Label-Free Monitoring of T-Cell Activation Using Laser Force Cytology™

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LumaCyte®

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Introduction

In the landscape of immunotherapy, Chimeric Antigen Receptor T-cell (CAR T) therapy stands out as a revolutionary approach in the treatment of cancer. CAR T therapies are engineered by harnessing a patient's own T cells and genetically modifying them *ex vivo* to attack tumor cells before reintroducing them to the patient. While CAR T therapy has shown remarkable success in clinical settings[1], many challenges remain, such as variability in donor T-cell quality, *ex vivo* senescence of T cells, and determining optimal activation conditions[2,3]. Industry guidance provided by the FDA underscores the importance of robust acceptance criteria for cellular starting materials in CAR T manufacturing due to significant lot-to-lot variability stemming from donor distinctions[4]. Successful T-cell activation is another pivotal aspect of the CAR T biomanufacturing cycle, most notably in the process of CAR gene insertion and the subsequent expansion of efficacious CAR T cells[5]. In the case of retroviral vectors, the CAR gene required for the cell to express the proper receptors to recognize the patient's tumor cells can only be transduced into an activated T cell[6]. T-cell activation is also a component of successful CAR T-cell proliferation and expansion for large-scale production before inserting the modified cells back into the patient[5]. Finally, excessively activated T cells can lead to Cytokine Release Syndrome (CRS)[7], an uncontrolled release of pro-inflammatory proteins. It is therefore important to closely monitor activation throughout the manufacturing process.

Because of the dynamic nature of the product and the complexity of the process, the biomanufacturing of CAR T cells can be a point of congestion for these promising therapies[8]. Current in-process testing methods lack consistency and comparability and can vary widely throughout each phase of CAR T manufacturing. Thus, there is an established need for process analytical technologies (PATs) that provide in-process or at-process monitoring to ensure quality of cell therapy products[6]. Different methods may be used in R&D, process development, and manufacturing, requiring costly assay development at multiple stages that lack comparison. A controlled, repeatable, and consistent method for in-process testing is required to streamline the manufacturing of CAR T therapies.

LumaCyte's label-free Laser Force Cytology™ (LFC™) instrument, Radiance®, offers a rapid and sensitive method for analyzing cellular starting material and monitoring T-cell activation throughout CAR T manufacturing, allowing for the development and monitoring of critical quality attributes (CQAs). By harnessing a combination of optical pressure and microfluidics, LFC™ discerns intrinsic biophysical and biochemical properties of cells, offering insights into cellular changes throughout a production process. With the use of advanced analytics, LFC™ and Radiance®

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can be used to characterize cells throughout the entire CAR T production process (**Figure 1**), relieving biomanufacturing bottlenecks and enhancing efficiency. Post-apheresis, LFC™ can be used for qualification and testing of initial patient samples ①. CMC Guidance dictates the need to assure product identity, quality and purity[4], which is heavily dependent on the starting materials. This is a crucial step for dictating subsequent processing steps as the initial quality of T cells can be highly variable. This will dictate specific steps to take to ensure a successful result. Enrichment and activation of the T cells occurs next ②, followed by viral gene insertion ③, wherein the specific genetic modifications are incorporated into the cell.

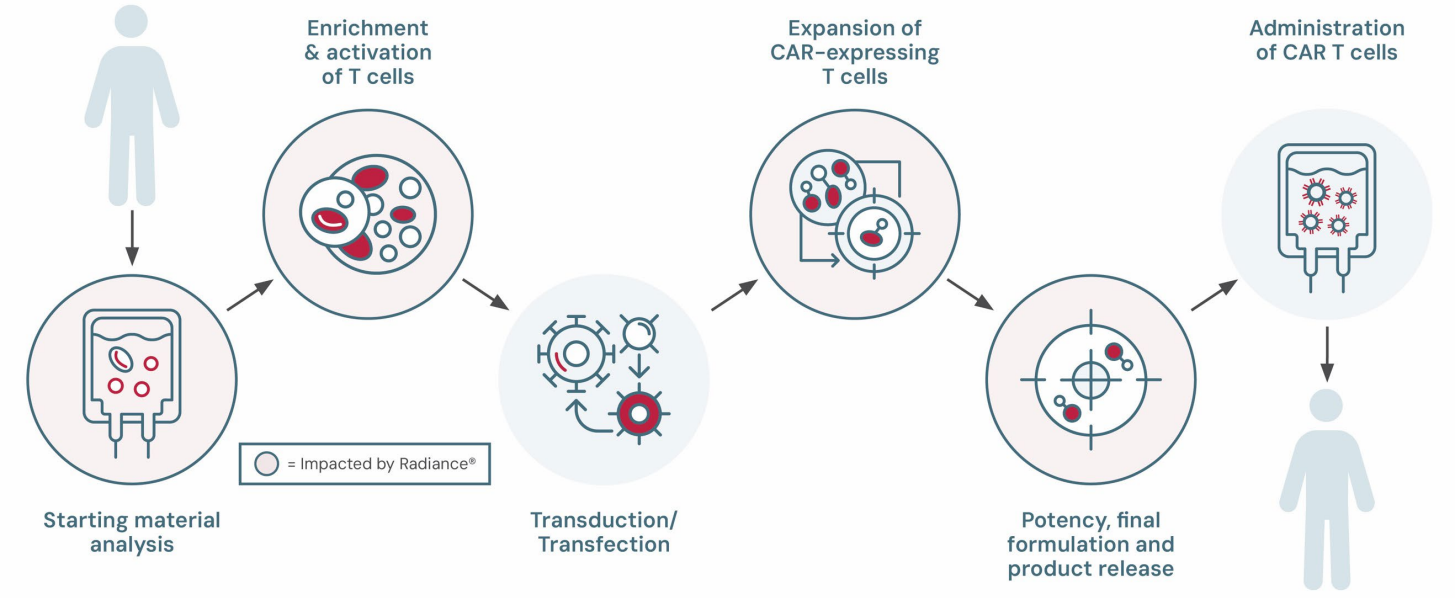

Figure 1: The CAR T-Cell Biomanufacturing Process

LFC™ can assess cell readiness for CAR gene insertion and track cell expansion ④, during which the modified T cells dramatically increase in number. Additionally, LFC™ evaluates T-cell potency through co-culture potency assays ⑤ against relevant cancer cell lines prior to treatment ⑥.

The capability of LFC™ to detect subtle phenotypic changes makes it ideal for assessing CAR T-cell potency by conducting cell-killing and other co-culture assays without the need for fluorescent labeling or other time-consuming steps. With its advanced label-free metrics, LFC™ offers a distinct advantage over conventional process monitoring technologies that could ultimately serve as a PAT to ensure critical quality attributes (CQA) in cell therapies.

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Experimental Results

To demonstrate LFC™'s ability to monitor T-cell activation, primary human T cells were seeded in a 96-well plate, activated, and then subsequently harvested at 24, 48, 72, and 96 hours for analysis. A second set of non-activated T cells were seeded, harvested, and analyzed at the same time. Principal component analysis (PCA) was performed on the single cell data using LFC™ metrics to differentiate the two populations.

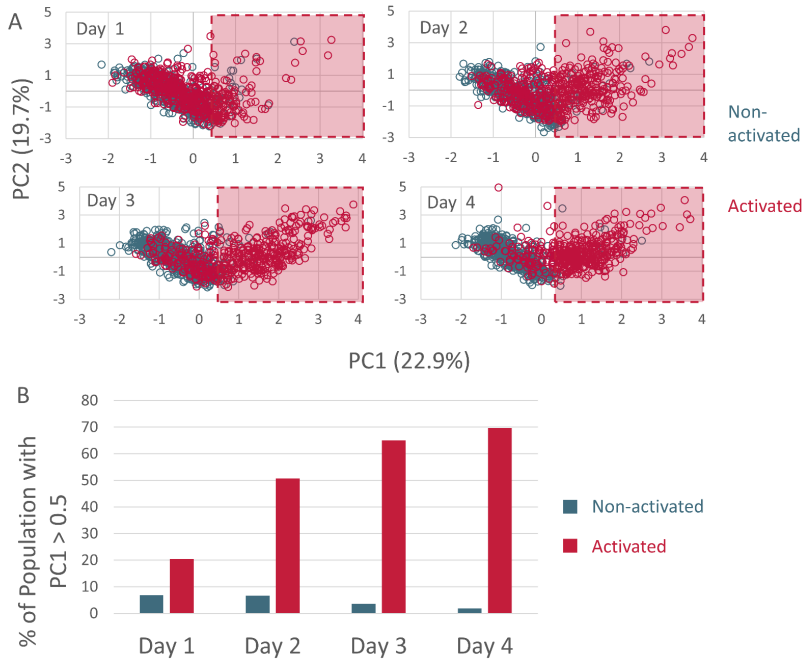

Figure 2: PCA analysis of treated vs non-treated T-cells over 96 hours. (A) PCA Scatter plots comparing the first principal component and the second principal component of the untreated and treated primary T-cells for four days. Red highlighted regions indicate progression of successfully activated T-cells, where greater than 80% of cells depicted in this region are from the activated sample. **(B)** PCA analysis of treated vs non-treated T-cells over 96 hours. Percentage of samples falling over a value of 0.5 for Principal Component 1.

As shown in the resulting PCA scatter plots in **Figure 2A**, differences are seen in both principal component 1 (PC1) and principal component 2 (PC2), as the non-activated population stays tightly clustered in the second and third quadrants, while the activated population broadens and moves towards the first and fourth quadrants. **Figure 2B** illustrates differences in the percentage of activated and non-activated cells with PC1 greater than 0.5. As can be seen in the graph, a significantly greater portion of cells from the activated group have a PC1 value of greater than 0.5 as the activated population increases. In addition to monitoring changes over time, differential responses between various types of T-cell activators can be measured using LFC™ (data not shown), illustrating the ability to measure subtle changes in cells during activation.

Figure 3 illustrates differences between the mean optical force index of the untreated and treated cell populations on each of the four days of analysis. As the time course progresses, there is an increasing difference between the untreated and treated samples, with the treated samples having a lower mean optical force index. This indicates that the optical pressure of the treated T cells is less than that of the untreated cells, potentially due to a lower overall effective refractive index than the untreated T cells. The optical force index is an important parameter when monitoring T-cell activation as changes to this parameter are a direct result of morphological and biochemical changes to the cell, independent of size. This means that key insights into cells' intrinsic properties could potentially be used throughout the CAR T-cell biomanufacturing process to predict and characterize many processes including viral gene insertion and expression, potency assays, and as these data show, success of T-cell activation.

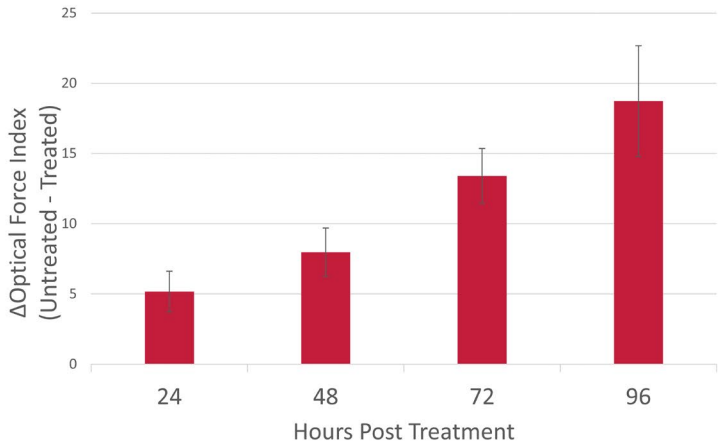

Figure 3: **Optical Force Index Changes.** Bar graph showing the difference in the population means of the treated and untreated samples. The delta optical force index is reported as the optical force index of the untreated cells minus the optical force index of the treated cells analyzed on LumaCyte's Radiance® instrument for four consecutive days.

One use case for Radiance® as a process analytical technology (PAT) is the tracking of cells along a specified path or roadmap throughout the manufacturing process to help ensure process and product consistency. To demonstrate this capability, additional experiments were conducted where T cells were activated and then either treated with a chemical stressor (doxorubicin) to interrupt the activation process or left untreated as control. **Figure 4** shows the results of monitoring T-cell activation across five independent experiments representing three different healthy donors, resulting in 25-28 replicates per timepoint. A consistent pathway is observed for control activated T cells sampled multiple times over a three-day period. Cells that have been successfully activated (**green**) follow a designated pathway within the PCA plot (increasing in PC1) as indicated by the process average (mean). The dashed lines indicate a process boundary, where cells within that boundary are in specification, representing 3.2 standard deviations of the mean in PC1 and 3 standard deviations in PC2 in either direction. Cell populations drifting outside of that boundary are therefore out of specification and may be identified early in the production process so that corrective can be taken.

The treated cell populations (**red**) shown in **Figure 4** failed to activate properly and can be shown to leave the in-specification pathway as early as day 1.

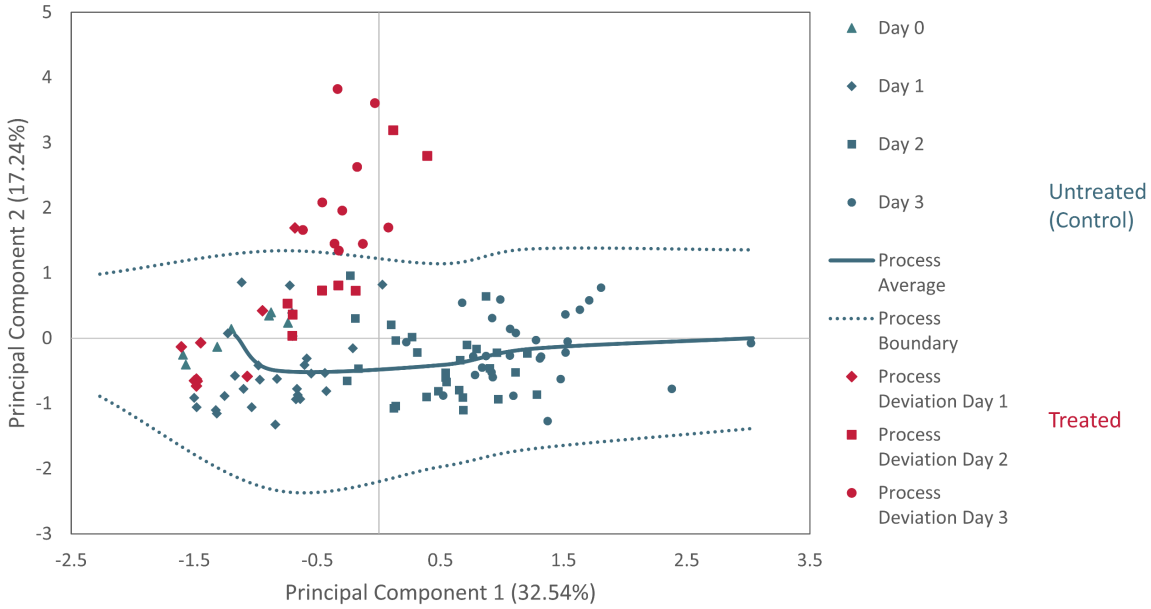

Figure 4: **Treated vs Untreated Cell Activation.** T-cells undergoing activation were treated with a chemical stressor (doxorubicin) to disrupt the activation process or left untreated. The untreated process average is outlined in **green**, while the treated (disrupted) cells are shown in **red**.

In summary, LFC™ can be used to discern between activated and non-activated T cells in a rapid, label-free manner – providing real-time monitoring throughout the manufacturing process, enabling rapid detection of anomalies, and facilitating sensitive adjustments for process improvement. LFC™ serves as a PAT to first develop a roadmap to describe a process and then to subsequently use that roadmap to detect deviations from the expected path. Future studies are underway to determine the specific effects of a wide variety of stresses that could result in deviations to classify the LFC™-based cell response, leading to an adaptive process capable of responding to and correctly identifying certain cell stressors and ensuring a consistent and high-quality cell therapy product.

From starting material analysis to final formulation, Radiance® swiftly tracks cell health and identifies specific shifts in cell populations, offering a comprehensive, sophisticated, and efficient solution that meets regulatory requirements and ensures potency throughout every phase of cell therapy development.

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Cell Culture and Experimental Methods

Negatively selected $CD3^+$ T cells were seeded in a 96-well plate at a density of 2E5/well in RPMI-1640 media (Gibco-Cat #A10491-01) supplemented with 10% fetal bovine serum (Corning-Cat #35-016-CV). Cells were activated shortly after seeding with StemCell ImmunoCult™ Human CD3/CD28 activator (Stemcell Technology Cat #100-0785) and incubated at 37°C in a humidified incubator with 5% CO2. Cells were either left as activated, non-activated/control or treated with 5µM doxorubicin at the time of activation for up to 4 days (Please refer to LumaCyte T-Cell activation protocol for more detail). Cells were then harvested on day 1, 2 and 3 after activation by centrifugation for 10 minutes at 250g for analysis. Pellet was then resuspended in 200 µl of LumaCyte sample dilution fluid and transferred to Radiance® 96-well plate for analysis with Radiance® instrument. Please contact your LumaCyte expert for more details.

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