Cell-based assays to provide functional confirmation of gene knockout

Introduction

The application of CRISPR-Cas9–mediated genome editing [1,2] to cell biology has revolutionized the efficiency at which targeted gene disruption can be carried out. This approach holds tremendous power—the function of a gene can now be easily studied with high-efficiency editing out of genes [3]. Successful gene deletion is often solely confirmed at the genetic level through sequencing or cleavage assays, or at the transcriptional level with qPCR assays. However, these methods are insufficient to accurately predict actual loss of the protein. The targeted region may not be fully disrupted in protein translation. In addition, the lifetime of the protein may be sufficiently long that there is residual protein retained within the cell, which necessitates the use of western blotting to measure protein levels within the edited population of cells. Furthermore, even if it is confirmed that the protein is absent, there are often redundant mechanisms as well as compensatory mechanisms that can occur. Functional assays that report the process impacted by the edited gene provide the ultimate "system-level" evaluation of genome editing.

Here we highlight this complete analysis of CRISPR-Cas9–mediated genome editing using a knockout (KO) of the gene for the glutamate-cysteine ligase modifier subunit *(GCLM)* [4]. *GCLM* is involved in the synthesis of glutathione. Subsequent reduction of glutathione to reduced glutathione (GSH) is an important mechanism to buffer against reactive oxidative species (ROS) in cytosol. Confirmation of successful editing with the genomic cleavage detection (GCD) assay and western blot (WB) was complemented with a functional assessment of the cellular levels of GSH with Invitrogen™ ThiolTracker™ Violet, a thiol-reactive probe used to detect intracellular GSH [5].

We used the targeted deletion of *GCLM* by CRISPR-Cas9 as a case study to demonstrate a complete workflow from guide RNA (gRNA) transfection through genomic and proteomic analysis to functional cellular assays (Figure 1). Taken together, the genomic, proteomic, and functional cellular assays provide the optimal conditions to confirm successful genome editing. Synthetic gRNA was designed using an online tool. The five optimized gRNA sequences were transfected via electroporation to U2OS cells stably expressing Cas9. An alternative method to delivering both gRNA and Cas9 is with transfection reagents such as Invitrogen™ Lipofectamine™ CRISPRMAX reagent [6]. Following transfection, cells were cultured and a subset analyzed for Cas9-mediated editing within the *GCLM* gene using the GCD assay.

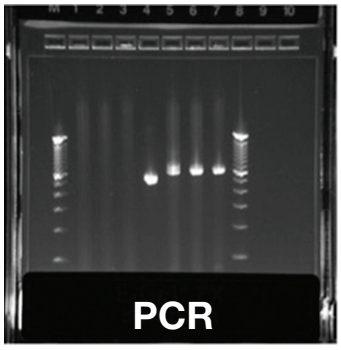
Figure 1. Workflow steps from designing gRNA to quantitating functional cellular assays.

Materials and methods

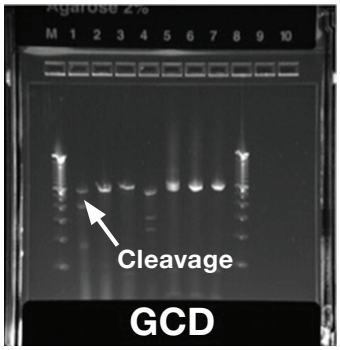
- Four gRNA sequences targeted at exons across the entire *GCLM* subunit gene were electroporated into U2OS cells stably expressing Cas9 protein, using the Invitrogen™ Neon™ Transfection System.
- As a control, scrambled gRNA sequences were electroporated into a different population of U2OS cells stably expressing Cas9 protein, using the Neon Transfection System.
- U2OS cells stably expressing Cas9 were cultured and expanded in Gibco™ McCoy's 5A Medium supplemented with 10% FBS and 5 mg/mL blasticidin.
- A subpopulation of the *GCLM* KO cells was harvested, and a GCD assay was performed using the Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit (Cat. No. A24372) to confirm that Cas9-mediated genome editing had occurred at the target sites.
- A subpopulation of the *GCLM* KO cells was harvested and used in a western blot to confirm GCLM was knocked out. Blots were imaged on the Invitrogen™ iBright™ Imaging System **(thermofisher.com/ibright)**.
- Wild type U2OS cells, scrambled gRNA, and *GCLM* KO cells were all plated in separate rows of a 96-well plate.
- The cells of all three conditions were labeled with 20 µM ThiolTracker Violet dye (Cat. No. T10096) at 37°C for 30 minutes in live-cell imaging solution (LCIS). Following labeling, the cells were washed twice in LCIS and imaged in LCIS.
- The cells of all three conditions were imaged on the Invitrogen™ EVOS™ FL Auto 2 Imaging System with identical exposure conditions.
- Analysis of all three conditions was performed with Invitrogen™ Celleste™ Image Analysis Software to quantify the intensity of ThiolTracker labeling, which measures the abundance of reduced glutathione within cells.

Results

Following PCR-based amplification (Figure 2), the GCD assay was performed to confirm editing within *GCLM* target 1. Following clonal selection and propagation of a subset of each clonal population—cells that were electroporated with scrambled gRNA, heterozygous *GCLM* KO cells, and parental cells—cells were lysed and the lysates were probed with anti-GCLM antibodies for western blot analysis of protein levels. Clone 5 showed complete ablation of *GCLM* gene expression compared to the two other clones (1 and 3) as well as heterozygous KO cells, parental cells, and those transfected with scrambled gRNA (Figure 3). Western blot analysis is a valuable complement to genomic detection (e.g., GCD or sequencing). It is important to note that protein lifetimes vary greatly among different gene products, and unless a protein is degraded quickly as part of its normal lifetime, disruption of the gene will not manifest itself immediately by its functional consequence, because competent protein will still remain within the cell. In our experiment, disruption of the *GCLM* gene using CRISPR-Cas9 resulted not only in cleavage in the GCD assay but also loss of the protein in at least clone 5. These data show successful CRISPR-Cas9–mediated genome editing at the genomic and proteomic levels.

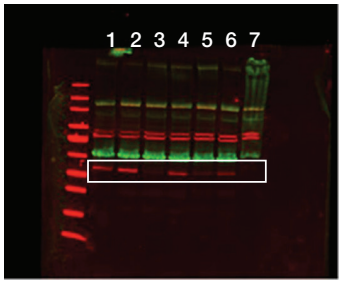


Lane M = ladder
Lane 1 = *GCLM* target 1
Lane 2 = wild type target 1
Lane 3 = scramble target 1
Lane 4 = control
Lane 5 = *GCLM* target 2
Lane 6 = wild type target 2
Lane 7 = scramble target 2
Lane 8 = ladder
Lane 9 = blank
Lane 10 = blank



Lane M = ladder
Lane 1 = *GCLM* target 2
Lane 2 = wild type target 2
Lane 3 = scramble target 2
Lane 4 = control
Lane 5 = *GCLM* target 2 negative control
Lane 6 = wild type target 2 negative control
Lane 7 = scramble target 2 negative control
Lane 8 = ladder
Lane 9 = blank
Lane 10 = blank

Figure 2. PCR followed by GCD assay to confirm *GCLM* edit. Successful cleavage within *GCLM* is seen in lane 1 of the GCD assay.



Lanes:
1. Wild type
2. Scramble
3. *GCLM* hetero
4. Control
5. *GCLM* clone 1
6. *GCLM* clone 3
7. *GCLM* clone 5

Figure 3. Western blot analysis of GCLM protein levels. Reduced GCLM is seen in the GCLM heterozygote (lane 3) as well as clones 1 and 5 (lanes 5 and 7). Each lane was loaded with 10 µg of total protein from the respective clone.

To ensure that the gene KO manifested itself through a functional consequence, cytoplasmic GSH levels were measured using a cell-based functional reporter, ThiolTracker Violet [5]. The fluorescence intensity of ThiolTracker Violet was compared between parental cells, *GCLM* KO clone 5, and those transfected with scrambled gRNA, using Celleste Imaging Analysis Software following imaging on the EVOS FL Auto 2 Imaging System (Figure 4). We show that CRISPR-Cas9–mediated gene editing of *GCLM* results in a significant reduction in ThiolTracker Violet fluorescence (Figure 4). We can therefore conclude that transfection of U2OS cells stably expressing Cas9 with 5 gRNAs against the *GCLM* gene results in decreased cytoplasmic levels of glutathione, which can be assayed using the fluorescent ThiolTracker Violet biosensor.

In the CRISPR-mediated knockout example highlighted above, we chose *GCLM*, a gene involved in the synthesis of glutathione, and demonstrated a complementary genomic and proteomic analysis with a cell-based assay for reduced glutathione. This paradigm can be easily adapted for other genes and other cellular processes. Table 1 highlights cell-based assays and their corresponding gene families that would provide an analysis across the multiple levels (genomic, proteomic, cellular) as described herein.

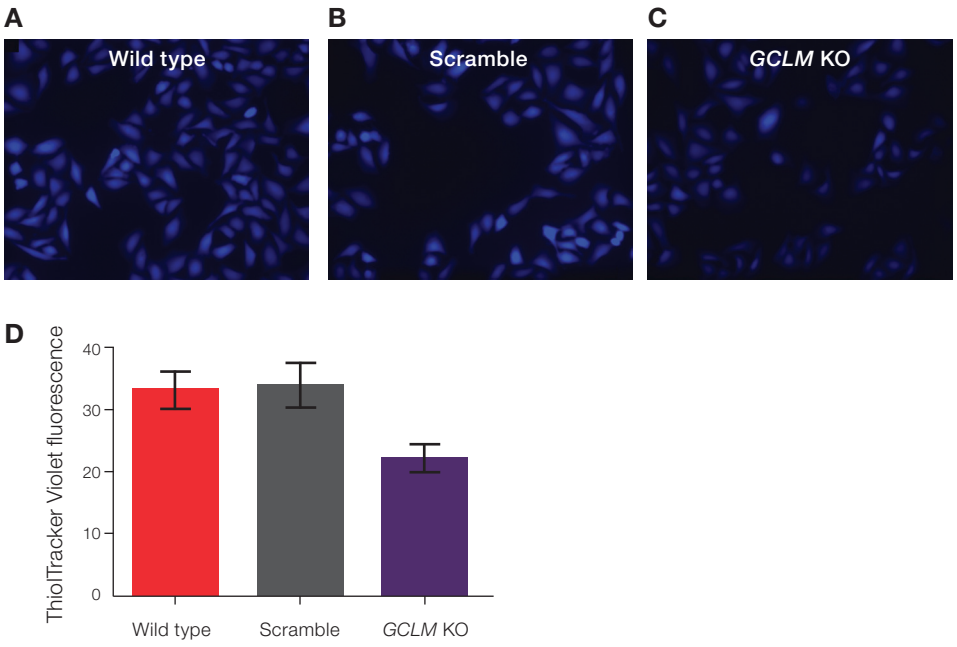


Figure 4. Measurement of cytoplasmic GSH using ThiolTracker Violet. (A–C) Wild type, cells transfected with scrambled gRNA, and *GCLM* KO cells were imaged using the EVOS FL Auto 2 Imaging System. **(D)** Quantitation of the ThiolTracker Violet fluorescence of the cells in **A–C**, using Celleste Image Analysis Software, showed lower staining intensity of *GCLM* KO cells than of wild type or cells transfected with scrambled gRNA, due to the reduced amount of glutathione in the KO cells.

Table 1. Suggested additional functional probes.

Process targeted	Reagents and kits	Reference compounds as controls
Apoptosis	CellEvent Caspase-3/7 Green reagent	Staurosporine
Viability	LIVE/DEAD imaging kit	Camptothecin
Oxidative stress	CellROX assay kit	Menadione
Cell proliferation	Click-iT EdU imaging kit	Taxol
Autophagy	Premo autophagy sensor kit	PP242 and/or chloroquine
Protein synthesis	Click-iT OPP assay kit	Anisomycin
Proteasomal function	Click-iT HPG assay kit	MG132
Calcium mobilization	Calcium imaging kit	Ionomycin
DNA damage	Click-iT TUNEL assay kit	Etoposide
Mitochondrial function	TMRM dye	CCCP
Lysosomal function	DQ BSA	Bafilomycin A1
Organelle structure	CellLight reagents	NA

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Conclusions

Through this case study on targeted deletion of *GCLM* by CRISPR-Cas9, we have established a complete workflow solution that demonstrates CRISPR-Cas9–mediated editing at both the genomic and proteomic levels. Furthermore, we successfully achieved "system-level" quantitation of gene knockout through a functional cell-based assay that measured the fluorescence intensity of ThiolTracker Violet as a function of cytoplasmic GSH level. This assay holds significant promise, as it can potentially be adapted to knock out any gene of interest and accurately predict loss of function of the related protein in downstream cellular processes.

References

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