Improved genome editing efficiency using carrier DNA in electroporation of CRISPR/Cas9 RNPs

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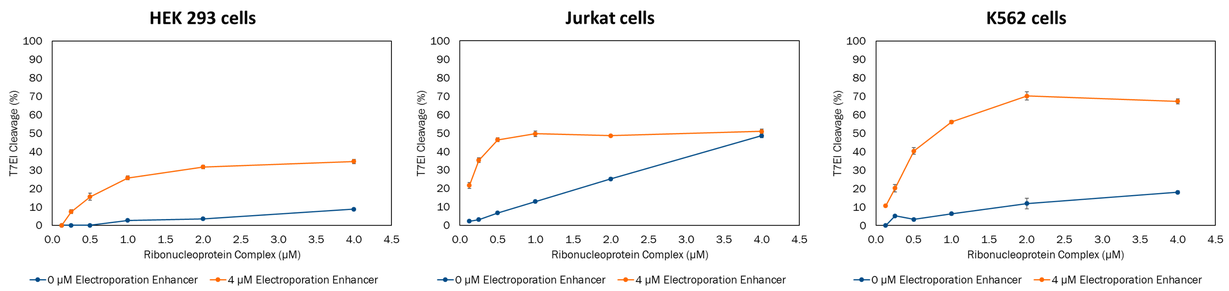
Introduction

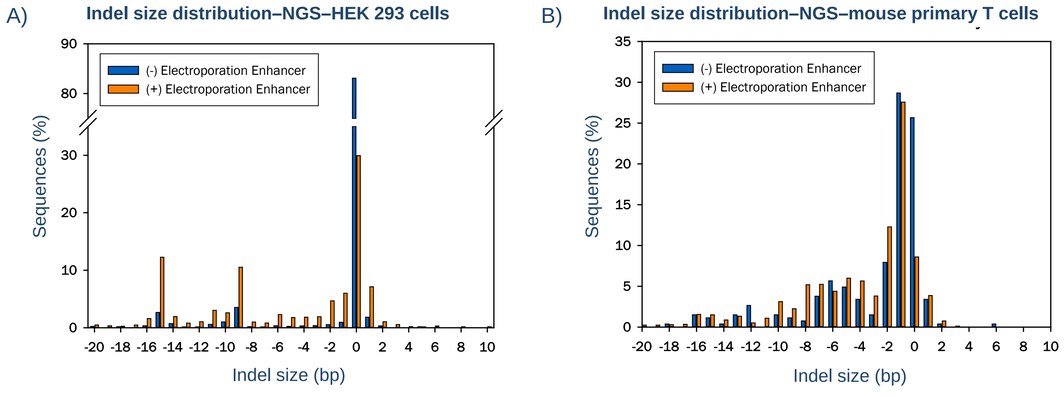
High levels of genome editing using the CRISPR/Cas9 system can be achieved by optimization of key variables such as protospacer sequence, chemical modification of the guide RNA complex, and the delivery mode. Transfection of the CRISPR/Cas9 machinery as a ribonucleoprotein (RNP) complex has a number of benefits, such as high on-target potency combined with reduced off-target effects due to exposure control, and no observed toxicity or innate immune responses. Here, we describe optimization of CRISPR/Cas9 ribonucleoprotein complex delivery by improving electroporation conditions. Optimal electroporation parameters result in high levels of editing efficiency as well as high levels of cell viability. The addition of a non-homologous, single-stranded oligonucleotide-based electroporation enhancer to the electroporation solution increases the frequency of editing events. Importantly, next-generation sequencing data demonstrates that the use of this Cas9 Electroporation Enhancer does not alter the editing 'fingerprint' specific for the on-target site and we find no evidence for incorporation of the Cas9 Electroporation Enhancer during the repair process. Transfection of primary mouse T-cells using these optimized conditions can routinely result in 80% or higher knockout of the T-cell receptor alpha locus leading to loss of the T-cell receptor complex on the cell surface.

Improved genome editing with Cas9 Electroporation Enhancer



Editing efficiencies increase significantly in the presence of Cas9 Electroporation Enhancer, and vary between cell lines. Alt-R™ RNP complexes were generated by combining the Alt-R™ Cas9 Nuclease 3NLS with a 2-part guide RNA complex, which consists of Alt-R™ crRNA and Alt-R™ tracrRNA. The Alt-R™ RNP was delivered at different concentrations using Amaxa® Nucleofector® System (Lonza) in 3 different cell lines. Addition of Cas9 Electroporation Enhancer increases genome editing efficiencies and allows the use of lower Alt-R™ RNP concentrations. Additionally, these results show cell-type dependent editing efficiencies.

No integration of Cas9 Electroporation Enhancer at target site



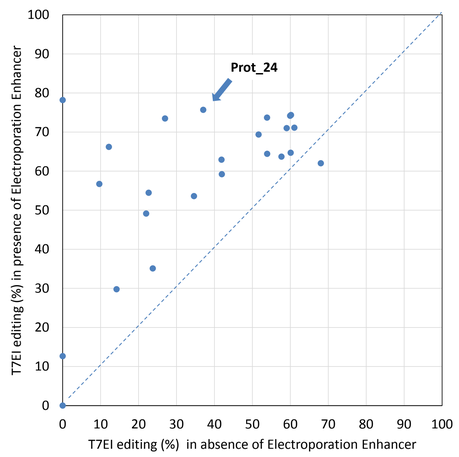
Similar profiles for indel size distributions in presence or absence of Cas9 Electroporation Enhancer. (A) HEK293 cells were electroporated with 4 µM Alt-R™ RNP with (+) or without (–) 4 µM Cas9 Electroporation Enhancer using Amaxa® Nucleofector® System. Editing events, including potential integration of the Cas9 Electroporation Enhancer at the target site, were determined by NGS and analyzed using CRISPResso. Overall editing levels were higher in the presence of Cas9 Electroporation Enhancer (71%) compared to absence (17%). Notably, the 2 indel profiles did not differ significantly, indicating similar editing patterns in the presence and absence of Cas9 Electroporation Enhancer. (B) Mouse primary T cells were electroporated using the Neon® Transfection System with 1.5 µM Alt-R™ RNP with (+) or without (–) 1.8 µM Cas9 Electroporation Enhancer. Again, NGS data, analyzed by CRISPResso, showed similar indel profiles. However, enhancer addition benefits were not as pronounced; raising from 74.3 to 91.8%.

Optimization of Neon® electroporation in Jurkat cells

	Editing efficiency (%)		*SD (%)*		*Cell density*			*Pulse*	
Test	**- enhancer**	**+ enhancer**	**- enhancer**	**+ enhancer**	**- enhancer**	**+ enhancer**	**Voltage (V)**	**Width (ms)**	**Number**
Prot_01	0.0	0.0	0.0	0.0	3	4	0	1	1
Prot_02	53.9	64.4	2.7	1.8	2	3	1400	20	1
Prot_03	59.0	71.0	0.5	4.5	2	3	1500	20	1
Prot_04	0.0	78.2	0.0	1.3	3	1	1600	20	1
Prot_05	60.1	64.7	3.1	13.2	2	2	1700	20	1
Prot_06	22.0	49.1	0.6	16.3	1	4	1100	30	1
Prot_07	41.9	62.9	3.2	6.8	3	4	1200	30	1
Prot_08	57.7	63.7	0.9	15.2	2	4	1300	30	1
Prot_09	60.2	74.4	2.4	3.1	3	2	1400	30	1
Prot_10	14.2	29.8	1.4	0.4	3	2	1000	40	1
Prot_11	34.6	53.6	0.9	0.1	4	1	1100	40	1
Prot_12	27.0	73.5	1.7	3.8	2	2	1200	40	1
Prot_13	22.7	54.5	1.3	4.6	2	2	1100	20	2
Prot_14	51.6	69.4	1.9	0.8	2	2	1200	20	2
Prot_15	59.9	74.2	3.0	0.7	2	2	1300	20	2
Prot_16	68.0	62.0	2.6	11.2	3	4	1400	20	2
Prot_17	0.0	12.7	0.0	0.3	4	4	850	30	2
Prot_18	23.7	35.1	0.9	2.0	3	2	950	30	2
Prot_19	9.7	56.7	0.6	3.1	3	3	1050	30	2
Prot_20	53.9	73.7	3.6	7.2	3	2	1150	30	2
Prot_21	41.9	59.2	1.4	1.4	3	4	1300	10	3
Prot_22	12.2	66.2	1.1	0.2	3	4	1400	10	3
Prot_23	61.1	71.1	1.7	2.2	3	2	1500	10	3
Prot_24	37.0	75.7	2.8	0.4	2	4	1600	10	3

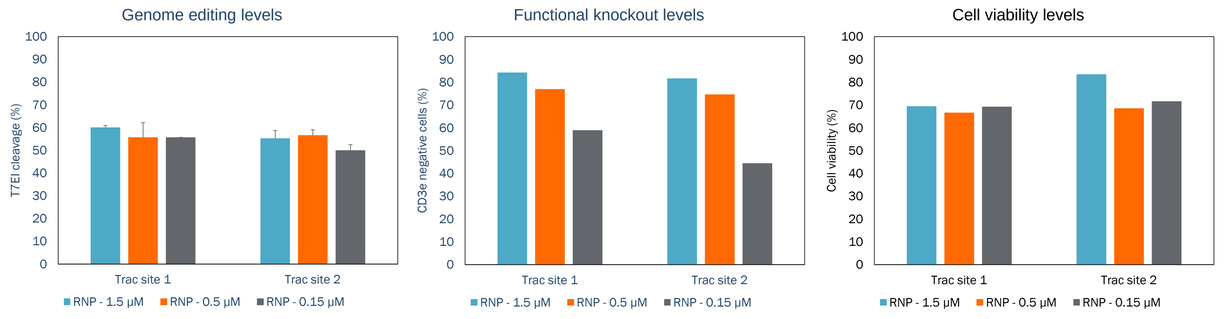
low editing — high editing

1, 2, 3, 4 — low cell density — high cell density



Optimization of electroporation parameters leads to high levels of genome editing and cell viability. Jurkat cells were electroporated with Alt-R™ RNP targeting the *HPRT* gene, in the presence and absence of Cas9 Electroporation Enhancer. Testing of different electroporation parameters (voltage, pulse width, number of pulses) determined the optimal conditions to obtain high levels of genome editing as well as high cell density. The optimal settings for electroporation of Jurkat cells using the Neon® Transfection System were found using protocol #24.

Highly efficient genome editing in mouse primary T cells



Functional knockout of the *Trac* gene in primary mouse T cells. Primary mouse T cells were electroporated with the Alt-R™ RNP + Cas9 Electroporation Enhancer at different concentrations, targeting 2 sites in the *Trac* gene using the Neon® Transfection System. Gene editing levels determined by a T7EI assay showed highly efficient gene editing efficiencies (~55%) even at lower Alt-R™ RNP concentrations (0.15 µM). Functional knockout levels of the T cell receptor complex were measured by determining levels of CD3e protein expression using flow cytometric analysis. Functional knockout was found to be dose-dependent. At 1.5 µM Alt-R™ RNP concentrations, T cell receptor expression was absent in ~85% of total cells. Overall, viability of cells after electroporation was 70–80%.

Conclusions

- Cas9 Electroporation Enhancer is a single-stranded Ultramer® Oligonucleotide
- Addition of Cas9 Electroporation Enhancer to the ribonucleoprotein (RNP) complex leads to increased levels of genome editing with electroporation-based methods
- Cas9 Electroporation Enhancer does not integrate into genomic DNA at the target site
- Optimized electroporation settings combined with the addition of Cas9 Electroporation Enhancer can lead to editing efficiencies >85% in primary T cells