	29	34	50	102	228	409	774
% Recovery	106%	98%	104%	101%	102%	114%	102%	97%
% CV	3.77	13.15	9.36	1.59	14.13	22.31	16.29	10.77

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Assay Performance (LLOQ) – Site 2								
Theoretical Beads	20	30	33	50	100	200	400	800
Measured Beads	28	28	37	51	117	163	366	689
% Recovery	141%	93%	111%	101%	117%	81%	91%	86%
% CV	17.21	17.32	28.41	17.34	10.38	11.55	6.61	5.22

Figure 3. Linearity and Sensitivity Determination for Residual Bead Assay. Assay performance was evaluated at the development site (Site 1) and transferred to partner site (Site 2). (A) Limit of Detection (LOD) was generated from 3 separate users across 3 different runs, at Site 1, and by a single user across 3 runs at Site 2, resulting in an LOD of 20 beads/mL for both sites, with a R^2 coefficient of 0.9964, and 0.9129, respectively. Linearity spans six serial dilution points following 2-fold serial dilution with final intermediate dilutions. (B) Similarly, sensitivity was evaluated at both sites by spiking in known quantities of Dynabeads into T-Cells. The lower limit of quantification (LLOQ) was determined to be 20 Beads and 30 Beads per million cells for Site 1 and Site 2, respectively. (C-D) LLOQ results across Site 1 and 2 include the theoretical bead count, mean measured bead count, the percent coefficient of variance (CV) within each sample, and the overall recovery of the sample. Results are presented as mean ± SEM, with shading on 99% prediction interval.

Deploy Residual Bead Assay

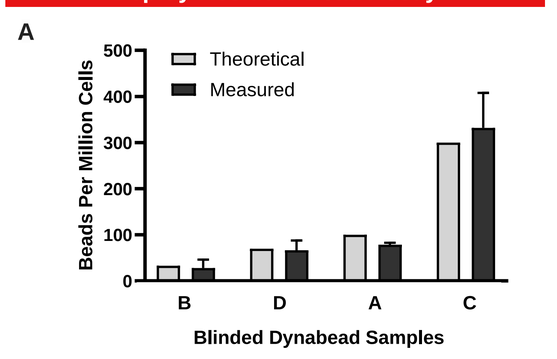

Figure 4. Deployment of Residual Bead Assay on Blinded Samples and CAR-T Drug Product. (A) Blinded test samples were created and tested by two users to determine the performance of the assay. The users were able to bin the results correctly with comparable results between theoretical and measured values. (B) CAR-T Drug product derived using CD3/CD28 Dynabeads was tested by a single operator with the developed protocol and resulted in the detection of only 1.8 beads per million cells, which is below our LLOQ detection limit and the FDA guidance on residual beads. Results are presented as mean ± SEM.

Conclusions

- Upon testing 26 different image analysis parameters, we found a gating scheme that leverages 5 parameters for residual bead detection.
- Limit of detection (LOD) with Dynabeads was determined to be 20 beads/mL with a coefficient of determination (R^2) of 0.99 and 0.91, respective to sites. Experiments at Site 2 are ongoing with additional users and runs to increase assay robustness.
- Lower Limit of Quantification (LLOQ) with Dynabeads and T-cells was determined to be as low as 20 beads per million cells.
- To further test our developed assay, blinded samples were run by two separate users, resulting in no substantial differences between theoretical and measured values.
- The assay was deployed on a CAR-T drug product and resulted in 1.8 beads per million cells, a measure below the LLOQ.

Overall, this approach improves on the current methodology for residual bead detection, as image-based flow offers a more sensitive and quantitative approach. Our next steps to further enhance this assay will incorporate the use of an AI screening tool, creating a more automated method for detecting residual Dynabeads to be locked down and implemented for cGMP manufacturing of CAR-T products.

Acknowledgements

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