A Path to Universal iPSCs for Gene Therapy

Rapid iPSC Clone Screening of Protein Expression

Universal iPSCs promise to bring new treatments to more patients. In this App Note, we create a genetic knockout library in iPSCs and functionally validate target knockdown using the Simple Western™ platform from Bio-Techne, a critical step in creating universal iPSCs.

What Are Universal iPSCs and Why Are They Important?

Induced pluripotent stem cells (iPSCs) can differentiate into any cell in the body, and advancements in gene editing technologies promise revolutionary iPSC-derived gene therapies. However, clinical use of gene-modified iPSCs is at risk of rejection of allogeneic transplantation by the immune system.[1] To deliver inexpensive off-the-shelf iPSC-derived products to more patients, generating hypoimmunogenic iPSCs that can evade immune cell attack and become universal cell therapy treatments is necessary.

Immune rejection during allogeneic transplantation results from mismatching the human leukocyte antigen (HLA) between donor and patient. HLA is an important molecule in the immune system that differentiates between self and non-self components. Therefore, to generate universal iPSCs, deletion of HLA is required to prevent mismatching and rejection by the immune system.

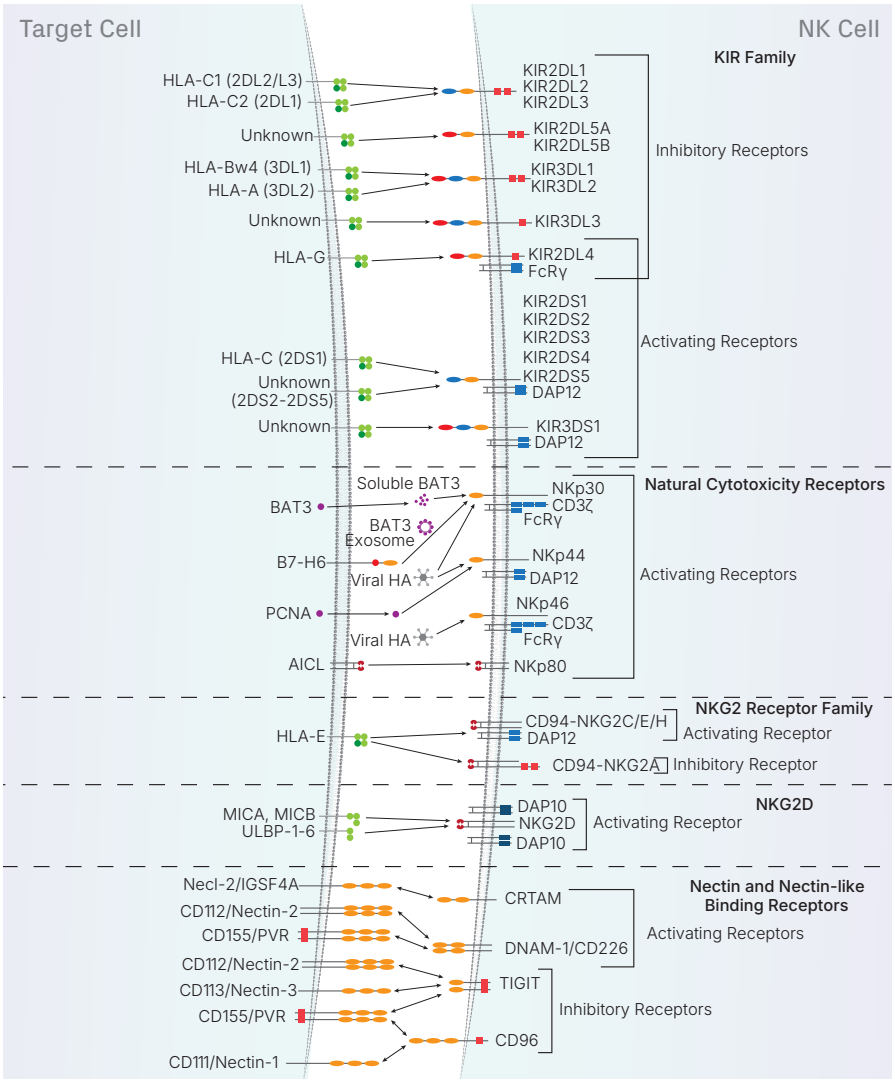
HLA is a large protein important to cell fitness, and deletion of HLA is not trivial. HLA comprises HLA class 1 and class 2, occupying a relatively large portion of the genome that is difficult to delete using a single gene editing strategy. Instead, deletion of the β_2M subunit can effectively suppress the expression of HLA-1.[2] In addition, deletion of the CIITA transcription factor suppresses HLA-2.[3] These two deletions lead to HLA suppression in iPSCs.

However, as HLA is important to cell fitness, unwanted NK cell attack is significantly enhanced by HLA-1 deficiency. Partial deletion of HLA-1 to keep HLA-C HLA-E/F/G can evade attack from NK cells,[4-5] but iPSCs with partial deletion of HLA-1 are only semi-universal. Thus, the biggest challenge in establishing a fully universal iPSC is effectively preventing attacks from NK cells.[1]

HLA-deficient iPSCs can avoid NK cell attack by suppressing NK-activating receptor (KAR) ligands. Deletion of both HLA and KAR ligands can lead to iPSCs that are fully universal. A recent study showed that deletion of CD155 and most of HLA with intact HLA-E largely escaped recognition by NK cells while maintaining anti-tumor activity.[6] However, NK cells have multiple KARs that bind to different ligands, and expression levels can differ between cell types (**FIGURE 1**).

FIGURE // 1

Natural Killer Cell Receptors



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Knockout or knockdown of protein-coding sequences requires validation by protein expression screening. However, intracellular protein detection with flow cytometry requires laborious permeabilization steps, and validating antibodies against intracellular targets is often more challenging because flow cytometry does not provide high-resolution subcellular localization information or insights into molecular weight. Likewise, ELISA lacks size separation information to verify antibody specificity, and matrices of complex samples may skew results. Traditional Western blot provides sized-based specificity but consumes large sample volumes, and traditional Western blot is time-consuming, semi-quantitative, and is often poorly reproducible.

Bio-Techne may help create off-the-shelf iPSCs that are universally applicable to more patients and help overcome rejection by the patient's immune system. In this App Note, we leverage Bio-Techne's **Genome Engineering Services**, **Pala Cell Sorter and Single Cell Dispenser**, and **Simple Western™** technology to screen an iPSC genetic knockout library, including knockdown of targets to suppress expression of HLA as well as several KAR ligands susceptible to NK cell recognition and attack.

Compared to flow cytometry, the Jess platform powered by Simple Western technology could readily detect membrane bound and intracellular targets and reproducibly quantify their expression relative to wild-type, needing only 3 µL of iPSC lysate samples. We also used the Simple Western platform to screen for residual Cas9 to ensure safety of engineered iPSCs. These screening assays using Simple Western technology process up to 25 samples in 3-hour hands-free run times, an easy high throughput Western blot method in screening applications

Materials and Methods

iPSC Strain Engineering

Wild-type iPSC cells were first electroporated with CRISPR/Cas9 targeting β_2M and CIITA and bulk sorted using the **Pala Cell Sorting and Dispensing instrument** from Bio-Techne, followed by CRISPR-Cas9 knockdown targeting BAT3, CD155, and MICA, and bulk sorted again by the Pala instrument. Then, we used limiting dilution to achieve single cell clones. Cells were analyzed by flow cytometry using the **H/M Pluripotent Stem Cell Multi-Color Flow Cytometry Kit** (Bio-Techne, FMC001).

Simple Western Analysis

All materials and primary antibodies used for analysis by the **Jess™** platform, powered by **Simple Western** technology, are listed in **TABLE 1** and **TABLE 2**, respectively.

iPSC wild-type and knockout clones were prepared at 1 mg/mL or 0.1 mg/mL. Clones with stock concentration <1.25 mg/mL were prepared at 0.8X. The **RePlex™** Total Protein (TP) assay was used for normalization. The TP-NIR for samples analyzed at 1 mg/mL, and the TP-HRP for samples analyzed at 0.1 mg/mL.

We denatured samples under reducing conditions with 1X Master Mix (MM) for 5 minutes at 95 °C. The blocking buffer was Antibody Diluent 2. We diluted primary antibodies in Antibody Diluent 2 and placed them on ice for 30 minutes. The ready-to-use (RTU) secondary antibodies were incubated on ice for 30 minutes.

Simple Western assays were set up according to the **Screening on Simple Western technology protocol**.

Simple Western data was normalized to **Total Protein** loaded. Simple Western technology on Jess and flow cytometry methods calculated the percent knockout or knockdown relative to the wild-type control, where 0% represents complete knockout. To normalize the data collected by flow cytometry, the signal from wild-type cells was subtracted from genetically modified clones. To quantify residual Cas9 in iPSCs, we generated a standard curve using the recombinant *S. pyogenes* **CRISPR-Cas9 protein** from Bio-Techne (9957-C9).

TABLE // 1

Simple Western materials used in this study

Name	Catalog #
12-230 kDa Separation Module	SM-W004
Anti-Rabbit Detection Module	DM-001
Anti-Mouse Detection Module	DM-002
RePlex™ Module	RP-001
Total Protein Module	DM-TP01
Streptavidin-NIR	043-816

All materials are from ProteinSimple, a Bio-Techne brand. All experiments were run on the Simple Western Jess™ platform.

TABLE // 2

Antibodies used in this study

Target	Catalog #	Simple Western Conc.
β_2M	NBP2-44471	20 µg/mL
BAT3	NBP2-38693	20 µg/mL
MICA	NBP1-32830	100 µg/mL
CD155	NBP1-88131	10 µg/mL
CIITA	NBP2-59074	95 µg/mL
Cas9	MAB10252	2 µg/mL

All antibodies are from Novus Biologicals, a Bio-Techne brand, except the Cas9 antibody is from R&D Systems, a Bio-Techne brand.

Results

We created a genetic knockout library to knockout HLA and KAR ligand targets (**TABLE 3**) using CRISPR-Cas9 genome editing technology. We screened clones from the genetic knockout library for the expression of β_2M and CIITA, which suppress HLA-1 and HLA-2, respectively (**FIGURE 2**, left panels). We used the RePlex™ module to detect total protein in the second probing cycle in the NIR fluorescence channel (**FIGURE 2**, right panels). Using a similar approach, we screened for expression of KAR ligands (**FIGURE 3**).

TABLE // 3

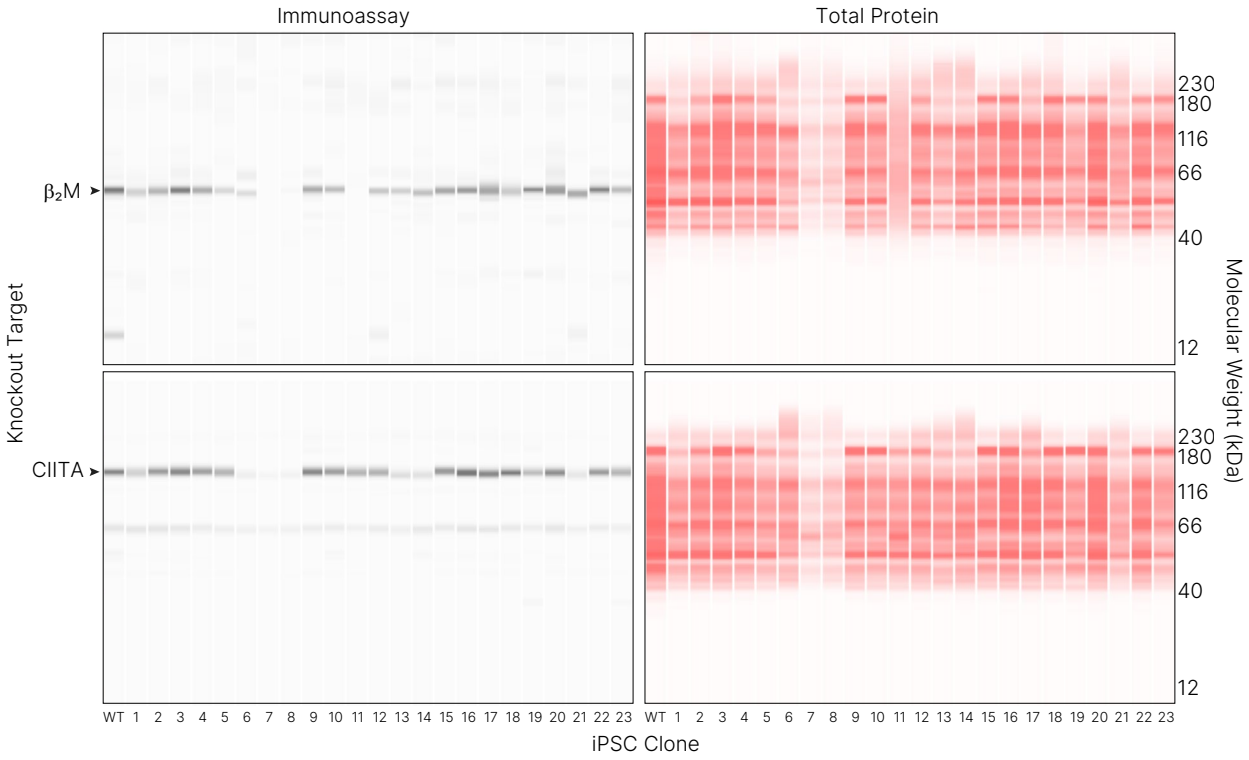
Targets for genetic knockdown in iPSCs

Target	Class	Subcellular Localization[8]
β_2M	HLA-1	Surface exposed
CIITA	HLA-2	Intracellular
BAT3	KAR ligands	Intracellular
CD155		Surface exposed
MICA		Surface exposed

Knockout of β_2M and CIITA suppress expression of HLA-1 and HLA-2, respectively. BAT3, CD155, and MICA are known KAR ligands. Two targets, CIITA and BAT3, are localized to the intracellular environment, and the remaining targets are surface exposed.[8]

FIGURE // 2

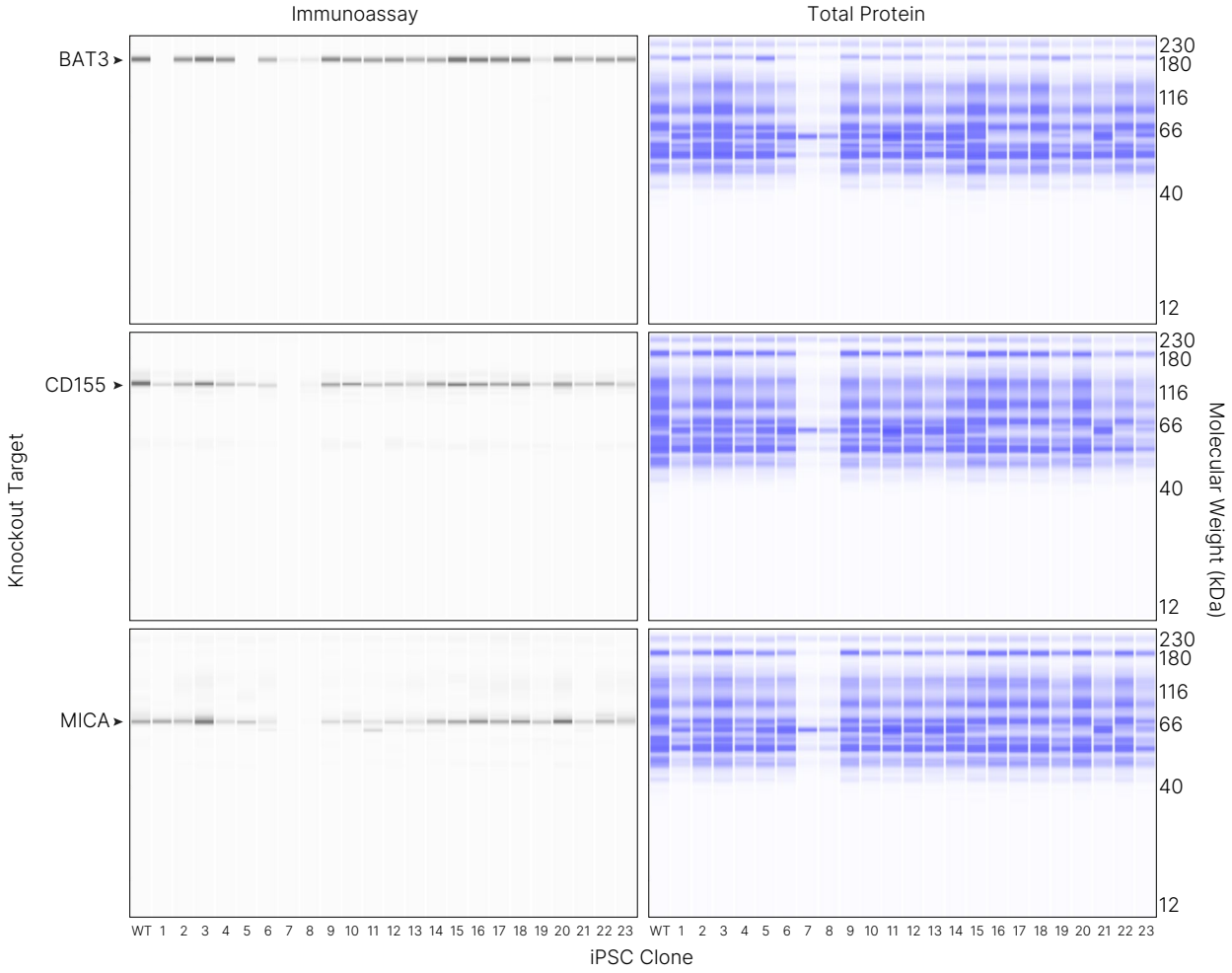
Simple Western screening of HLA knockouts in iPSCs



The RePlex™ Module was used to simultaneously detect β_2M and CIITA in Probe 1 (left) and total protein with NIR detection in Probe 2 (right).

FIGURE // 3

Simple Western Screening of KAR Ligand Knockouts in iPSCs



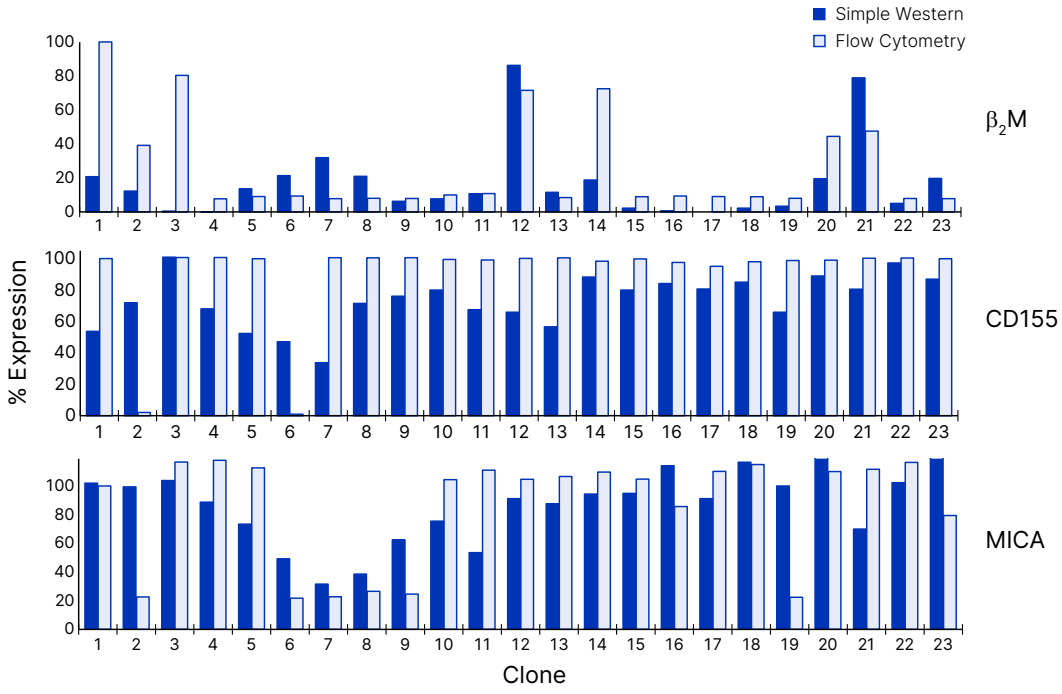
The RePlex™ Module was used to simultaneously detect BAT3, CD155, and MICA in Probe 1 (left) and total protein with chemiluminescence detection in Probe 2 (right).

Our study involved a comparison between the Simple Western method and flow cytometry. We quantified the percent knockdown in each clone relative to wild (**FIGURE 4**). It's worth noting that flow cytometry, while a widely used method, was not able to reliably detect CIITA or BAT3, likely due to their intracellular subcellular localization (TABLE 3). This limitation underscores the need for alternative methods. In contrast, the Simple Western analysis, a method we found to be more effective, readily detected these intracellular targets, enabling quantification of expression levels relative to the wild-type control (**TABLE 4**).

Interestingly, Simple Western analysis showed higher target knockdown rate than flow cytometry for most clones screened (**FIGURE 4**, **TABLE 4**). Ultimately, the results with Simple Western indicate that Clone #7 had the highest percentage knockdown of all 5 targets, with all targets showing less than 50% expression relative to the wild-type control (**FIGURE 4**, **TABLE 4**), except for BAT3, which appeared to be expressed at 69% relative to wild-type (**TABLE 4**).

FIGURE // 4

Target Knockdown Measured by Simple Western & Flow Cytometry Methods



Values represent percent expression relative to the wild-type control, where wild-type expression equals 100% and complete knockdown equals 0%. Targets CIITA and BAT3 are not shown because flow cytometry was not able to reliably detect these targets, likely due to their intracellular localization. The quantification of % knockdown of CIITA and BAT3 are shown in TABLE 4 instead.

TABLE // 4

Knockdown of Intracellular Targets that were Unreliably Detected by Flow Cytometry were Measured by the Simple Western Platform

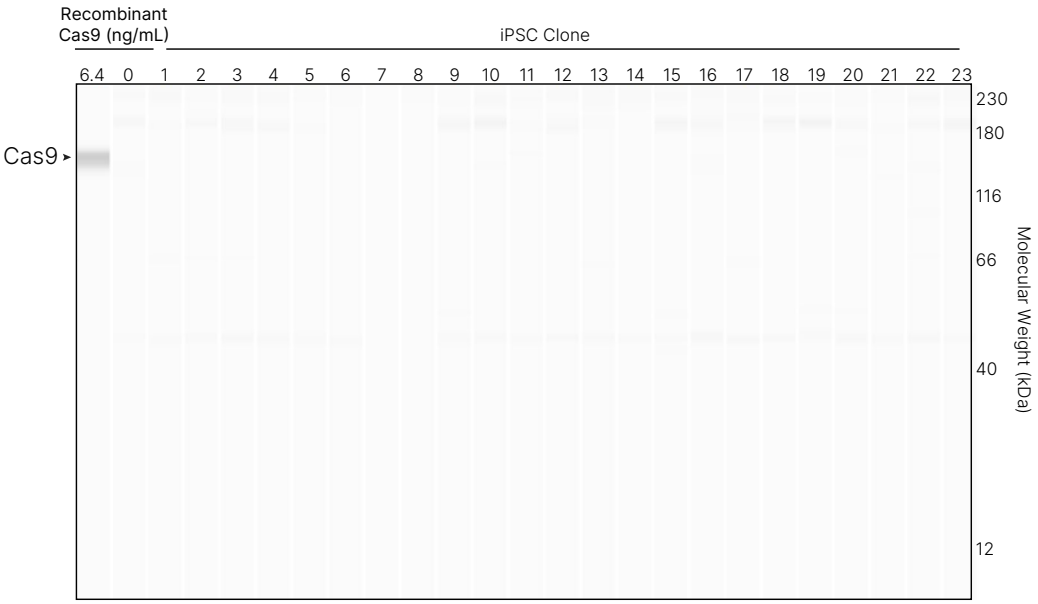
Clone	CIITA	BAT3	Clone	CIITA	BAT3
1	101	2	13	73	89
2	126	85	14	68	82
3	138	94	15	131	98
4	129	96	16	141	111
5	130	0	17	128	109
6	48	90	18	130	95
7	43	69	19	106	53
8	48	69	20	123	109
9	147	94	21	76	86
10	139	96	22	119	91
11	125	86	23	132	94
12	121	84			

Values represent percent expression relative to the wild-type control, where wild-type expression equals 100% and complete knockdown equals 0%. Protein expression was measured using the Simple Western platform. These targets were not readily detectable by flow cytometry under the conditions tested here.

Finally, we screened for residual Cas9 protein in iPSC clones, which were engineered for target knockdown by the CRISPR-Cas9 gene editing system. Residual Cas9 in engineered cells is known to cause off-target effects, toxicity, and must be closely monitored for the safety of therapeutics.[9] Here, Cas9 was not detectable in any of the clones above the recombinant Cas9 protein control, which was loaded at loaded at 6.4 ng/mL and shown in Lane 1 (**FIGURE 5**).

FIGURE // 5

Simple Western screening for residual Cas9 in engineered iPSCs



Recombinant Cas9 protein and iPSC clones engineered with CRISPR-Cas9 technology were probed with an anti-Cas9 antibody using the Simple Western platform.

The Simple Way to Protein Expression Screening

iPSCs promise to usher in new cell and gene therapies, screen small molecule drugs, and model diseases. Genetically modified iPSCs to become universal hypoimmunogenic donors must be functionally validated at the protein level. For this step, Simple Western technology overcomes many of the shortcomings of traditional methods for protein analysis.

- Flow cytometry is a workhorse method for cell engineering, primarily for cell surface proteins. The detection of intracellular proteins by flow cytometry adds laborious permeabilization steps and can make results unreliable or difficult to interpret. Simple Western instruments measure whole cell lysates, surface proteins, and purified proteins alike, all in a single hands-free 3-hour run.
- Custom assay development with Simple Western technology is easy. Traditional Western blot assays are **easily transferred to Simple Western platforms**. As a capillary electrophoresis immunoassay, Simple Western technology provides size-based specificity and antibody-based detection of targets, with a growing catalog of over **5,000 validated antibodies**.
- Simple Western instruments don't require extended cell culture because Simple Western instruments only require 3 µL of lysate for analysis.
- Simple Western is the only platform on the market that provides a fully automated western blotting workflow, offering high reproducibility, sensitivity, and quantification, in addition to time and cost savings. As an open capillary western blot platform, Simple Western users may choose any commercial or custom antibody for specific detection of target proteins and protein isoforms.

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