Validate CRISPR-edited cells using imaging and western blot detection on a microplate reader

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Benefits

- **Measure transfection efficiency by accurately identifying GFP-positive cells vs non-transfected cells**
- **Validate CRISPR-mediated gene knockdown using western blot analysis on protein bands**
- **Capture high-quality western blot images**

Introduction

Genome editing has been widely used to study gene expression and protein function, but many of these methods are labor intensive and inaccurate[1]. The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 system has become a very popular tool for editing genes due to its high accuracy and ease of use[2]. It originated as part of the innate immune system of bacteria and archaea and was used to protect against foreign DNA[3,4]. Short sequences of known foreign DNA are expressed as CRISPR targeting RNA (crRNA), which guide the Cas9 enzyme to cleave any foreign DNA containing a similar sequence. By harnessing this system, researchers can study the effects of gene silencing and transcription regulation, and they can potentially alleviate genetic disorders in diseased cells[5].

A guide RNA (gRNA) similar to a crRNA is designed to target a region in the gene, and the Cas9 enzyme can create double-strand breaks in this specific region of the host cell's genome (Figure 1). After a double-strand break is made, the cell will undergo one of two repair pathways: the nonhomologous end joining (NHEJ) pathway or the homology-directed recombination (HDR) pathway. The NHEJ pathway is commonly used to disrupt genes via base insertions or deletions (indels), while the HDR pathway can be used to knock in a reporter gene or an edited sequence by exchanging sequences between two similar or identical molecules of DNA[6].

Validation of gene editing is necessary to determine if the introduced mutation knocked down the gene of interest. Usually, genomic PCR and genetic sequencing tests are performed to confirm successful deletions or insertions, however, measuring protein expression is a more definitive measure of gene knockdown, avoiding signal from in-frame deletions/mutations[7].

Here, we demonstrate how the SpectraMax i3x Multi-Mode Microplate Reader and ScanLater Western Blot Detection System can be used to validate CRISPR/Cas9 genome editing. We used
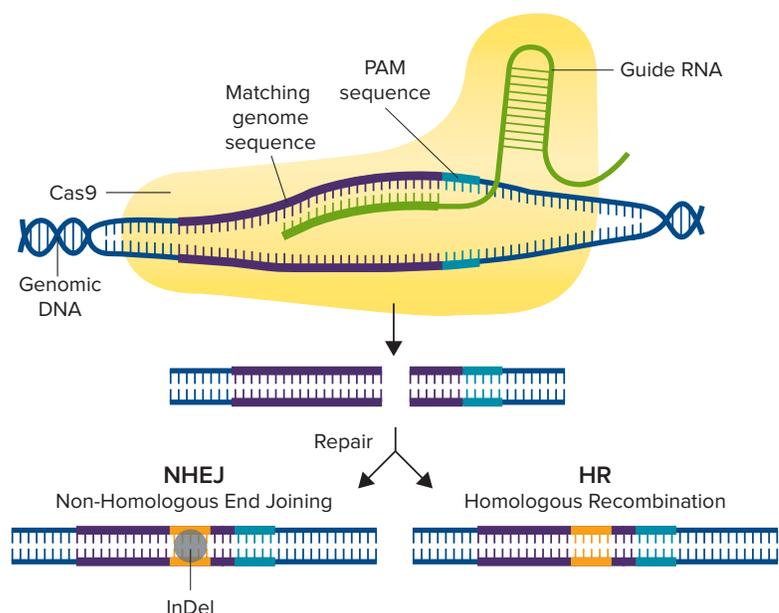
Figure 1. CRISPR/Cas9 Mechanism. The Cas9 enzyme is activated by first binding to a guide RNA, then binding to the matching genomic sequence that immediately precedes 3-nucleotide PAM sequence. The Cas9 enzyme then creates a double-strand break, and either the NHEJ or the HDR pathway is used to repair the DNA, resulting in an edited gene sequence.

Origene's ATG5 human gene knockout kit via CRISPR kit to knock down Autophagy Related 5 (ATG5) protein expression in HEK293 cells and knock in green fluorescent protein (GFP) and puromycin resistance genes. Knockdown was verified with ScanLater western blot analysis.

Materials

- ATG5 Human CRISPR Knockout kit via CRISPR (Origene cat. #KN210563)
- FUGENE HD Transfection Reagent (Promega cat. #E2311)
- Opti-MEM Reduced Serum Media (ThermoFisher cat. #31985062)
- Rabbit Polyclonal Antibody against ATG5 (Origene cat. #TA301481)
- ATG5 HEK293T cell transient overexpression lysate (Origene cat. #LC401532)
- pCas-Guide-EF1a-GFP Plasmid (Origene cat. #GE100018)
- HEK293 cells (ATCC cat. #CRL-1573)
- SpectraMax® i3x Multi-Mode Microplate Reader (Molecular Devices cat. #i3x)
  - ScanLater™ Western Blot Detection System (Molecular Devices cat. #0200-7027)
- SpectraMax® MiniMax™ 300 Imaging Cytometer (Molecular Devices cat. #5024062)
- ScanLater: Eu-Streptavidin Evaluation Kit (Molecular Devices cat. #R8200)
- ScanLater: Anti-Mouse Evaluation Kit (Molecular Devices cat. #R8201)
- Pierce BCA Protein Assay Kit (Thermofisher cat. #23225)
- Puromycin (InvivoGen cat. #ant-pr)
- TGX 12% SDS-PAGE Gel (Bio-Rad cat. #1620174)
- TransBlot Turbo Mini-size LF PVDF membranes (Bio-Rad cat. #1620174)
- Trans-Blot® Turbo™ system (Bio-Rad cat. #1704150)

Methods

Cell transfection

HEK293 cells were seeded in Costar tissue culture-treated 6-well plates at 300,000 cells per well. Cells were transfected with (1) a guide vector (AAGATGTGCTTCGAGATGTG) and (2) a donor vector containing sequences for puromycin resistance and GFP. In parallel, three wells of cells were transfected
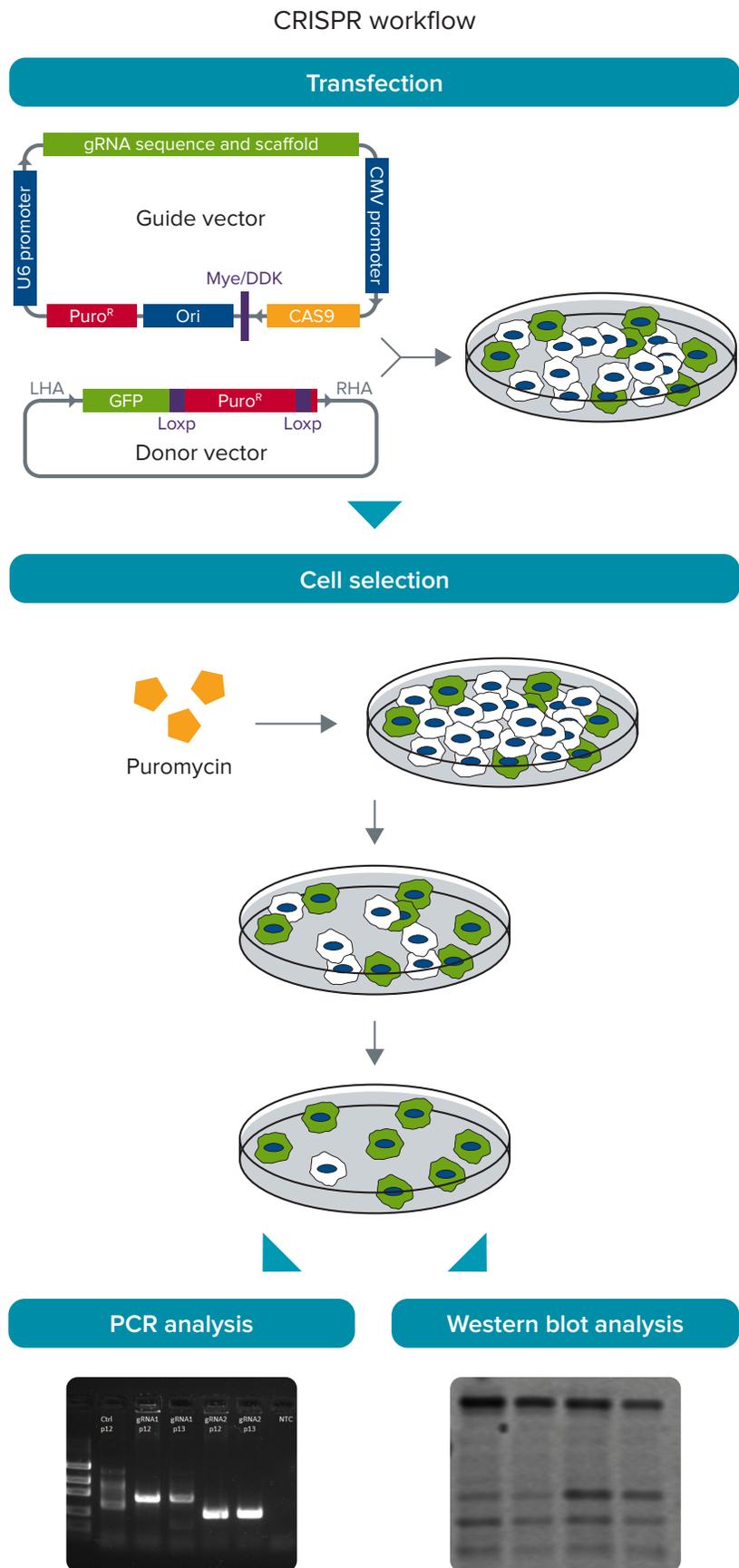
CRISPR workflow

Figure 2. CRISPR/Cas9 experimental workflow. HEK293 cells were transfected with the guide and donor vectors. Cells are then selected with 1.0 µg/mL puromycin to enrich for cells with gene insertion. DNA and protein are collected from the selected cells, and genomic PCR and western blotting are performed to verify gene insertion and knockdown, respectively.

with the pCas-Guide-EF1a-GFP vector that transiently expresses GFP as a transfection control. 6 µL of FUGENE HD transfection reagent and 2 µg of total DNA (3:1 ratio) were used to transfect cells. Afterwards, cells were incubated at 37°C overnight. Transfected cells expressed GFP and were identified and counted in the green fluorescence channel of the MiniMax cytometer. Total cell count was calculated using StainFree analysis, and transfection efficiency was measured by dividing the number of GFP-positive cells by the total number of cells using SoftMax Pro Software.

Cell selection

Transfected cells were cultured in medium containing 1.0 µg/mL puromycin to select for cells containing an inserted puromycin-resistance gene. Afterwards, cells were lysed, and genomic DNA and total protein were collected. Genomic DNA was sent to GENEWIZ for PCR analysis to verify correct gene insertion. Protein concentrations were quantitated using the Pierce BCA assay read on the SpectraMax i3x reader, and results were analyzed using a preconfigured protocol in SoftMax Pro Software.

Western blot

5 µg and 10 µg of total protein from both CRISPR-edited cells and unedited cells were prepared by diluting cell lysates in Laemmli sample buffer and boiling at 100°C for 10 minutes. Samples were then loaded onto a 4-20% pre-cast gel, and SDS-PAGE was performed. Proteins were then transferred to a PVDF membrane using the Trans-Blot Turbo system's semi-dry method.

The membrane was incubated in ScanLater Blocking Buffer at room temperature for one hour. The loading control, ladder, and ATG5 region lanes of the membrane were then cut into separate pieces and incubated in their respective primary antibodies (see Table 1) at 4°C overnight. Following three washes in ScanLater Wash Buffer, the blot pieces were then incubated with ScanLater Eu-labeled secondary antibodies at room temperature for one hour. The antibodies and their dilutions are shown in Table 1.
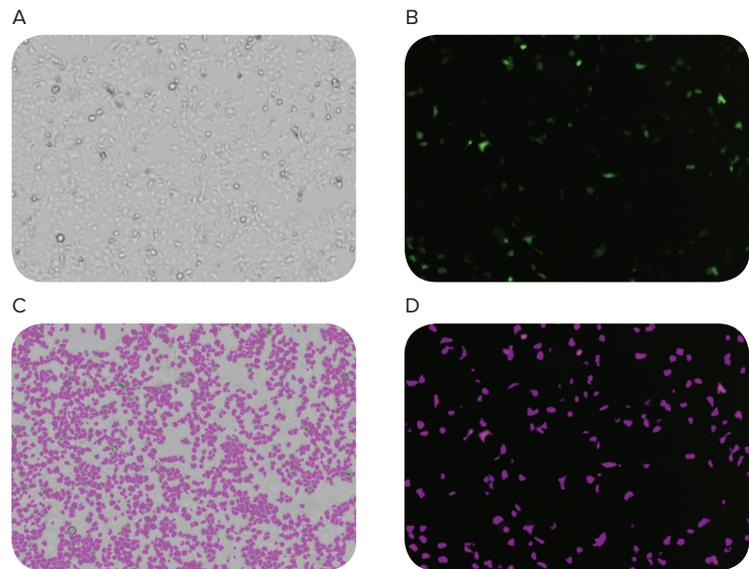
Figure 3. Images of cellular transfection. HEK293 cells were transfected with a pCas-Guide-EF1a-GFP vector in parallel with CRISPR/Cas9-transfected cells to calculate transfection efficiency. The MiniMax cytometer was used to image cells in the bright-field channel **(A)** and the green fluorescence channel **(B)**. SoftMax Pro Software identified and counted cells in both channels **(C and D)**, and a 13% transfection efficiency was calculated by dividing GFP-positive cells by total cell count.
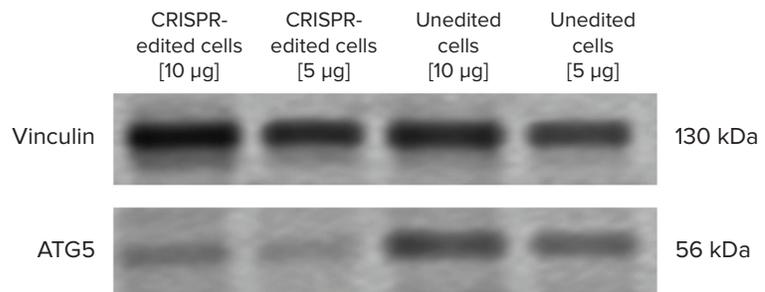
Figure 4. ATG5 western blot experiment. ATG5 protein expression was visibly lower in CRISPR-edited cells compared to unedited cells. 5 µg and 10 µg of sample were loaded onto the SDS-PAGE gel to account for over/under sample loading. Vinculin was used as a loading control to normalize for variable sample loading. The western blot image was exported to ImageJ software to measure band density.
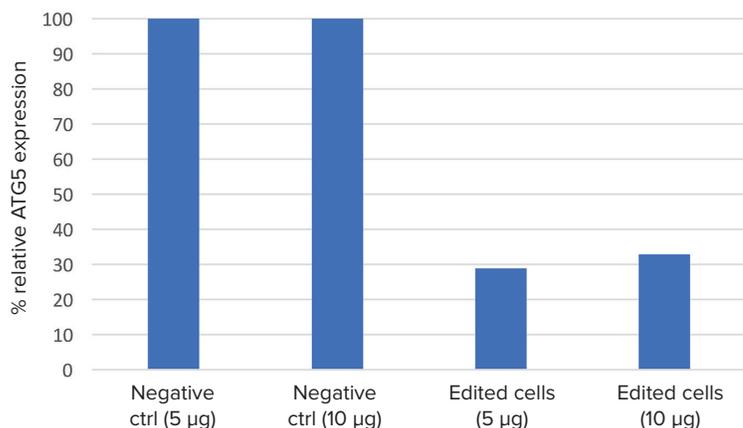
Figure 5. Relative ATG5 protein expression. The western blot image from Figure 4 was exported to ImageJ software, and band density was analyzed. ATG5 protein band density was normalized to their respective loading controls. CRISPR-edited cells had consistently lower ATG5 protein expression compared to non-edited cells.

Following the secondary antibody incubation, the membrane was washed three times in ScanLater Wash Buffer, dried, and reassembled before reading with the ScanLater Western Blot Detection System.

	Primary antibody	Secondary antibody
Sample blot	1:2500 rabbit anti-ATG5	1:10000 Goat Anti-Rabbit
Loading control	1:10000 rabbit anti-Vinculin	1:10000 Goat Anti-Rabbit
ScanLater Western Blot Protein Ladder		1:10000 Eu-Streptavidin

Table 1. Antibody dilutions used for western blot.

Data analysis

Data were exported from SoftMax Pro Software into ImageJ software, and band densities were measured to quantitate relative ATG5 protein expression. Vinculin, a 117-kDa cytoskeletal protein associated with cell-cell and cell-matrix junctions, was used as a loading control to normalize for sample loading variation.

Results

The MiniMax cytometer captured high quality images of both transfected and non-transfected cells, and SoftMax Pro Software was able to identify and quantitate the number of GFP-positive cells and total number of cells. A 13% transfection efficiency was calculated (Figure 3).

PCR analysis of genomic DNA samples determined that the correct-sized insertion had been made in the ATG5 region of the HEK293 cell genome (data not shown).

In the western blot image, visible decrease in ATG5 protein band intensity was seen between the CRISPR-edited cells and the unedited cells (negative control) (Figure 4). Using ImageJ software to calculate protein band density, CRISPR-edited cells had roughly 60% knockdown in ATG5 protein expression compared to the unedited cells (Figure 5).

Conclusion

CRISPR gene editing technology requires careful monitoring of the entire process to ensure accurate results. The SpectraMax i3x Multi-Mode Microplate Reader provides a complete solution for analyzing results of a CRISPR-editing experiment from initial transfection to confirmation of protein knockdown. With the MiniMax cytometer, researchers can assess transfection efficiency by comparing total unlabeled cell counts to counts of fluorescence-expressing tranfected cells. The ScanLater Western Blot Detection System enables sensitive detection and quantitative analysis of proteins of interest in control and CRISPR-edited cells.

References

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