Prioritizing Safety in CAR-T Therapy: Patient Monitoring with Cerba Research's Testing Portfolio

Cerba Research

Transforming research, advancing health together.

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Navigating the Frontier: Addressing the Need for Patient Safety Monitoring

Recent strides in knowledge and technology have significantly enhanced our capacity to precisely manipulate cells and genes for therapeutic purposes, ushering in the era of Cell and Gene Therapy (CGT).

We suggest a novel approach to patient safety monitoring by combining breakthrough flow cytometry and genomics solutions.

A major breakthrough CGT has been the chimeric antigen receptor T (CAR-T) cells to treat cancer. Cell and gene therapies entail certain risks requiring careful attention and monitoring to safeguard patient safety. Retroviral vectors (RVV) and lentiviral vectors (LVV) used to produce CAR-T cells, are modified from wild viruses, and do not replicate. It is theoretically possible for LVV/RVV to recombine with other viruses genetically, thereby restoring their ability to replicate. These potentially harmful viruses are referred to as replication-competent lenti-/retroviruses (RCL/RCR).

Another potential risk in CAR-T is insertional oncogenesis. In this scenario, vectors integrate upstream of cancer-associated genes, triggering gene expression. Hypothetically, this could lead to the development of secondary blood cancers—an ongoing concern of CAR-T therapies currently surveilled by the FDA. Accordingly, one must understand the exact integration site of the vector within the genome and the quantity of copies integrated into cells.

These two complications demand meticulous monitoring and are mandated by the FDA to ensure safety in CAR-T therapy manufacturing and clinical trials. In response, Cerba Research has formulated a comprehensive suite of services to accurately identify the integration of viral vectors, specifying the location and quantity of copies within malignant cells of patients experiencing secondary blood cancers[†] following CAR-T therapy.

In this article, we highlight several of our capabilities in this field, including:

- Vector copy number (VCN) testing by droplet digital PCR (ddPCR)
- Viral integration site (VIS) analysis by sequencing
- Replication competent virus testing by quantitative PCR (qPCR)
- Enumeration of CAR+ T cells in blood circulation and immunophenotyping of CAR+ T cells and CAR- T cells by flow cytometry (FCM)

[†]Cerba Research can apply the methods discussed in this white paper to monitor all secondary malignancies.

Duplex VCN Assay (ddPCR): Detecting Low Vector Copies in Cells

As outlined above, understanding the number of integrated viral vector copies within patient cells is crucial. It is widely accepted in CGT and by the FDA that integrated CAR copies should be below five copies per cell to minimize the risk of insertional oncogenesis.

By employing digital droplet polymerase chain reaction (ddPCR), Cerba Research's vector copy number (VCN) testing is the most sensitive method for precisely determining the number of integrated vector copies in cells.

In ddPCR, samples of DNA or RNA are partitioned into approximately 20,000 droplets, and the subsequent PCR process identifies the presence of targets through fluorescence within these droplets. This innovative approach significantly improves the likelihood of detecting very low levels of target analytes, and its efficacy remains unaffected by potential inhibitors present in samples, such as blood.

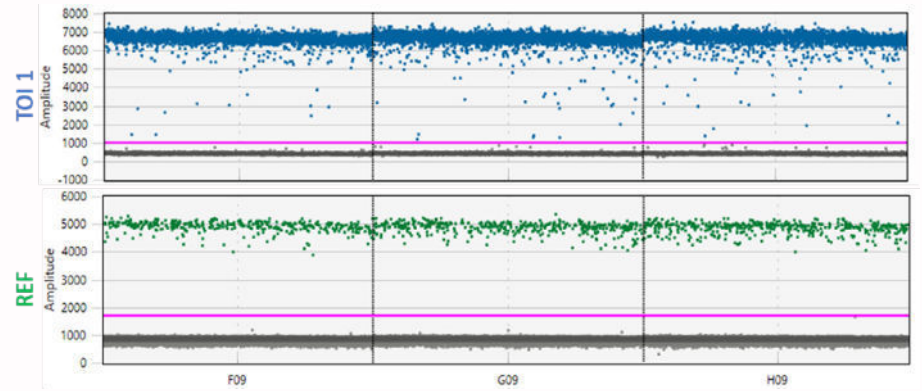
With a theoretical limit of up to one copy per reaction, ddPCR is a reliable method for VCN-testing, meeting and exceeding the stringent requirements set by the FDA.

Cerba Research utilizes ddPCR for DNA and RNA analyses, which has allowed the company to develop a duplex vector copy number assay, combining a generic target derived from publicly available lentiviral vector sequences with albumin as an internal reference (see Figure 1A).

To qualify the VCN assay, Cerba Research has used WHO reference material (Zhao Y, 2017) and spike-in of synthetic targets to reference human genomic DNA (gDNA).

Illustrated in Figure 1B, our assay boasts a linear quantification range, spanning from 10 to 10,000 copies per 100 ng of gDNA. This signifies the company's capability to precisely detect vector copy numbers, reaching a sensitivity of 5.89 copies per 100 ng of gDNA.

A.



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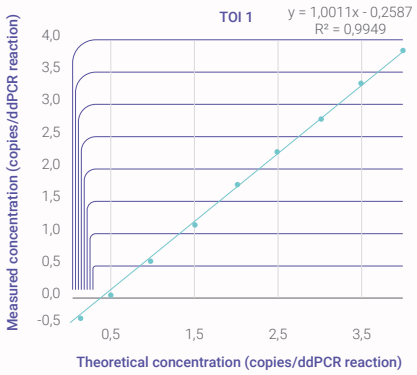


Figure 1: ddPCR quantification of a generic lentiviral vector target. A. Utilizing WHO reference material as a control, this scatter dot plot displays fluorescence intensity for the target of interest (TOI) in blue, albumin (ALB) in green, and negative droplets in grey (no fluorescence). The purple line represents the fluorescence intensity threshold. B. The linear relationship between theoretical (x-axis) and measured (y-axis) copies of TOI (log10 transformed) in ddPCR quantification of a synthetic generic lentiviral vector target spiked-in background of human gDNA.

VIS Analysis: Applying CRISPR-Cas9 and Oxford Nanopore Technology to Characterize Viral Vector Integration Sites

In gene therapy, viral vectors play a crucial role in integrating therapeutic genes, such as CARs, into the host's genome.

The FDA's concerns surrounding the potential oncogenic risks associated with this integration, specifically activating proto-oncogenes, underscore the need for rigorous safety testing. Examining the integration of viral vectors into the host genome is essential to ensure the secure placement of CARs within the genome.

Moreover, Cerba Research's assays are adept at characterizing the integration sites of viral vectors, if any, within the genome of malignant host cells and in CAR-positive malignant cells. This analysis enhances our ability to safeguard the integrity of gene therapy interventions.

Cerba Research's team has applied cutting edge technology to analyze lentiviral vector integration sites, harnessing the power of CRISPR-Cas technology for targeted enrichment, coupled with Oxford Nanopore sequencing to precisely pinpoint these integration sites within the sequenced DNA.

The development of this assay was facilitated by utilizing the WHO control sample for lentiviral vector integration site analysis (WHO/BS/2019.2373, 2019), which contains pre-defined LV integration sites.

Given that LV integration is a stochastic process with an average of 10 reported integration sites, Cerba Research has resourcefully crafted guide RNAs designed to target LV and potentially enrich the 10 predetermined integration sites.

This innovative methodology enhances Cerba Research's ability to unravel and comprehend the intricacies of lentiviral vector integration in a precise and controlled manner.

Utilizing its Viral Integration Site Analysis methodology, it has successfully identified nine out of the ten viral integration sites (see Table 1; Figure 2). All nine identified sites precisely align with the anticipated chromosomal locations, as outlined in Table 1.

The integration of the viral vector into chromosome 3 is depicted in Figure 2. As one site was unidentifiable, Cerba Research's team is actively redesigning guide RNAs to enhance and optimize this VIS assay.

Table 1 – LV Integration Sites WHO control sample.
Nine out of ten defined LV integration sites were identified.

Chromosome	Start (approx.)	Gene	Identified
21	43867986	AGPAT3	✓
6	34299319	NUDT3	X
3	50145157	SEMA3F	✓
4	93625425	GRID2	✓
8	39055454	ADAM9	✓
12	57274964	R3HDM2	✓
9	163465	CBWD1	✓
7	114508378	FOXP2	✓
22	39825823	ENTHD1	✓
17	1821489	SMYD4	✓

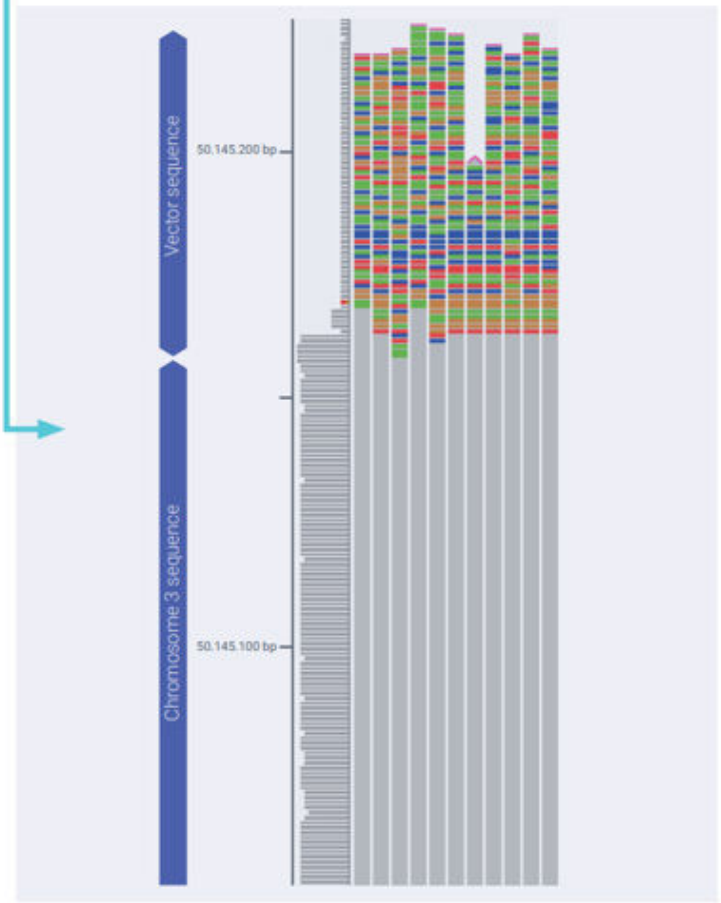


Figure 2: IGV plot shows the integration of the viral vector in chromosome 3. Grey sections represent human sequencing reads that match the human reference genome (hg13), while the colored blocks indicate vector sequences that do not match the human reference genome.

Replication Competent Virus Testing (qPCR): Regulatory-Ready RCL/RCR Assays for Use in Clinical Trials

Genetically modified retro-/lentiviruses have paved the way as viral vectors in patient therapies, showcasing their potential for safe use. However, a theoretical concern looms— the possibility of these viral vectors recombining with wild-type viruses during manufacturing or within the patient, potentially giving rise to RCL/RCRs.

Although CAR-T clinical trials have demonstrated the safety of viral vectors, addressing these theoretical concerns demands robust assays for detecting the presence of replication-competent viruses (FDA, Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up, 2020).

Crucially, the genetic sequence of the VSV-G* envelope serves as the most commonly targeted element in RCL/RCR assays. Furthermore, it is important to note that the viral vectors used in T-cell transduction and CAR-T cell production lack the genetic sequence for the envelope (e.g. VSV-G), and without an envelope there is no active RCL/RCR. The presence of VSV-G or any other envelope sequence in genomic DNA from a patient's sample clearly indicates RCL/RCR.

Cerba Research has validated qPCR assays to identify VSV-G (or GALV**) sequences in patient genomic DNA, adhering to established guidelines and its high-quality standards (see Figure 3).

Our comprehensive testing includes assessing matrix interference from whole blood and peripheral blood mononuclear cells (PBMCs) to account for potential PCR inhibitors. The VSV-G assay, as conducted by our team, meets regulatory agency criteria for clinical testing, ensuring the detection of replication-competent retroviruses (RCL/RCR) when utilizing genomic DNA from whole blood and PBMCs.

Remarkably, Cerba Research's qPCR exhibits a limit of detection below 5 copies/100 ng of gDNA, with a broad quantification range spanning from 31.6 to 1,000,000 copies/100 ng of gDNA. This signifies its capability to detect RCL/RCR incidents in CAR-T therapy products sensitively and aligns with FDA requirements for detecting ≤ 50 copies/µg of gDNA (FDA, Long Term Follow-Up After Administration of Human Gene Therapy Products, 2020).

With these achievements, we present a validated RCL/RCR assay primed for clinical sample testing. Furthermore, our GALV assays mirror the development and validation processes applied to the VSV-G assays above.

*VSV-G: Vesicular Stomatitis Virus-G protein

**GALV: Gibbon Ape Leukemia Virus

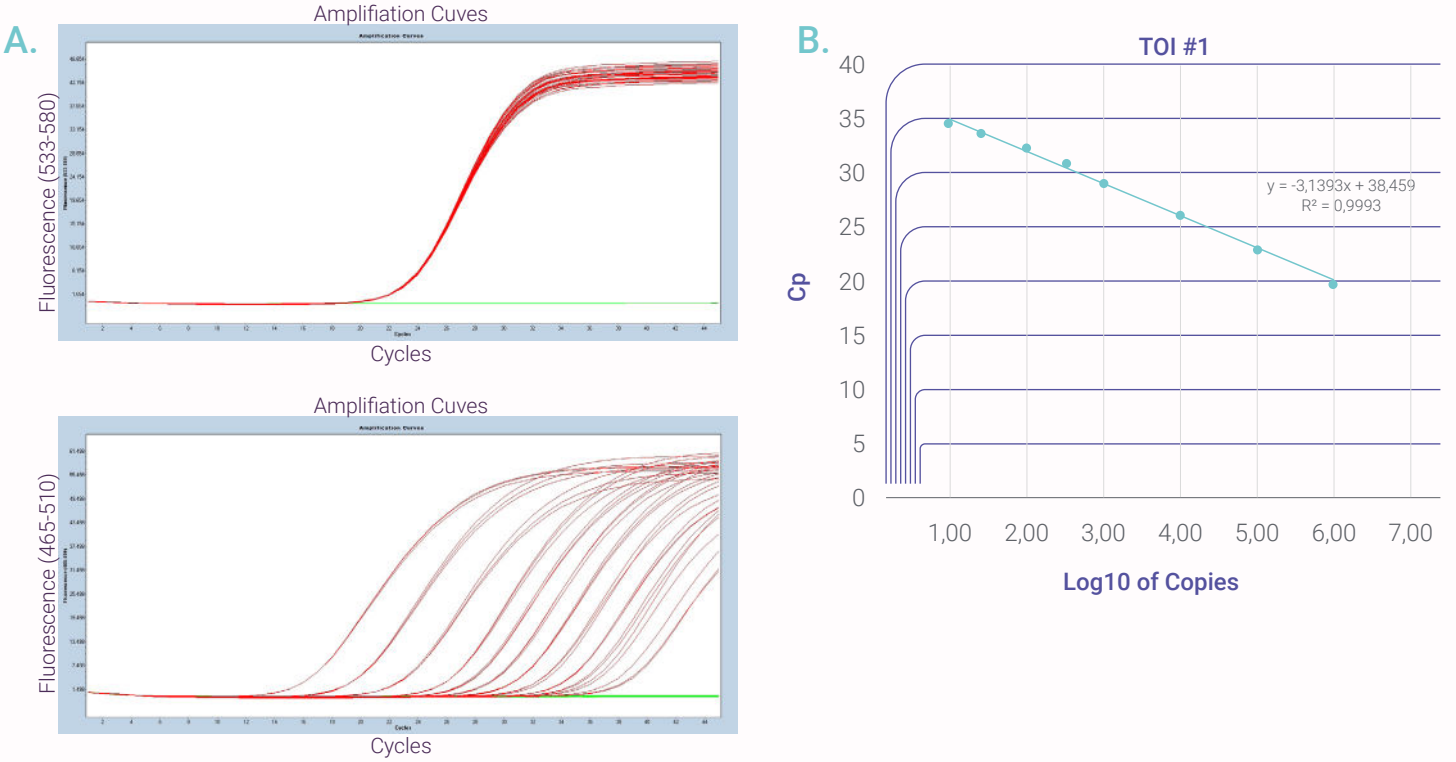


Figure 3: qPCR data from a dilution series of VSV-G (TOI #1) synthetic sequence spiked-in background of reference genomic DNA for determining the sensitivity of the duplex RCL assay. A. Representative qPCR curves from a dilution series with VSV-G assay (FAM) duplexed with the reference assay (HEX) B. Linear relationship of spiked-in copies of TOI (log10 transformed) (in x-axis) versus Cp values measured (in y-axis).

Flow Cytometry: Dynamically Monitoring the CAR+ T Cell Population

With the FDA warning of potential secondary lymphoma development in specific CAR-T therapy recipients, a thorough evaluation of CAR+ T cell expansion and persistence has become a crucial component of long-term follow-up protocols.

Cerba Research has seamlessly incorporated flow cytometry assays to quantify and immunophenotype CAR+ T cells across diverse clinical trials, encompassing autologous and allogeneic CAR-T trials.

Given the unique nature of most CARs, CAR detection demands a sponsor-specific approach. Therefore, flow cytometry panels are meticulously customized for each CAR-T product, with the sponsor's detection reagent (anti-idiotype antibody) for assay validation and patient testing. We present a sample flow cytometry panel for CAR+ T enumeration in Table 1.

Fluorochrome Detector	FL1	FL2	FL3	FL4	FL5	FL6
*Tube 1 (FMO)**	*Viability*	*CD45*	*CD3*	*CD4*	*CD8*	
Tube 2	*Viability*	*CD45*	*CD3*	*CD4*	*CD8*	*CAR*
*Tube 3***		*CD45*				

**FMO = Fluorescence-Minus-One (FMO) Tube; used to verify gating*
*** Trucount tube for calculation of absolute count*
Table 1: Flow panel configuration for CAR+ T enumeration. Based on fluorochrome for CAR antibody (provided by sponsor), fluorochromes for other markers will be selected to optimize the panel.

Enumerating CAR+ T Cells By Flow Cytometry

Cerba Research has successfully implemented a CAR+ T cell enumeration assay, incorporating absolute cell counts in whole blood for secondary end point assessment in clinical trials. This can be achieved with a dual-platform approach, utilizing a hematology analyzer and flow cytometry assay, or the streamlined single-platform option with the flow cytometry assay alone, ensures flexibility and accuracy. Both platforms are validated and ready for testing clinical samples, offering a robust solution for autologous and allogeneic CAR-T trials.

Across laboratories worldwide, Cerba Research has successfully validated and implemented flow cytometry assays for autologous and allogeneic CAR-T therapies.

Recognizing the distinct nature of these CAR-T products, whether autologous or allogeneic, necessitates defining a gating strategy. Autologous CAR-T cells, sourced from the patient, can be gated through CD3-positive T cell selection (see Figure 4). In contrast, allogeneic CAR-T cells are engineered to knock out the TCR gene to reduce graft-vs-host disease risks. As such, CD3 is not expressed, requiring allogeneic CAR-T cells to use an alternative gating strategy.

As exemplified in Figure 4, a specific tag expressed with the CAR antigen or target protein (fluorescently labeled) can be employed to identify CAR+ T cells. This choice depends on the CAR-T product of each sponsor, and Cerba Research scientists who will determine the optimal approach for detection and data analysis for each clinical trial.

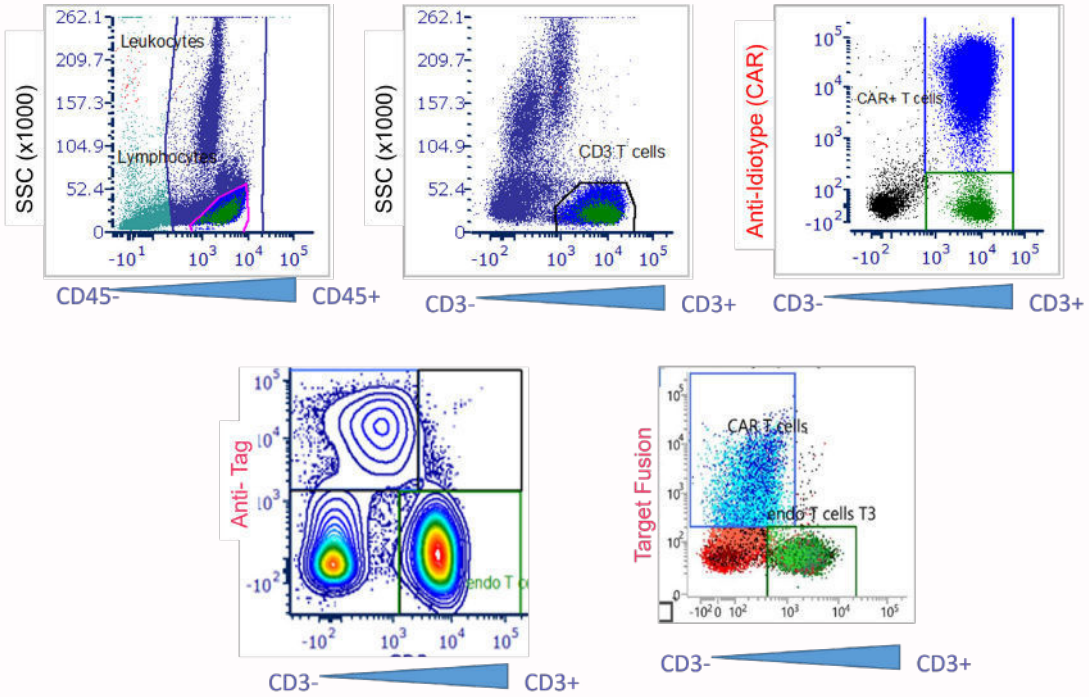


Figure 4: CAR+ T cell detection in autologous (top) and allogenic CAR-T trials (bottom). Top: scatter plots show the sequence of gating strategy. First plot is gated for CD45+ leukocytes, and second plot is gated for CD3+ T cells. Data are derived from CD3+CAR+ T cells. Last plot shows lymphocytes and is plotted between CD3 (x-axis) and CAR (y-axis) to differentiate CAR+ and CAR- T cells. Bottom: anti-Tag antibody (left) or target fusion protein (fluorescently labeled) is used to detect CAR+ T cells, which lack CD3 expression. X-axis shows CD3 and y-axis shows CAR.

Immunophenotyping of CAR+ T Cells by Flow Cytometry

In addition to CAR+ T cell enumeration, Cerba Research offers a diverse array of validated assays for determining the immunophenotype of CAR+ T cells. This encompasses the identification of memory subsets, assessment of activation status, and evaluation of exhaustion status.

These custom-developed assays are tailored to each sponsor's unique requirements, with its team of scientists collaborating closely to design panels that precisely characterize the requested immunophenotype of CAR+ T cells. These assays could reveal any abnormal expansion within specific subsets of CAR+ T cells or CAR- T cells (endogenous).

Identifying T-cell lymphoma in CAR+ T cell-treated patients is challenging, as there are no specific markers for T-cell lymphoma. Although T-cell clonality or a specific T-cell lymphoma phenotype is not currently reported in CAR+ T cell-treated patients, it has proven valuable in diagnosing T-cell malignancies. Leveraging TRBC1 (T-cell Receptor Constant β Chain-1) in flow cytometry assays provides a cost-effective, robust, and highly specific test that detects clonality of immunophenotypically distinct T-cell populations (Horna P, 2021).

While TRBC1 is useful, it may not be sufficient to test for T-cell lymphoma. Cerba Research seamlessly integrates its flow approach with cutting-edge molecular assays to address this. The company recently developed TCR sequencing[1], complementing its flow assays and enabling the profiling of the CAR+ T-cells within a patient's T cell receptor population.

This next-generation sequencing (NGS) technology allows the characterization of any emerging clonal subset of CAR-T receptors.

The T-cell clonality or specific T-cell lymphoma phenotype identified by NGS can subsequently serve as flow markers for a newly developed flow immunophenotyping assay.

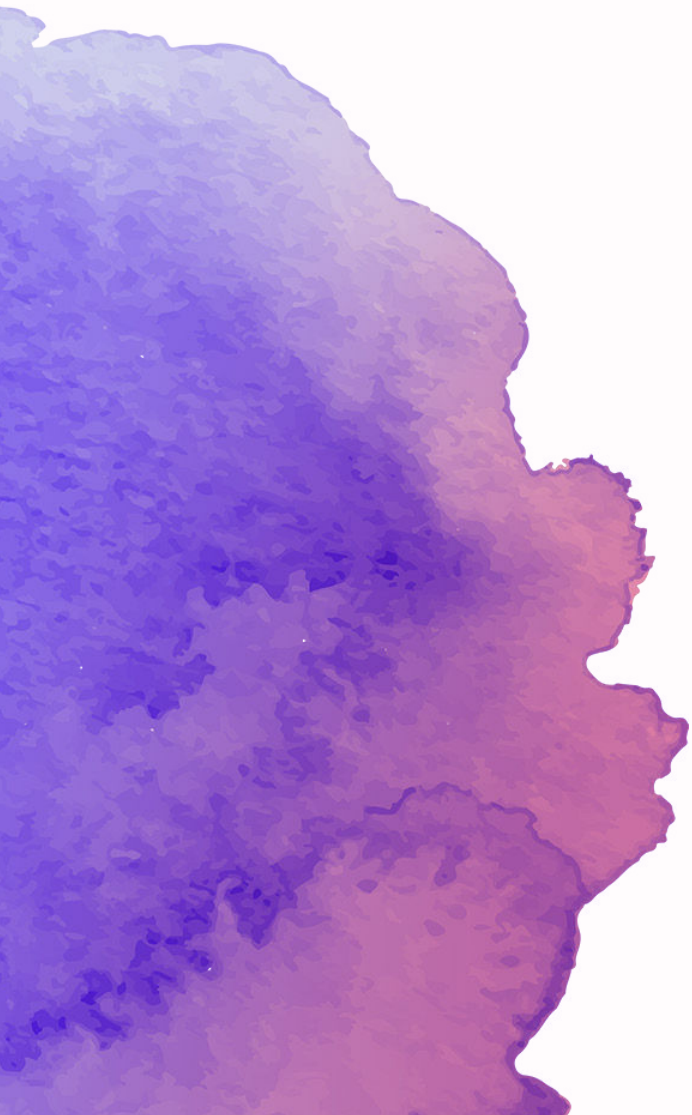
Cerba Research: Ensuring Safety, Elevating Confidence

As human lifespans extend, the challenges presented by diseases such as cancer and autoimmune disorders become more pronounced. Cerba Research emerges as a reliable solution provider to monitor the safety and effectiveness of CAR-T cell therapy.

Based on regulatory standards, its extensive testing portfolio offers a source of reassurance and includes ultra-sensitive testing methods, cutting-edge flow cytometry[2], biomarker services and leading genomics solutions.

These advanced methods can be combined with our off-the-shelf cytokine and chemokine assays, which the FDA/EMA requires in case of adverse events in a study.

Embrace the future of healthcare with Cerba Research—together we can deliver the promise of precision and predictive medicine.



Immerse yourself in further reading below or visit our resources library: cerbaresearch.com/resources/

References and Further Reading

[1]Visit our resources library and learn more about our TCR sequencing capabilities: https://cerbaresearch.com/app/uploads/2023/04/Cerba-Research_White-paper_Decoding-a-T-Cell-fingerprint.pdf

[2]Download our E-Book on flow cytometry in CAR-T Therapy here: CAR+T Drug Development: The Critical Role of Flow Cytometry (cerbaresearch.com)

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