Validating Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 Genomic Editing Experiments Using Molecular Devices Instruments

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ABSTRACT

The CRISPR/Cas9 gene editing system is a very popular tool for studying gene function due to its relative ease of use and accuracy. Additionally, the system has enormous potential for treating hereditary diseases. Validation of CRISPR/Cas9 gene editing is necessary to ensure that genes of interest are successfully knocked down or knocked out. Here, we demonstrate how Molecular Devices' family of instruments can be utilized in gene editing experiments by using CRISPR/Cas9 to knockdown autophagy-related protein 5 (ATG5) in HEK293 cells. Cells successfully transfected with the CRISPR/Cas9 plasmid expressed GFP, and a 13% transfection efficiency was calculated automatically using the SpectraMax® i3x Multi-Mode Microplate Reader with MiniMax™ 300 Imaging Cytometer. The same platform was used to quantitate DNA and protein concentrations for subsequent validation experiments. We observed about a 70% decrease in ATG5 protein expression in CRISPR-edited cells compared to non-edited cells using the ScanLater™ Western Blot Detection System. Finally, autophagosome formation was measured in CRISPR-edited vs. non-edited cells via high-content imaging using the ImageXpress® Micro Confocal High-Content Imaging System.

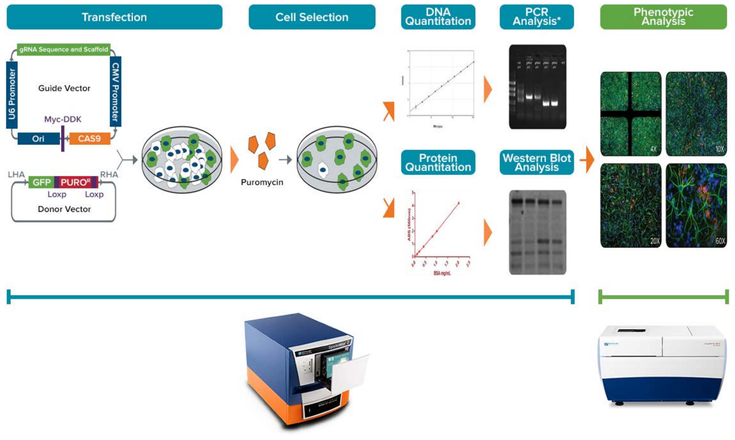

Figure. 1 Shown above is a CRISPR/Cas9 gene editing experimental workflow. The SpectraMax i3x reader was used to calculate transfection efficiency, quantitate DNA and protein concentrations, and validate gene edits through ScanLater Western Blot analysis. In parallel, PCR analysis was done to confirm proper sequence insertion occurred. Afterwards, the ImageXpress® Micro Confocal High-Content Imaging System was used to assess any changes to autophagosome accumulation in edited cells.

METHODS

Cell Transfection

HEK293 cells were seeded in tissue culture-treated 6-well plates and were transfected with (1) a guide vector containing the Cas9 enzyme sequence and a gRNA sequence targeting ATG5 (5' AAGATGTGCTTCGAGATGTG 3'), and (2) a donor sequence for puromycin resistance. In parallel, a set of HEK293 cells were transfected with a pCas-Guide-EF1a-GFP vector that transiently expresses GFP to approximate transfection efficiency. The vectors were provided from Origene's ATG5 Human Gene Knockout Kit (CRISPR). Transfected cells expressing GFP 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 calculated by dividing the number of GFP-positive cells by the total number of cells using SoftMax Pro Software.

Cell Selection

Transfected cells were treated with 1 µg/mL puromycin antibiotic to select for successfully edited cells. Cells were then passaged several times to ensure that cells did not contain episomal puromycin resistance from the donor vector.

DNA and Protein Purification and Quantitation

Edited cells and non-edited cells were lysed and genomic DNA and total protein were collected. Afterwards, the SpectraMax i3x reader was used to quantitate DNA concentration and protein concentration using ultraviolet (UV) absorbance quantitation and the BCA protein assay respectively.

CRISPR/Cas9 Gene Edit Validation

Polymerase Chain Reaction (PCR) was performed to confirm that the donor sequence was inserted into the proper genome region (not shown). A protein ladder, 5 µg and 10 µg total protein from edited and unedited cells were loaded onto a 4–20% TGX gel, and SDS-PAGE was performed. Proteins were then transferred to a PVDF membrane and separated into 3 parts: Scan-Later ladder, loading control, and the ATG5 region. The ladder was treated with 1:10000 Europium (Eu)-Streptavidin. The loading control was incubated with 1:10000 rabbit anti-Vinculin primary antibody, and the ATG5 region was incubated with 1:2500 rabbit anti-ATG5 primary antibody. Blots were washed three times after incubation. Afterwards, the loading control and ATG5 region were incubated with 1:10000 goat anti-rabbit Eu-bound secondary antibodies. Blots were washed three times before being reassembled. The blots were scanned using the SpectraMax i3x reader with the ScanLater Western Blot detection system. Afterwards, the scanned western blot data were exported into ImageJ software, and relative ATG5 protein expression was calculated from western blot band densities.

Autophagosome Quantitation

Edited and non-edited cells were plated into a 96-well cell culture-treated plate, and they were subsequently treated with a dilution series of chloroquine for 18 hours. Afterwards, autophagosomes were stained using Enzo's CYTO-ID® Autophagy detection kit, and nuclei were stained with Hoechst. Images were acquired with a 40x PA (0.95NA) objective in the FITC and DAPI channels on the ImageXpress Micro Confocal High-Content Imaging System. The number of autophagosomes per cell was quantified using MetaXpress® Software and a pre-configured module for detecting granularity. Data was exported to SoftMax Pro software for generating graphs.

TRANSFECTION EFFICIENCY

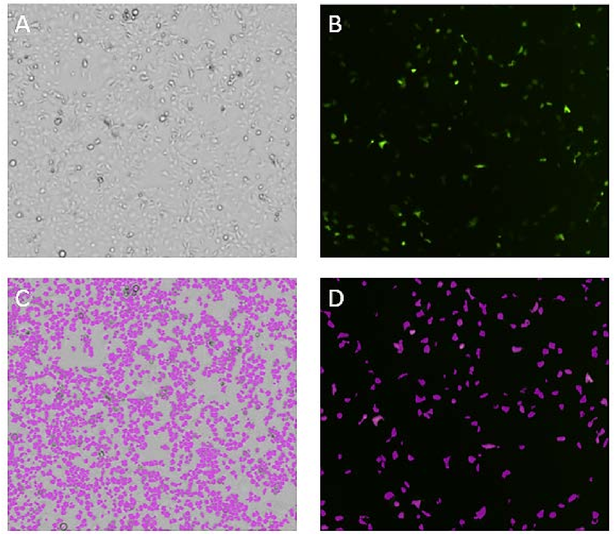

Figure 2: Transfection efficiency allows researchers to approximate CRISPR/Cas9 gene editing events, providing an early indication for successful gene editing experiments. The MiniMax cytometer was used to acquire high quality images with the Transmitted Light channel (**A**) and with the green fluorescence channel (**B**). SoftMaxPro software was able to automatically identify and count every cell (**C**), and GFP-positive cells (**D**). Afterwards, a 13% transfection efficiency was calculated from the cell counts.

ATG5 PROTEIN EXPRESSION

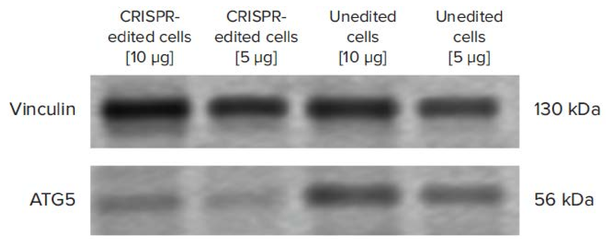

Figure 3. The ScanLater Western Blot detection system generated clear protein bands using time-resolved fluorescence scanning of Eu-bound secondary antibodies. The CRISPR-edited cells demonstrated reduced ATG5 protein expression in comparison to non-edited cells. Vinculin protein was clearly visualized as well. Both sample loading volumes are visible and are not saturated – allowing for quantitative analysis.

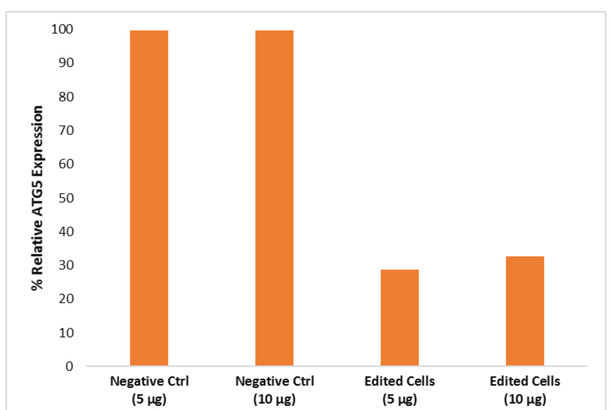

Figure 4. Edited cells showed decreased % relative ATG5 protein expression compared to non-edited cells based on ImageJ analysis. Both loading volumes displayed similar results, implying that there was no overloading/underloading of protein samples.

AUTOPHAGOSOME QUANTITATION

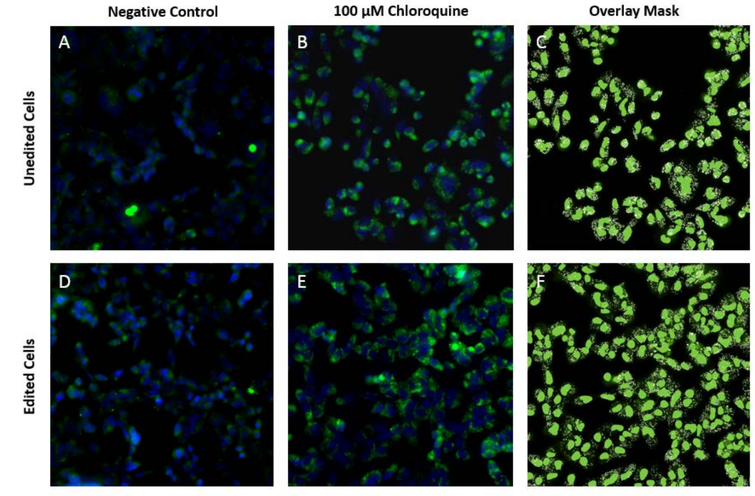

Figure 5. Autophagosome formation can be imaged and quantified through the use of the ImageXpress Micro Confocal Imaging system and MetaXpress software. Vehicle controls for the chloroquine-treated cells are shown in **A** and **D** respectively, and both demonstrated very little FITC signal**.** Increased FITC signal was seen in cells treated with 100 µM chloroquine – evidence of increased autophagosome accumulation (**B** and **E**)**.** A premade granularity analysis package was used to identify nuclei and autophagosomes. The white dots represent the autophagosomes, and the light-green blots represent the nuclei in figures **C** and **F**. Data was subsequently transferred to SoftMax Pro software for analysis.

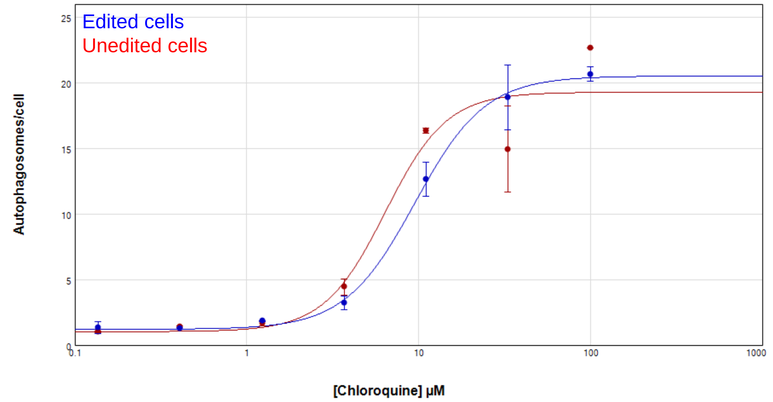

Figure 6. Autophagosome accumulation did not differ between the edited cells and unedited cells when treated with chloroquine. The calculated EC_{50} values were 9.47 and 6.37 µM for the edited and unedited cells respectively. A possible explanation is that the cells overcompensated using a different autophagic pathways[1].

CONCLUSION

- Molecular Devices' family of instruments can effectively be used to perform experiments ensuring the success of CRISPR/Cas9 gene edits.
- The SpectraMax® i3x Multi-Mode Microplate Reader can be used to assess transfection efficiency, monitor cell growth, quantitate DNA & protein, and validate CRISPR/Cas9 edits through ScanLater Western Blot analysis.
- High quality images of autophagosomes can be acquired using the ImageXpress Micro Confocal System.
- MetaXpress® Software was able to identify and quantitate individual autophagosomes from every cell allowing us to analyze phenotypic changes occurring from the CRISPR/Cas9 gene edits.
- Unexpectedly, there was no difference in autophagosome formation between the edited and non-edited cells. This could be due to compensation from a parallel autophagic pathway[1]. We would like to repeat this autophagy assay with an earlier passage of cells. Additionally, it might be necessary to knockdown other ATG-related proteins to actually affect autophagy.

1. Staskiewicz, Leah, et al. "Inhibiting autophagy by shRNA knockdown: cautions and recommendations." (2013): 1449-1450.

MOLECULAR DEVICES