invitrogen

ADVANCED DELIVERY TECHNOLOGIES FOR IMPROVED CRISPR-based GENOMIC EDITING IN STEM CELLS FOR DISEASE MODEL GENERATION

Nektaria Andronikou, Xin Yu, Xiquan Liang, Sean Essex, Mahalakshmi Sridharan, Uma Lakshmipathy, Jason Potter, Xavier de Mollerat du Jeu - Thermo Fisher Scientific – 5781 Van Allen Way, Carlsbad, CA 92008

ABSTRACT

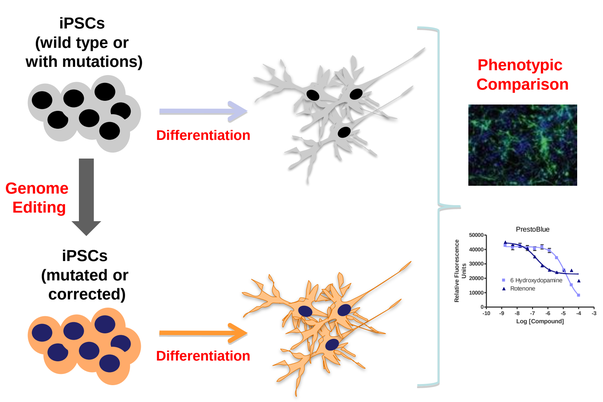
Stem cells, specifically induced pluripotent stem cells (iPSCs), hold promise for the future of regenerative medicine and therapeutic treatments. More recently, manipulation of stem cells and progenitor lines has been achieved utilizing TALENs and CRISPRs for genome engineering purposes and disease model generation. However, the lack of advanced technologies has been hindering the current pace of research and discovery. Improved delivery can help accelerate research for emerging new therapies and help the understanding of disease pathways and mechanisms.

We've demonstrated that Lipofectamine® 3000, an improved DNA delivery reagent, can achieve optimal transfection efficiency of various sizes of plasmid DNA with low toxicity in both embryonic stem cells (ESCs) and iPSCs, which have been traditionally hard to transfect. Alternative to DNA, transfection of mRNA requires that the cargo enters only the cell cytoplasm, not the nucleus, and therefore mitigates the risk of integration and more importantly greatly improves the transfection efficiency. In a recent study, we utilized an mRNA specific delivery reagent, Lipofectamine MessengerMAX™, and showed successful genetic cleavage with Cas9-mRNA and gRNA complexes, in embryonic stem cells and iPSCs. The Cas9 RNA approach had improved nuclease-mediated %indel rates compared to a plasmid Cas9 approach.

Most recently, we have shown that improved indel rates directly improve multiplexed targeting and reduce off-target effects. A newly developed workflow, consisting of a Cas9-ribonucleoprotein and specifically developed transfection reagent, Lipofectamine CRISPRMAX™, can yield greater than 80% indel rates in Jurkat T-cells and iPSCs. The Cas9 RNP complex can act immediately upon cell entry, since transcription and translation are not required. Moreover, the complex is rapidly cleared from the cell, minimizing the chance for off-target cleavage events. Taken together these advancements in delivery greatly improve downstream workflows, enable easier stem cell manipulation, and enhance the generation of knock-in or knock-out cell models and transgenic small animal models.

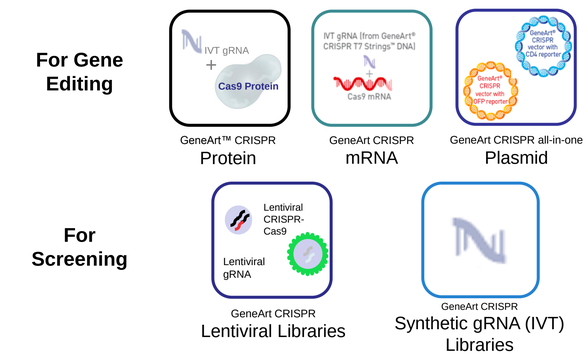
INTRODUCTION

Patient-derived iPSCs offer exciting potential in both cell therapy and *in vitro* disease modeling by enabling access to cell populations that are otherwise unavailable from living donors. With the recent discovery of site-specific gene editing, this true power is fast approaching. Having the ability to develop two cell models with isogenic background, except for the site specific edit, gives researchers the potential to study the true effects of a single mutation in a pathway or syndrome and in turn develop advanced therapies and treatments.



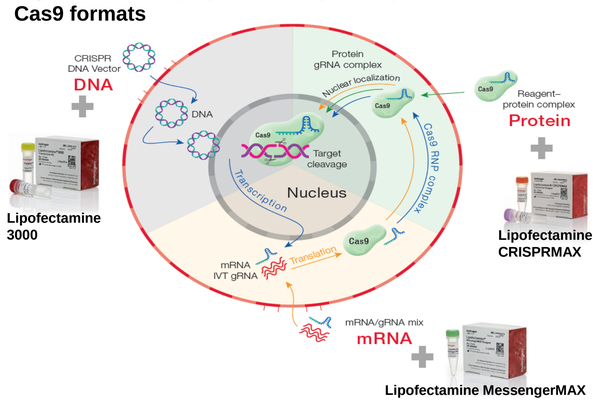
MATERIALS AND METHODS

Figure 1. Available CRISPR-Cas9 Formats



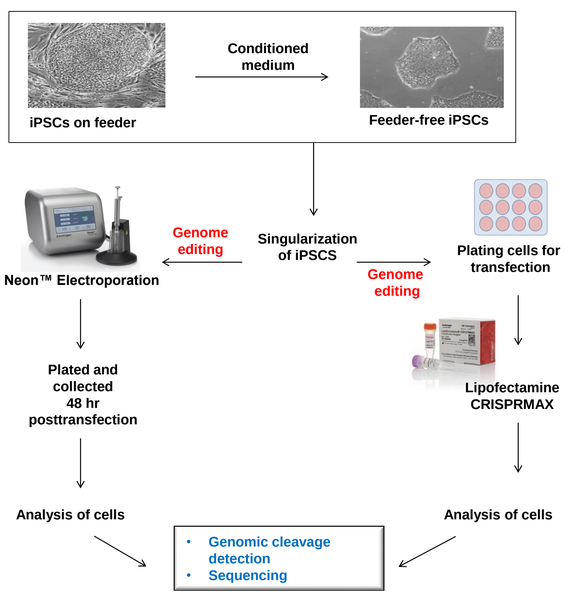
There are four different formats of Cas9 available for various applications. The all-in-one CRISPR/Cas9 plasmid, which also contain constructs for enrichment such as an OFP or CD4 reporter; the Cas9 mRNA, purified Cas9 protein and two different formats of CRISPR libraries for screening: purified gRNA libraries and CRISPR Lentiviral libraries.

Figure 2. Delivery options and pathways for various CRISPR-Cas9 formats



Efficient delivery of each of the CRISPR-Cas9 systems is required for efficient genetic cleavage. The Cas9 mRNA or Cas9 protein format, streamline the gene engineering process by eliminating the required transcription or translation steps. Additionally, the Cas9:gRNA complex of the protein system, formed in a controlled in vitro environment, protects the gRNA and prevents degradation.

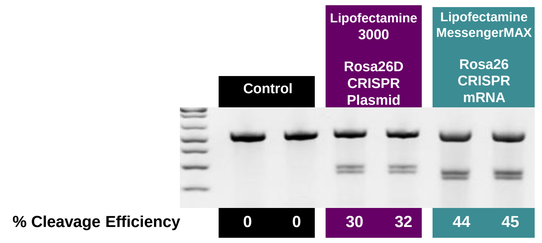
Figure 3. Stem Cell Gene editing workflow



Schematic diagram outlining the steps for a gene editing workflow in stem cells. ESCs/iPSCs that have been cultured with a feeder system, should first be transitioned to a feeder free culture system in conditioned medium. Cells are harvested and single cell suspensions are either electroporated or plated for transfection reagent mediated genome editing. We analyze cells 72 hours post transfection by genomic cleavage detection assay or by genetic sequencing.

RESULTS

Figure 4. Plasmid vs. mRNA CRISPR editing in mouse ESC



Cleavage efficiency obtained with CRISPR DNA and mRNA formats when delivered using transfection reagents. DNA CRISPR was transfected using Lipofectamine 3000 and mRNA Cas9 with IVT gRNA was transfected using Lipofectamine MessengerMAX. As shown, CRISPR DNA format resulted ~30% cleavage whereas mRNA format of CRISPR resulted in higher cleavage, ~45%.

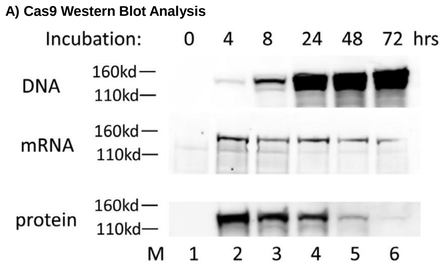
Table 1. Plasmid, mRNA vs RNP CRISPR editing in various cells

Cell Types	Plasmid (GCD%)	mRNA (GCD%)	Protein (GCD%)
	Lipofectamine 3000	*Lipofectamine MessengerMAX*	*Lipofectamine CRISPRMAX*
Mouse ESCs	30	45	75
Human ESCs (H9)	0	20	n/a
Human iPSCs	5	66	55
N2A	66	66	70
Jurkat	0	0	19
K562	0	0	20
A549	15	23	48
HEK293FT	49	70	75
U2OS	15	21	55

Cleavage efficiency obtained with CRISPR DNA, mRNA and protein formats when delivered using respective transfection reagents. As shown, CRISPR protein format resulted in most efficient cleavage in the widest range of cell types. In certain cell types, such as Jurkat and k562, the only method that was able to achieve genetic cleavage was the Cas9 protein at 19% and 20% respectively.

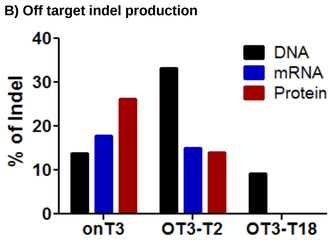
Figure 5. Cas9 Protein reduces off-target effects

A) Cas9 Western Blot Analysis



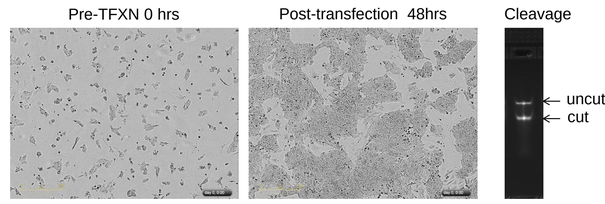
A) HEK293FT cells were transfected with DNA, mRNA and RNP using transfection reagents. Sample collection for western blot analysis performed at indicated time points (4-72hrs). Plasmid format of Cas9 has increasingly elevated protein levels from 4hrs to 72hrs; whereas mRNA and protein format is cleared from the cells faster, and reduces the potential of off-target effects from continuous Cas9 expression.

B) Off target indel production



B) Off-target mutation of VEGFA T3 target caused by Cas9 plasmid DNA, mRNA or protein transfection. Percentages of on-target mutation as well as OT3-2 and OT3-18 off-target mutations were determined by sequencing. onT3 = on target. OT3 = off target site for T3 gRNA.

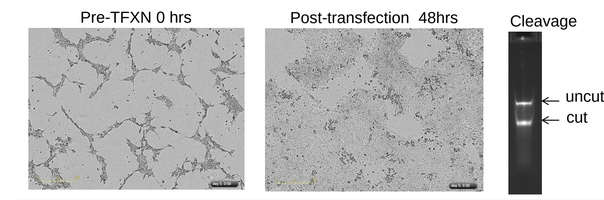
Figure 6. Protocol details for mESC genome editing with CRISPRMAX and Cas9 protein



Cell Type	Cell Density		Locus	% Cleavage
	Cell Numbers per Well	% Confluence at time of transfection		
mESC	1.0 x 10^5	27%	Rosa26	75±3%

Mouse ES cells were plated in a 24-well format at 1x10^5 cells per well. Confluency image is shown to help determine proper cell density that is required for successful transfection and cleavage. Cas9:gRNA complexes were formed with 500ng of GeneArt Platinum™ Cas9 Nuclease and 125ng of gRNA targeting the Rosa26 locus in Opti-MEM™ I Serum free media with 2ul of Lipofectamine CRISPRMAX and 1ul of Cas9 PLUS™ Reagent. Cleavage was determined with the GeneArt Genomic Cleavage detection kit 72 hours following transfection.

Figure 7. Protocol details for human iPSC genome editing with CRISPRMAX and Cas9 protein



Cell Type	Cell Density		Locus	% Cleavage
	Cell Numbers per Well	% Confluence at time of transfection		
iPSC	0.4 x 10^5	30%	HPRT 1	55±5%

Gibco® human iPS cells were plated in a 24-well format at 0.4x10^5 cells per well. Confluency image is shown to help determine proper cell density that is required for successful transfection and cleavage. Cas9:gRNA complexes were formed with 1000ng of GeneArt Platinum Cas9 Nuclease and 250ng of gRNA targeting the HPRT-T1 locus in Opti-MEM I Serum free media with 1.5ul of Lipofecatamine CRISPRMAX and 6ul of Cas9 PLUS Reagent. Cleavage was determined with the GeneArt Genomic Cleavage detection kit 72 hours following transfection.

CONCLUSIONS

Efficient gene editing in stem cells for disease modeling and drug development is critical for improved downstream workflows and high throughput screening. The Cas9 protein nuclease, in combination with the newly developed Lipofectamine CRISPRMAX transfection reagent has shown to be the most efficient system for high-throughput genetic cleavage in many cell models with reduced off target effects, low cell toxicity and multiplexing capabilities.

REFERENCES

1. X Liang, Potter J, Kumar S et al. (2015) Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. J Biotechnol. 208:44–53. doi:10.1016/j.jbiotec.2015.04.024.
2. X Yu, Liang X, Xie H et al. (2016) Improved delivery of Cas9 protein/gRNA complexes using Lipofectamine CRISPRMAX. Biotechnology Lett. DOI 10.1007/s10529-016-2064-9.

ACKNOWLEDGEMENTS

The transfection team at Life Technologies™, Thermo Fisher Scientific would like to thank the synthetic biology team for their support in providing the required CRISPR editing tools and expertise in design and genome engineering techniques. We would also like to extend a special thanks to the stem cell team for their support in providing all the necessary cell models for many of the above referenced experiments.

ThermoFisher SCIENTIFIC