HIGH THROUGHPUT AMENABLE GENE EDITING TOOLS FOR FUNCTIONAL GENOMICS AND ENGINEERED CELL LINE DEVELOPMENT

Maryam Mafreshi, Yanfei Zou, Julia Braun, Natasha Jacobsen, Jonathan D. Chesnut, Namritha Ravinder.
Synthetic Biology, Biosciences Division, Thermo Fisher Scientific, Carlsbad, California, USA

ABSTRACT AND INTRODUCTION

Latest advancements in genome editing technologies has revolutionized gene therapy and opened up new opportunities for treating diseases related to perturbations in protein expression and mutations in the genome. With editing tools like CRISPR-Cas9 researchers are able to efficiently knockout genes of interest and study its functions or knock in a specific change into the genome and correct a disease relevant mutation. However there is a significant need for easy-to-use and efficient high throughput (HTP) gene editing workflows which will enhance the capability of these tools in challenging and disease cell types including primary T-Cells and stem cells. The work described here includes

1. HTP amenable tools and optimized workflows including multiplexed gene editing using pre-complexed CRISPR-Cas9 ribonucleoprotein (RNP) and
2. Application data using genome-wide functional knockout screening tools such as CRISPR and siRNA libraries.

To date, we have observed up to 80% editing efficiency with multiplexed RNP complex targeted to simultaneously edit six different genes in T Cells. Data also shows that the pre-complexed CRISPR- Cas9 RNP complex described here is stable for at least six months without compromising the editing efficiency when tested in T-Cells giving the user the flexibility to reuse the mix for subsequent experiments. The HTP tools and protocols developed through this work will expand the toolbox capability for disease modeling and drug discovery by enhancing overall productivity thereby accelerating biotherapeutic research.

MATERIALS AND METHODS

All experiments described here were performed using

1. TrueCut™ Cas9 v2 protein, TrueGuide™ synthetic guide RNA and Invitrogen™ LentiArray™ CRISPR gRNA Lentivirus for gene editing applications,
2. Neon ™ Transfection System
3. GeneArt™ Genomic Cleavage Detection Kit (GCD), Sanger Sequencing or Ion™ GeneStudio S5 systems for editing efficiency analysis (NGS)
4. Real-time TaqMan® PCR reagents for gene expression knockdown and
5. Attune™ NXT Flow Cytometry for protein knock down efficiency using protocols described in the product manual or white papers found on thermofisher.com.

Figure 1: Multiplexed gene editing workflow using CRISPR-Cas9 RNP

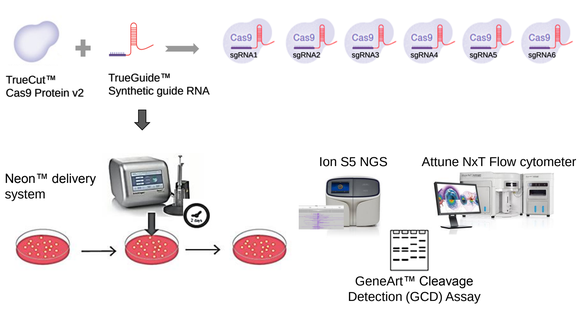

Figure 2: CRISPR-Cas9 RNP dosage optimization

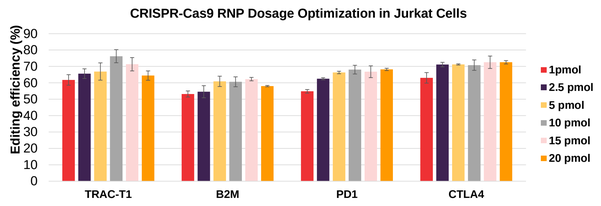

Figure 2A. RNP Dosage optimization in Jurkat Cells in 24 well format using 10µl tip Neon™ delivery format. High knock out efficiencies can be achieved with up to 20 pmoles Cas9/sgRNA complex without compromising cell viability (data not shown). The molar ratio of sgRNA and Cas9 Protein is 1:1. Data suggests there is broad dosage range to accommodate multiplexed editing with 6 or more targets.

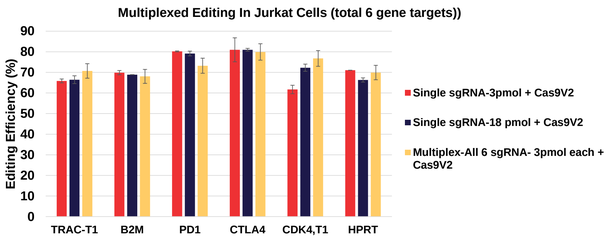

Figure 2B. Editing efficiency for multiplexed gene editing with CRISPR-Cas9 RNP was measured by GCD. Results showed that multiplexed RNP gave the same high efficiency as single plexed RNP for each gene target tested.

Figure 3: Ready-to-use CRISPR-Cas9 RNP mix is stable up to six months

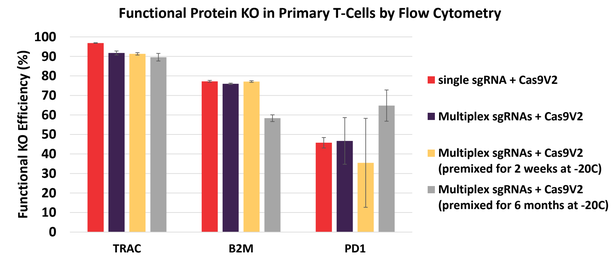

Figure 3A. Functional Knockout (KO) Efficiency with multiplexed CRISPR RNP KO mix was measured by antibody staining followed by Flow Cytometry. RNP premix stored at -20ºC showed the same high protein knockout efficiency as fresh RNP mix in primary T cells. RNP KO mix was made of Cas9 and gRNA at molar ratio 1:1. The Mix is stable up to 6 months at -20C.

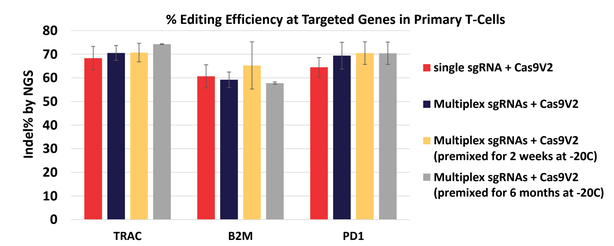

Figure 3B: Editing efficiency with multiplexed RNP KO mix simultaneously targeting T-Cell receptor (TRAC), PD1 and B2M genes. Genomic cleavage efficiency measured by next-generation sequencing (NGS). Results show pre-complexed RNP mix stored at -20ºC up to 6 months showed the same high editing efficiency as freshly complexed RNP mix in primary T cells.

Figure 4: Feasibility test of ready-to-use CRISPR-Cas9 RNP mix with HDR donor DNA

Figure 4A. gRNA targeting T-Cell Receptor alpha chain (TRAC) has been designed for specific cleavage site (T5) within TRAC gene. Donor DNA was designed to add 4 nucleotides at the cleavage site to generate frame shift in TRAC gene and thereby knockout TRAC protein.

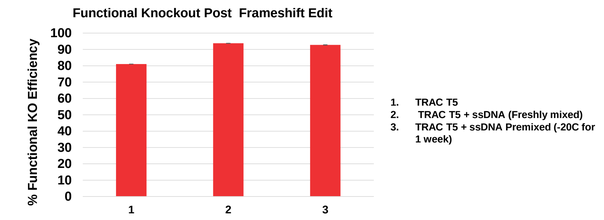

Figure 4B: Functional Knockout Efficiency post HDR mediated frame shift edit was measured by antibody staining followed by Flow Cytometry. RNP mix with donor DNA showed high TRAC knockout efficiency in primary T-cell. The mix stored at -20ºC for one week showed similar knock-in edit efficiency as the freshly complexed RNP.

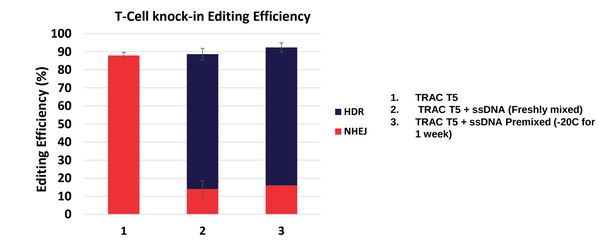

Figure 4C: HDR and NHEJ efficiency was measured by sequencing. RNP mix with donor DNA showed high HDR efficiency in primary T-cell. Pre-complexed mix stored at -20ºC for one week showed similar efficiency as the freshly complexed RNP.

Figure 5: Gene Knockout with LentiArray CRISPR Libraries in iPSC

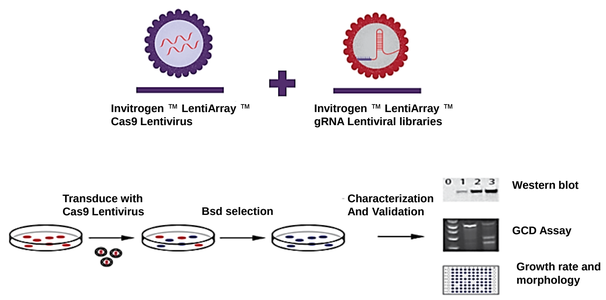

Figure 5A. Gene editing workflow using LentiArray CRISPR Libraries.

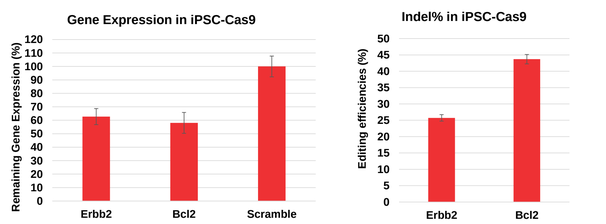

Figure 5B. The expression of genes targeted by LentiArray CRISPR sgRNAs were measured by TaqMan Real-time PCR assays. The edited samples showed decreased gene expression in iPSC-Cas9 cell compared to negative control (scrambled) gRNA. (MOI 2, N=3)

Figure 5C. Editing efficiencies at targeted genes were measured by GCD assay. Results confirmed editing at targeted loci.

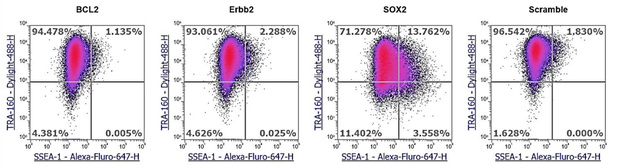

Figure 5D. The pluripotency of iPSC-Cas9 cell post gene editing was measured by SSEA1 and TRA-160 staining followed by Flow Cytometry. LentiArray CRISPR sgRNA targeted to ERBB2 or BCL2 didn't change the pluripotency of iPSC.
LentiArray gRNA targeting transcription factor SOX2 that is essential for pluripotency was used as a positive control for loss of pluripotency and scrambled gRNA was used as a negative control. As expected SOX2 knockout samples showed decreased pluripotency compared to negative control gRNA targeted sample.

Figure 6: CRISPR RNP could be used to generate disease-related cell models

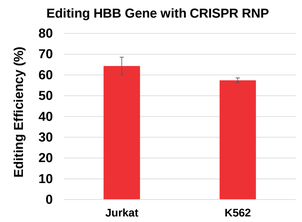

Figure 6: The HBB gene encodes a protein called beta-globin. Beta-globin is a component (subunit) of a larger protein called hemoglobin, which is located inside red blood cells. Beta thalassemia is a disease associate with dysfunctional beta-chain gene. A person with beta thalassemia has abnormal red blood cell shape (see the picture on the right) since beta chain is reduced or absent in the red cells. With CRISPR RNP, I can efficiently edit HBB gene in Jurkat and K562 cells. This enables the capability of generating disease related cell models using CRISPR RNP.

CONCLUSIONS

1. Pre-complexed Cas9+gRNA ribonucleoprotein (Cas9 RNP) are stable and functional over a significant period of time offering workflow flexibility for a number of approaches including general editing of single genes to multi-genes in parallel or whole pathway interrogations. We have demonstrated here that premixed Cas9 RNP could be stored at -20ºC up to 6 months without compromising the cleavage efficiency.
2. At Thermo Fisher Scientific, we offer LentiArray CRISPR libraries to enable genome wide screening across broad cell types. We are currently optimizing workflows with LentiArray CRISPR tools in primary T cells.

REFERENCES

1) Invitrogen™ TrueCut™ Cas9 Protein v2. www.Thermofisher.com
2) Invitrogen™ TrueGuide™ Synthetic gRNA. www.Thermofisher.com
3) Invitrogen™ LentiArray™ CRISPR gRNA Lentivirus. www.Thermofisher.com
4) Achieve functional knockout in up to 90% of human primary T cells. www.Thermofisher.com

ACKNOWLEDGEMENTS

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TRADEMARKS/LICENSING