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Scientific Review

Driving CAR T-Cells in the Fast Lane to Market

How Leading Cell Therapy Manufacturers Use Simple Western for Faster & Superior Protein Analysis

Chimeric antigen receptor (CAR) T-cell therapy is a revolutionary pillar in cancer treatment that demonstrated remarkable success in treating subsets of B-cell leukemia and lymphoma. Now much attention is focused on expanding CAR T-cell therapy to other hematologic malignancies and solid tumors. For cell therapies to reach patients, protein expression analysis is essential to finding new targets, arming T-cells to attack cancer cells and avoid immunosuppression, and characterizing treatment responses. Accurate protein quantification is necessary, often with limited and complex sample types.

Researchers have a choice of methods to characterize protein expression and modification in cells, but each method brings a set of unique limitations that can slow development. For example, flow cytometry is a common choice for signaling pathway analysis, but flow cytometry is best suited for surface-exposed proteins and many intercellular signaling proteins go undetected underneath the surface. ELISA can measure both secreted cytokines and intercellular proteins but requires specific antibodies to differentiate phospho-protein isoforms, complicating assay development. Western blot, nearly half a century old, is still part of the status quo despite severe challenges with reproducibility and scalability.

Contents

New Strategies for Targeting Immune Cells for Cancer Therapy 3

CAR T-Cells with New and Improved Features 4

More Mileage for CAR T-cells with Less Exhaustion 5

Linking Antigen Expression with Potency Assays for Cell Therapy Products 6

Looking Under the Hood: Cell Signaling Pathway Analysis 6

Running Red Lights with Immune Checkpoint Inhibitors 7

Pedal to the Metal: Simple Western Assays Bring Speed and Scalable Automation

There is high demand across the cell therapy industry for automated methods capable of generating analytical-grade results that meet regulatory standards, are easy to transfer, and are amenable to scale with manufacturing. Current methods like flow cytometry and ELISA fall short of meeting this demand, so cell therapy manufacturers are turning to alternative protein analysis solutions for their workflows, like the capillary electrophoresis-based immunoassay platform, Simple Western™. Supported by the growing number of patent citations and research articles by leading cell therapy R&D groups,[1-12] Simple Western better meets industry needs for a robust protein characterization assay with several advantages critical to cell therapy manufacturing including speed and scalable automation **(TABLE 1)**.

By highlighting real-world examples across the development pipeline, this review seeks to connect researchers with solutions in protein analysis using Simple Western platforms that can be leveraged in cell therapy manufacturing workflows, arming them with faster alternatives and accelerating time to market.

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TABLE 1.

	Simple Western	Sandwich ELISA	Intracellular Flow Cytometry	Western blot
Sample Volume	3 µL	100 µL	>100 µL	50 µL
Hands-On Time	30 minutes	90 minutes	120 minutes	>180 minutes
Scalable Automation	Yes	No	No	No
MW Information	Yes	No	No	Yes
Custom Assay Development	Easy	Challenging	Challenging	Moderately Challenging

TABLE 1. A comparison of commonly used methods for protein analysis in cell therapy manufacturing.

New Strategies for Targeting Immune Cells for Cancer Therapy

Advances in cell therapy have made it possible to reprogram a patient's T-cells to target antigens presented on cancer cells. Genetically engineered CAR T-cells contain an artificially generated receptor with a surface-exposed single-chain variable fragment (scFv) linked to intracellular CD3ζ or co-stimulatory molecules in its intracellular domain. CAR T-cell therapy has been used most successfully to treat hematological malignancies like leukemia and lymphoma. While the FDA has already approved several CAR T-cell therapies targeting the CD19 antigen, researchers are exploring new avenues to broaden CAR T-cell therapies to more cancers by targeting other antigens on tumor cells.

ROR1 is considered a promising target for cancer therapy due to putative tumor-specific expression, and multiple groups are developing antibodies and modified T cells to target ROR1. On-target, off-tumor toxicity is challenging for most nonmutated tumor antigens; however, prior studies suggest that ROR1 is absent in most normal tissues. Researchers at Fred Hutchinson Cancer Institute showed that published antibodies lack sensitivity to detect endogenous levels of cell surface ROR1 by immunohistochemistry (IHC) in formalin-fixed, paraffin-embedded tissues.[8]

Instead, they used Simple Western to develop a ROR1-specific monoclonal antibody (mAb) targeting the carboxy terminus of ROR1 **(FIGURE 1)**. They leveraged Simple Western's serology assay to screen mouse sera to produce antibodies that bind the C-terminal portion of RORI. Simple Western's size separation enabled the selection of particular antibody clones, which would have been challenging to perform with traditional serology assays like ELISA. With the help of Simple Western analysis, a patent was issued for the new anti-ROR1 antibody, including its use in CAR T-cell therapy.

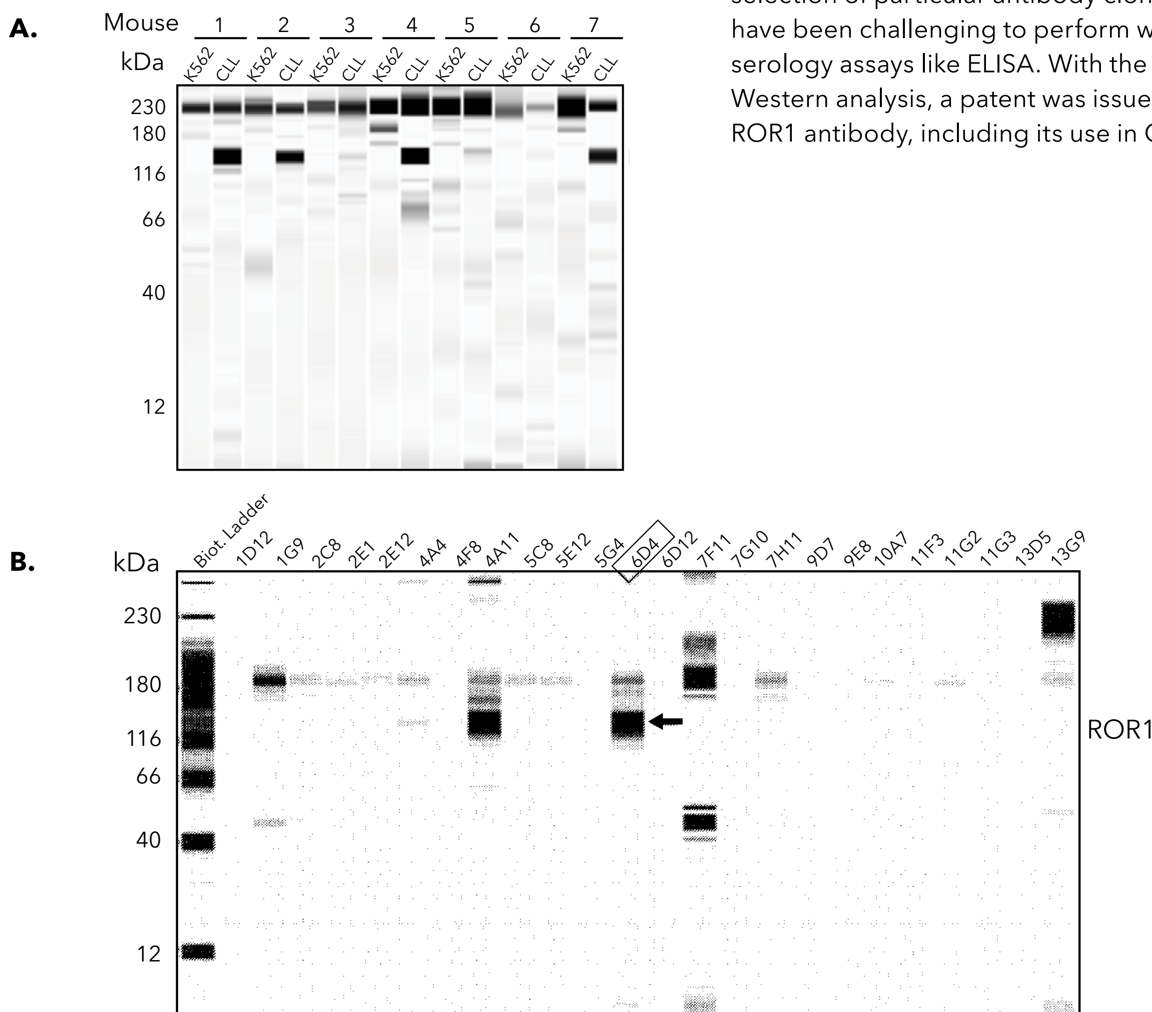

FIGURE 1. Simple Western serology assay to screen polyclonal mouse sera and hybridoma clones to produce antibodies that bind to the C-terminal portion of RORI. (A) Multiple polyclonal mouse sera were screened against RORI$^-$ K562 cells and RORI$^+$ CLL cell lysate by Simple Western analysis to detect 130 kDa RORI protein. **(B)** Results of screening hybridoma clones by immunoblot analysis for production of antibodies capable of binding only full-length RORI expressed in CLL cells. Adapted from Ashwini et al.[2]

CAR T-Cells with New and Improved Features

The fusion of genome engineering and adoptive cellular therapy holds immense promise for treating genetic diseases and cancer. Multiplex genome engineering using targeted nucleases can increase the efficacy and broaden the application of such treatments. Still, multiplex genome engineering calls for multiplex protein expression assays to validate knockouts or transgene expression. Researchers at the University of Minnesota used Simple Western to engineer new base editors with improved efficacy and specificity in T cells.[9] Following electroporation, they used Simple Western to monitor the protein expression of base editors directly in T cells **(FIGURE 2)**. Simple Western's sensitivity and small sample volume requirements minimized the amount of human T cells required for analysis.

CAR T-cells have recently been developed in which the target antigen is expressed within the CAR T-cell, like CD3ζ. To avoid self-killing (e.g., fratricide), the CAR T-cells also express a protein expression blocker (PEBL) that serves to reduce the expression of the target antigen on the cell surface **(FIGURE 3)**. To measure the expression of both CAR and PEBL, researchers used Simple Western's multiplex capabilities. Simple Western analyzed cell lysates from primary T cells transduced with the indicated retroviruses for β-actin, Myc-tagged PEBL, CAR, and endogenous CD3ζ expression.

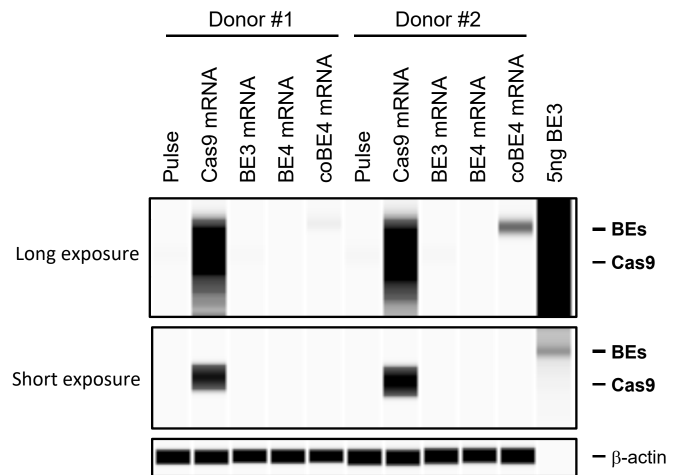

FIGURE 2. Highly efficient multiplex human T-cell engineering without double-strand breaks using Cas9 base editors. Adapted with permission (CC BY 4.0).[9]

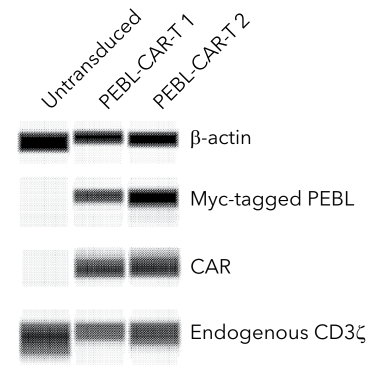

FIGURE 3. Two-gene vectors for generating CAR T-cells were transduced in primary T-cells and CAR transgene expression was measured with Simple Western. Adapted from Rizal et al.[6]

More Mileage for CAR T-Cells with Less Exhaustion

In a 2023 study published in Nature Biomedical Engineering, researchers used Simple Western in their stem cell engineering workflow to create iPSC-derived CAR T-cells with improved antitumor activity.[10] Knockout of DGK, which inhibits antigen-receptor signaling and enhances proliferation and persistency of CAR T-cells in tumors, was achieved by electroporation of iPSCs with Cas9-gRNA ribonucleoprotein complexes targeting DGK, followed by differentiation into iPSC-derived CAR T-cells. Simple Western confirmed the knockout of DGK isoforms DGKα and DGKζ in iPSC-derived CAR T-cells **(FIGURE 4)**.[10] Simple Western provides protein expression readout directly in cells with minimal sample preparation and needs only 3 µL of sample, conserving precious iPSC-derived CAR T-cells. In addition, Simple Western is faster than sequencing, which is only partially predictive of protein expression and function.

Researchers at CRISPR Therapeutics used Simple Western to engineer T-cells with improved proliferation and persistency.[4] By targeting genes involved in apoptosis, self-renewal, and T-cell exhaustion, they created a T-cell bank with enhanced expansion, proliferation, and activation capacity and reduced apoptosis. Recombinant AAV encoding of an anti-BCMA CAR was delivered with Cas9 to activate allogeneic human T-cells. About one week post electroporation, cells were assessed for TET2 protein knockdown using an anti-TET2 polyclonal antibody. The results obtained from Simple Western assays confirm no detection of TET2 in TET2 KO (TET2−) anti-BCMA CAR T-cells, while TET2 expression was detected in control cells and TET2 WT (TET2+) anti-BCMA CAR T-cells.[4] These mutations leading to TET2 gene knockout or protein truncation increased proliferation and expansion in primary human T-cells.

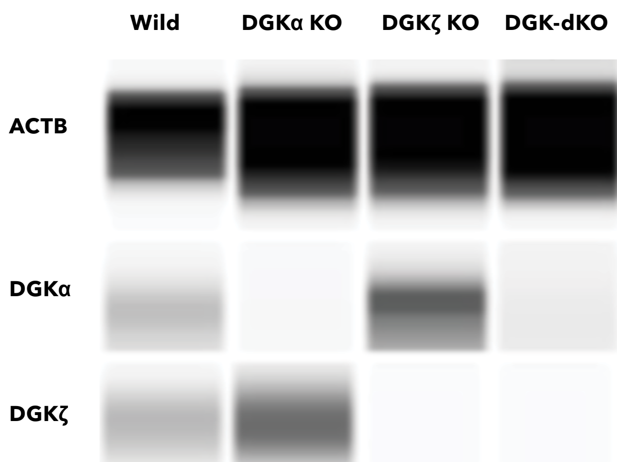

FIGURE 4. Simple Western confirmation of knockouts for the optimization of the proliferation and persistency of CAR T-cells derived from human induced pluripotent stem cells. ACTB was used as a control. Adapted with permission (CC BY 4.0).[10]

Linking Antigen Expression with Potency Assays for Cell Therapy Products

A protein called FLT3 is presented on most leukemia cell isolates in patients with acute myeloid leukemia and is limited to healthy individuals. Thus, researchers at Amgen hypothesized that FLT3 would make an attractive target for CAR T-cell immunotherapy.[11] They developed a CAR T-drug product that specifically targets FLT3, then correlated the expression of FLT3 with cytotoxicity by relying on Simple Western. The data showed a strong positive correlation between cells that express FLT3 and the cytotoxic activity of CAR T-cell treatment. In the study, the authors explicitly justify their decision to use Simple Western:

> *"This single platform assay has more quantitative, reproducible results and a wider dynamic range than a traditional Western analysis."*[11]

Looking Under the Hood: Cell Signaling Pathway Analysis

The characterization of CAR T-cells signaling cascades is essential to understanding their mechanism of action, but presents several challenges. Quality antibodies are needed to measure signaling molecules in complex intracellular and extracellular environments. Traditional immunoassays like Western blot and ELISA often need to be improved to measure protein expression in the limited and challenging sample types required for analysis, like tumor tissue biopsies. Western blot is poorly reproducible and requires large amounts of sample. ELISA does not provide molecular weight size information to monitor antibody specificity to target proteins and isomers and often suffers from interference by the sample matrix. FACS can label CAR T-cells in several ways, but drawbacks include antibody cross-reactivity, limited antibody availability, and difficulty detecting intracellular proteins.

Because signaling networks are complex, a high-throughput method is often needed to measure all protein targets involved, including phosphorylated and non-phosphorylated protein forms of each protein in the signaling cascade. Traditional Western blots are commonly chosen for signaling studies over other immunoassay approaches since the provided molecular weight information paired with immunodetection increases assay specificity, which can be challenging when looking at phosphorylated protein isoforms or other post-translational modifications. Nonetheless, traditional Western blots are typically limited in their throughput and ability to profile more than 1 or 2 proteins in the signaling pathway at a time.

In a study published by a team from Janssen BioTherapeutics, Simple Western was instrumental in revealing how the TIM-3 receptor inhibits T-cell activation. TIM-3 is a negative regulator of T-cell function by suppressing anti-CD3/CD28- induced T cell receptor (TCR) mediated activation, but the suppression mechanism has remained elusive. To address this lack of understanding, the authors relied on Simple Western to comprehensively analyze the TIM-3 signaling cascade **(FIGURE 5)**. Their results identified a very interesting signaling complex involving TIM-3 and several kinases involved in TCR signaling. In the inactivated state, they placed the association of Vav-1, Akt, SLP-76, ZAP-70, Syk, P85α-PI-3K, Fyn, and the adaptor proteins 3-BP2 and SH2D2A (T cell-specific adaptor protein) with TIM-3 **(FIGURE 5, left lane)**. Conversely, TIM-3 is no longer associated with those kinases in an activated state but with the Src family kinase, Lck, and enhanced association of PLC-γ1. Furthermore, they did not see an association of TIM-3 with the P85α-PI-3K subunit in the activated state **(FIGURE 5, middle lane)**. Therefore, the sequestration of both Lck and PLC-γ1 by TIM-3 could serve as an inhibitory mechanism in which TIM-3 negatively regulates NF-kB/NFAT signaling pathways.

Running Red Lights with Immune Checkpoint Inhibitors

To prevent T-cells from indiscriminately engaging in a dangerous autoimmune attack, CAR T-cells encode immune checkpoint proteins that allow them to recognize healthy cells. However, cancer cells can disguise themselves by exploiting these proteins to evade immune system attacks. Thus, targeting these checkpoint proteins with checkpoint inhibitors is an essential anti-cancer strategy. For example, the checkpoint protein PD-1 on the surface of T-cells can bind PD-L1 on the surface of cancer cells, inactivating the cytotoxic activity of T-cells. Checkpoint inhibitors that downregulate PD-L1 are a promising anti-cancer strategy. In a seminal study featured in Nature Medicine, Simple Western was used to monitor the regulatory pathway of PD-L1 in melanoma cells, leading to potential new treatment strategies.[13]

In cell therapy, PD-L1 is an adoptive T-cell therapeutic agent that affects adoptive T-cell transfer. Anti-PD-L1 antibodies that prevent PD-1 binding represent a promising strategy for improving CAR T-cell therapy. However, blocking PD-L1 on healthy cells can cause a toxic autoimmune response. Therefore, an activatable anti-PD-L1 antibody with localized antigen-binding activity in the tumor microenvironment is highly desirable. The design of such activatable antibodies, Prodrugs, includes a protease cleavage motif cleaved explicitly by proteases in the tumor microenvironment. Simple Western is the accepted standard in clinical trials to monitor cleavage and activation of activatable anti-PD-L1 antibodies and other Prodrugs because Pb-Tx activation can be observed in a target-agnostic manner using anti-idiotypic antibodies. This allows for the identification of cleavage events and specific quantification of the activated light chain without needing additional detection antibodies.

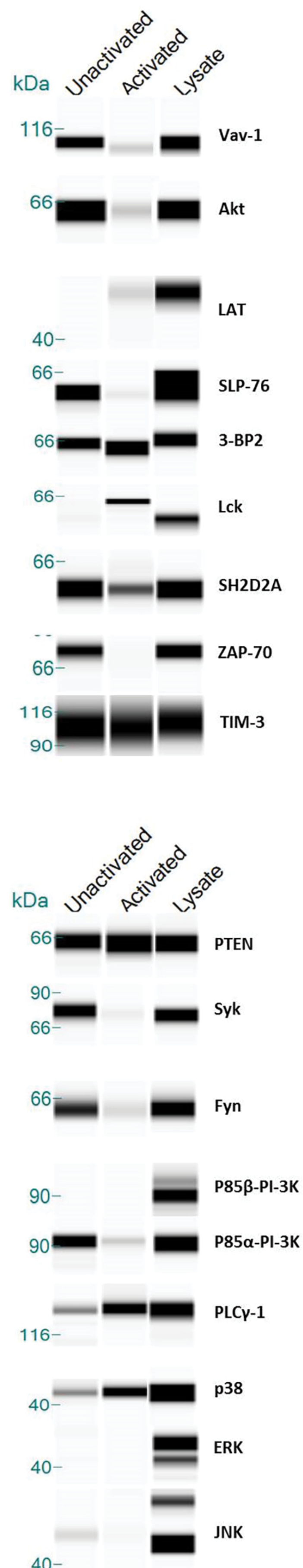

FIGURE 5. Simple Western analysis reveals the TIM-3 signaling web in inactivated and anti-CD3/CD28-activated T-cells. Cleared lysate served as a loading control for individual antibody reactivity. Figure adapted with permission (CC BY 4.0).[12]

Simple Western Is an Engine Driving CAR T-Cell Development

The use of living cells as therapeutics brings unique challenges compared to non-living drugs. Without the right analytical tools, understanding the complex network of proteins that create safe and effective CAR T-cells can jam the development pipeline and prevent therapies from reaching the final destination - a patient in need of treatment. It is clear from the track record of patents and publications that Simple Western is driving innovation of new CAR T-cell technology by simplifying assay development and providing more quantitative, reproducible results and scalable automation than competing immunoassay methods.

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