BioResearch

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Genome Editing using Nucleofector™ Technology

Technical Reference Guide

This guideline provides a brief background on various genome editing tools and describes how to establish Lonza's Nucleofector™ Technology for genome editing applications in hard-to-transfect cell types, such as pluripotent stem cells.

1. Introduction to Genome Editing

The wealth of genomic sequence data now available to researchers has laid the foundation for a revolution in genetic modification technology. This technology, termed genome editing, provides the means by which heritable DNA alterations can be made at pre-determined specific sites in the genome.

In general, there are various options to modulate gene expression, be it on the DNA, RNA, or protein level. Many of these options only result in a transient modulation that might be sufficient or even advantageous for some approaches. However, prior to genome editing, a stable, heritable DNA modification was accomplished either by random integration of plasmids, transposons, or viruses or via homologous recombination. The latter method results in site-specific integration but is a very time-consuming and inefficient process. With the introduction of genome editing tools, site-specific stable modifications can now be performed easily. Zinc Finger Nucleases (ZFN) and Transcriptional Activator-like Effector Nucleases (TALEN) technologies were established over the last decade as useful tools for site-specific genomic modifications but, with the recent discovery of the CRISPR (clustered regularly interspaced short palindromic repeats) technology another potent alternative has emerged.

2. Applications

Genome editing technology has been applied in a wide variety of ways to effect genetic modifications in basic and applied research. Loss-of-function mouse knockout studies formerly accomplished by homologous recombination methods can now be performed rapidly and with greater efficiency due to the 10-100-fold increases in genetic modification rates with genome editing[1, 2]. The fidelity and magnitude of gene expression decrease provided by genome editing has been shown to be superior to RNAi-based methods[3,4]. In addition RNAi-based methods only provide a transient knockdown. Several genome wide loss-of-function screens in tumor lines were recently carried out demonstrating the robustness of the technology[3,4]. Transgene insertions that site-specifically add a fluorescent protein, luciferase, or other reporter molecule have facilitated cell homing and lineage tracing studies that rely on preserving native cell function[5]. Cell models have also been created for monogenic diseases either by using patient-derived iPSCs or incorporating well-characterized mutations in iPSCs from normal individuals[6]. In addition to pre-clinical applications, therapeutically relevant cells have been modified with genome editing. For example, genome edited T-cells have been used in AIDS trials where the HIV-resistant form of the CCR5 gene replaced the normal allele[7].

3. Basics on Genome Editing Process and Tools

This chapter gives a brief introduction of the process and the main tools used. For more details please refer to the various reviews available (e.g. Gaj T *et.al.* 2013[8]).

For genome editing, engineered nucleases are used to delete, insert, or replace a gene at a targeted genomic location. Such engineered nucleases are typically comprised of two elements: an endonuclease DNA cleavage module, and a sequence-specific DNA binding domain.

The nuclease cleaves double-stranded DNA creating a double-strand break (DSB) (Figure 1). The DSB induces the cellular DNA repair process. There are two types of repair processes that can occur. Without a homologous donor fragment available – be it the corresponding allele or an external donor DNA – the broken ends will be re-joined. This process is called non-homologous end joining (NHEJ) and is often accompanied by a mutation that may cause a deletion of a functional element of the gene.

If a partially homologous donor sequence is present, e.g. the genomic allele or foreign donor DNA, an insertion or replacement of a gene can take place via homology-dependent repair (HDR). The frequency of NHEJ versus HDR depends on the individual experimental setting, e.g. the cell-type and the donor amount.

The combination of such nucleases with a sequence-specific DNA binding domain that can be customized to recognize virtually any sequence facilitates these repair processes in a targeted manner. The predominant DNA binding domains used in genome editing are zinc finger (ZF) proteins, transcriptional activator-like effector (TALE) proteins or CRISPR-guideRNAs (gRNA)

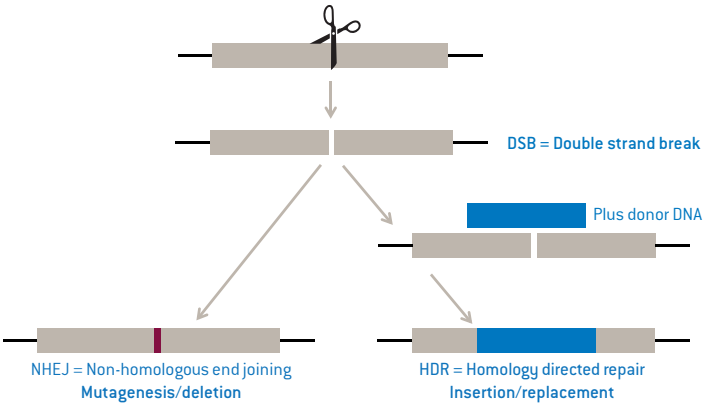

Figure 1. Cellular repair processes following the nuclease-induced double-strand break (simplified scheme).

3.1 Zinc Finger Nucleases (ZFN)

Zinc finger (ZF) proteins are the most abundant and versatile DNA binding motif in nature[9]. An individual zinc finger domain binds 3 DNA base pairs. Because of their modular structure, they provide an ideal framework for designing an artificial sequence-specific binding molecule. This can be fused to an endonuclease which together mediate sequence-specific cleavage. Since its first proof-of-principle in 1996 by Kim *et al.*[10], ZFN-based genome editing technology has further evolved. The current generation of ZFNs utilizes 5 to 6 ZF domains, which recognize a genomic DNA stretch of 15-18 bp and are fused to Fok I nuclease. Two such zinc finger-nuclease fusion proteins work in combination to bind the sense and antisense strand of the targeted DNA sequence (Figure 2). Once both partners have bound, the Fok1 nuclease can form an active dimer and induce the double-strand break that leads to subsequent cellular repair processes. Since ZFNs target a total of 30-36 bp they provide a highly specific genome editing tool.

3.2 Transcriptional Activator-Like Effector Nucleases (TALEN)

In 2009 transcriptional activator-like effectors (TALEs) were discovered to provide a simpler, modular DNA recognition code[11, 12]. TALEs are naturally occurring proteins from the plant pathogenic bacteria genus Xanthomonas, and contain DNA-binding repeats, each recognizing a single base pair. Compared to the triplet-based DNA binding of zinc fingers, this single base recognition mode of TALE–DNA binding repeats enables greater flexibility in design but also holds some cloning challenges. Thus, except for the binding mode, the principle of targeting is very similar. Again sequence-specific, engineered TALEs are typically fused to Fok1 nuclease* to build the TALE-nuclease fusion (TALEN). As with ZFNs, a pair of TALENs must be generated for each target with each monomer binding 13 or more base pairs on the sense or antisense strand of the targeted DNA (Figure 2).

*Also other effector enzyme combinations might be used.

3.3 CRISPR/Cas9 System

Clustered regulatory interspaced short palindromic repeats (CRISPRs), discovered in 1987 in *E. coli*, were recently shown to provide an even simpler genome editing tool[13, 14, 15, 16]. The CRISPR pathway is part of the bacterial immune system to defend against invading viruses. This system has been adapted for use in eukaryotic cells. The specificity is driven by a so-called "guide RNA", which typically binds to a complementary stretch of 18-20 base pairs in the targeted DNA (Figure 2) and has some additional sequence motifs that help in forming a complex with the Cas9 nuclease (CRISPR-associated nuclease). For successful binding of Cas9, the genomic target sequence must also contain a correct Protospacer Adjacent Motif (PAM) sequence immediately following the target sequence. The PAM is an NGG motif adjacent to the binding site. In contrast to ZFNs and TALENs, for CRISPR-based genome editing the DNA binding domain and the nuclease are not fused, since the DNA binding part is an RNA and not a protein. This feature makes it much easier to design a new guide RNA addressing a new target and also allows for multiplexed targeting[16].

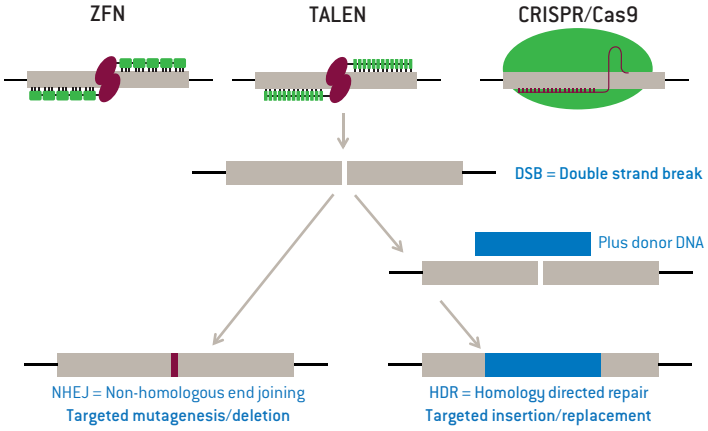

Figure 2. Sequence-specific induction of a double-strand break and subsequent repair processes (simplified scheme).

3.4 Comparison of ZFN, TALEN and CRISPR

Table 1 summarizes the main features of the three different genome editing tools. Briefly, ZFNs and TALENs require the generation of fusion proteins, thus making it more laborious to create a new engineered nuclease for another target site. For the CRISPR system only a new guide RNA needs to be generated to target another sequence. In addition, with CRISPR multiple targeting can be performed by combining the Cas9 nuclease with several guide RNAs.

On the other hand, currently ZFNs and TALENs are more specific than CRISPR and thus carry a lower risk for off-target effects. This is primarily due to their targeting of longer DNA stretches and the requirement for two partner molecules to form the final active nuclease dimer. To overcome this liability, some researchers have mutated the CRISPR Cas9 nuclease to a "nickase" which can then be used in conjunction with paired sense and antisense gRNAs thus providing enhanced specificity[17].

Most importantly, with the universal recognition of the potential of CRIPSR as a cutting edge technology much research is taking place to optimize the tool to suit specific applications.

Table 1. Brief Comparison of Genome Editing Tools

	ZFN	TALEN	CRISPR
Nuclease	Fok 1	Fok 1	Cas9
DNA binding via	ZF protein	TALE protein	GuideRNA (gRNA)
Type	Fusion protein – High effort to modify for new targeting site	Fusion protein – High effort to modify for new targeting site	Protein + RNA – Easy to modify Multiple targeting possible
Binding site	2 sites (15 or 18 bp each) – High specificity – Low risk for off-target effects	2 sites (≥ 13 bp each) – High specificity – Low risk for off-target effects	1 site (18-20 bp + 3bp NGG) – Lower specificity – Higher risk for off-target effects

3.5 Co-transfection

One feature that is common to all three tools is the need to co-transfer several substrates (plasmids, mRNAs, or oligonucleotides) into the cell type of interest for successful modification of genomic DNA (Figure 3). Co-transfection can be challenging, especially when it comes to hard-to-transfect cell types such as primary T cells, human embryonic stem cells (hESC) or induced pluripotent stem cells (iPSCs). This challenge is overcome by Lonza's non-viral Nucleofector™ Technology, which has been shown to work as a reliable and efficient method for transferring the required DNA-, RNA-, or even protein-based components into various cell lines, primary cells, and stem cells. It has proven to work with any of the genome editing technologies described above (Table 5).

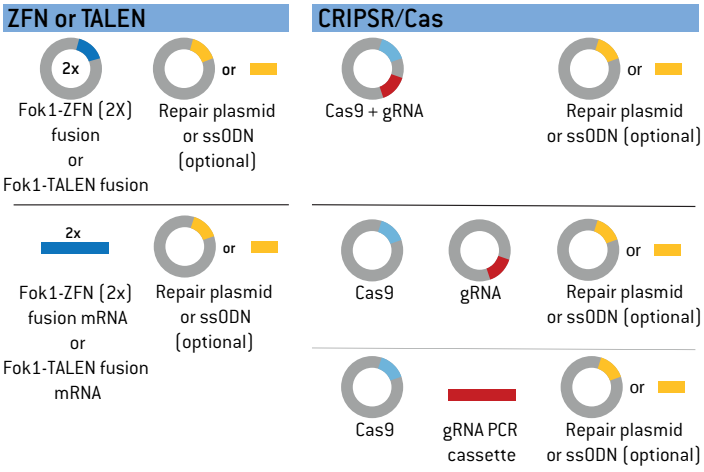

Figure 3. Possible co-transfection scenarios for ZFN, TALEN or CRIPSR/Cas9. The scheme shows some substrate type combinations (plasmids, mRNAs, or oligonucleotides) that have been described in the literature. However, additional scenarios may apply, e.g. transfection of proteins (see 4.4).

4. Using Nucleofector™ Technology for Genome Editing

4.1 Establish/Verify Nucleofection Conditions with pmaxGFP™ Vector

Lonza offers ready-to-use Optimized Protocols for a broad range of cell types (www.lonza.com/protocols) including hard-to-transfect cell lines and primary cells. Before performing a genome editing experiment we highly recommend to transfect our pmaxGFP™ Positive Control Vector to verify that the optimal conditions we identified also work well in the user-specific setting.

In case no ready-to-use protocol is available for a certain cell type, one can easily determine the optimal Nucleofection conditions using the pmaxGFP™ Vector by following the respective optimization protocol for a certain cell group or our general optimization protocols for primary cells or cell lines. For embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), for example, we recommend using our "Basic Stem Cell Protocol", since each ESC or iPSC clone may require slightly different transfection conditions. Once the optimal conditions have been determined, they remain the same whether DNA- or RNA-based substrates (or both together) are transfected.

4.2 Determination of Optimal Substrate Amounts

For successful genome editing it is important to determine the optimal substrate amounts. Tables 2 – 3 provide some example ranges for the different genome editing tools derived from published data. The ranges are given in amount per microliter to account for the different Nucleofection formats available (20 and 100 µL).

Table 2. Substrate Ranges Published for ZFN

Substrate	Range (per µl Nucleofection volume*)
ZFN plasmid (each)	0.01 – 0.05 µg/µL each
ZFN mRNA (each)	0.02 – 0.2 µg/µL each
Donor plasmid	0.04 – 0.2 µg/µL

*Note: Depending on the Nucleofection volume, ranges have to be multiplied by 20 or 100.

Table 3. Substrate Ranges Published for TALEN

Substrate	Range (per µl Nucleofection Volume*)
TALEN plasmid (each)	0.01 – 0.1 µg/µL each
Donor plasmid	0.05 – 0.2 µg/µL
Donor dsDNA (lin)	0.1 µg/µL
Donor ssODN	10 µM

*Note: Depending on the Nucleofection volume, ranges have to be multiplied by 20 or 100.

Table 4. Substrate Ranges Published for CRISPR/Cas9

Substrate	Range (per µL Nucleofection Volume*)
Cas9/gRNA plasmid	0.025 µg/µL
Cas9 plasmid	0.02-0.05 µg/µL
gRNA plasmid	0.02-0.05 µg/µL
gRNA PCR Cassette	0.5 ng/µL
Donor dsDNA (lin)	0.02-0.1 µg/µL
Donor ssODN	0.5-10 µM
Cas9/gRNA ribonucleoprotein	See references 23 and 24

*Note: Depending on the Nucleofection volume, ranges have to be multiplied by 20 or 100.

4.3 Transfection of mRNA

Due to its shorter half-life the use of mRNA instead of plasmids might be beneficial when aiming to minimize the presence time of the nuclease and avoid multiple events. mRNA may also provide higher integration frequencies[18, 19].

When working with mRNA, the same protocol and program can be used that is optimal for the transfection of DNA into the respective cell type. However, there are a few additional things that should be considered:

- The mRNA should be capped and poly-adenylated
- As with plasmids the optimal mRNA amount has to be titrated, but it might be higher than for plasmid DNA (Table 2)
- If higher amounts are required, the total volume added to the transfection reaction should not exceed 10% of the total sample volume
- When collecting the cells for the transfection experiment you may want to include an additional wash step with PBS to get rid of serum-derived RNAses[19]
- Keep mRNA on ice prior to addition to the sample
- To avoid any degradation, e.g. due to prolonged contact with cells, the mRNA might be transferred directly into the empty cuvettes before adding the cell-solution mix on top and transfection should be performed immediately

4.4 Transfection of Protein

The Nucleofector™ Technology is also suited to transfect peptides[20,21,22] and proteins. As a starting condition we would recommend using the established optimal conditions for nucleic acids, but some program fine tuning might be required. Kim *et al.* (2014)[23] recently reported the transfection of Cas9-gRNA ribonucleoprotein using the 4D-Nucleofector™ System. They transfected K562, BJ or H9 cells with Cas9 protein pre-mixed with in vitro transcribed gRNA. A similar approach was used by another research group who transfected Cas9-gRNA ribonucleoprotein into HEK293T cells, primary neonatal fibroblasts and H9 cells. For protein ranges used please refer to the publications.

4.5 Factors Influencing Genome Editing Results

Besides the transfection efficiency, there are various factors that may influence the outcome of a genome editing experiment. For example, the integration frequency differs depending on the cell type selected[18]. In addition, as with any other substrate transfected, the quality of the genome editing tool used can have a major impact on the editing results. Tools from various non-commercial and commercial sources have been successfully tested in combination with the Nucleofector™ Technology (see Table 5).

When aiming for insertions via HDR, either double-stranded DNA or single-stranded oligonucleotides (ssODN) can been used as repair template. The latter provides an effective method for introducing single mutations and a simple format for screening approaches[18, 25].

5. Post Nucleofection – Selection and Expansion

Clonal selection can be started between 24 h and 7 days post transfection. The optimal time point has to be determined depending on the individual experimental setting.

One option to increase the number of clones is transfecting a vector that co-expresses a fluorescent protein, which would allow enrichment of transfected cells by FACS sorting.

For cells that do not like to be grown as single cells (e.g. ESCs or iPSCs) FACS sorting might also be an alternative to the limiting dilution process.

6. Analysis of Editing Events

Genome editing events can be analyzed by various means. Typically used methods comprise one or more of the following: PCR or RT-PCR, sequencing (e.g. deep sequencing, next generation sequencing), Southern blot, Northern Blot or mutation frequency assays (mismatch assays like e.g. Cel1 assay, T7 endonuclease I assay, SURVEYOR™ Nuclease Assay, or RFLP analysis) or Western blot (to analyze protein knockout). For iPSCs, Yang *et al.* (2014)[25] have developed a robust and user-friendly system (genome editing assessment system) using next-generation sequencing to screen for both HDR and NHEJ events.

7. Summary

The Nucleofector™ Technology is a very versatile method for transfection of multiple substrates in hard-to-transfect cell types. Here we provided some general recommendation about important factors to consider when using the technology for ZFN-, TALEN- or CRISPR-mediated genome editing. For more specific recommendations on a certain cell-tool combination you may refer to the respective publication (see Table 5). For example, Ran *et al* (2013)[26] gives comprehensive background information about CRISPR technology and provides a detailed protocol how to use Lonza's 4D-Nucleofector™ X Unit for CRISPR-based genome editing in HUES9 (a human stem cell line) and HEK293 cells. It also includes protocols for functional analyses, tips for minimizing off-target effects and FAQs. You may also contact our Scientific Support Teams for any specific guidance. (www.lonza.com/researchsupport).

Table 5. Selected publications for genome editing using the Nucleofector™ Technology

Tool	Authors	Citation	Year	Nucleofector™ Platform	Cell type
ZFN	Chen F *et al.*	Nat Meth 8(9):753-5	2011	Nucleofector™ II/2b Device	K562, HCT116, U2OS, HEK293, HepG2 and MCF7
	Fung H *et al.*	PLoS ONE 6(5):e20514	2011	Nucleofector™ II/2b Device	hESC
	Genovese P *et al.*	Nature 510:235ff	2014	4D-Nucleofector™ System	hCD34
	Hansen K *et al.*	J Vis Exp (64):e3304	2012	Nucleofector™ II/2b Device	K562
	Liu X *et al.*	PLoS ONE 7(5):e37071	2012	Nucleofector™ II/2b Device	hES
	Ou W *et al.*	PLoS ONE 8(11):e81131	2013	Nucleofector™ II/2b Device	iPSC
	Qu X *et al.*	Nucleic Acids Res 41:7771-7782	2013	Nucleofector™ II/2b Device	HIV-infected PBL and CD4 T cells
	Piganeau M *et al.*	Genome Res 23:1182-1193	2013	Nucleofector™ II/2b Device	hESC and Jurkat cells
	Richter S *et al.*	PLoS ONE 8(6):e65267	2013	Nucleofector™ II/2b Device	HTC116 and H460
	Robbez-Masson LJ *et al.*	PLoS ONE 8(11):e78839	2013	Nucleofector™ II/2b Device	MCF7
	Samsonov A *et al.*	PLoS ONE 8(7):e68391	2013	Nucleofector™ II/2b Device	A549
	Schjoldager K	PNAS 109:9893-9898	2012	n.d.	HepG2
	Torikai H *et al.*	Blood 119(24):5697-705	2012	Nucleofector™ II/2b Device	Human T cells
	Toscano MG *et al.*	Dis Model Mech 6:544–554	2013	Nucleofector™ II/2b Device	K562
	Wang J *et al.*	Genome Res 22:1316-1326	2012	Nucleofector™ II/2b Device and 96-well Shuttle™ Add-On	K562
	Zou J *et al.*	Blood 117:5561-5572	2011	Nucleofector™ II/2b Device	iPSC
	Zou J *et al.*	Blood 118:4599-4608	2011	Nucleofector™ II/2b Device	iPSC
	Yan W *et al.*	Scientific Rep 3:2376	2013	Nucleofector™ II/2b Device 4D-Nucleofector™ System	iPSC
TALEN	Piganeau M *et al.*	Genome Res 23:1182-1193	2013	Nucleofector™ II/2b Device	hESC and Jurkat cells
	Zhu F *et al.*	Nucleic Acids Res 10.1093/nar/gkt1290	2014	4D-Nucleofector™ System	iPSC and H9 hESC
	Yan W *et al.*	Scientific Rep 3:2376	2013	Nucleofector™ II/2b Device 4D-Nucleofector™ System	iPSC
	Yang L *et al.*	Nucleic Acids Res 41:9049-9061	2013	4D-Nucleofector™ System	iPSC
	Mussolino C *et al.*	Nucleic Acids Res 42(10):6762–6773	2014	Nucleofector™ II/2b Device	human newborn foreskin fibroblasts, K562
CRIPSR	Fu Y *et al.*	Nat Biotechnol 31(9):822–826	2013	4D-Nucleofector™ System	U2OS, K562
	Kim S *et al.*	Genome Res 24:1012–1019	2014	4D-Nucleofector™ System	K562, BJ fibroblasts
	Lin S *et al.*	eLife 3:e04766	2014	96-well Shuttle™ Add-On	HEK293T, human primary neonatal fibroblast and H9 hESC
	Petit CS *et al.*	J Cell Biol 202:1107-1122	2013	Nucleofector™ II/2b Device	HeLa
	Ran FA *et al.*	Cell 154:1380–1389	2013	4D-Nucleofector™ System	HUES62
	Ran FA *et al.**	Nat Prot 8(11):2281–2308	2013	4D-Nucleofector™ System	HUES9 and HEK293
	Yang L *et al.*	Nucleic Acids Res 41:9049-9061	2013	4D-Nucleofector™ System	iPSC

For more publications please refer to **www.lonza.com/citations.**

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