Guide-it™ Products for Successful CRISPR/Cas9 Gene Editing

Q&As

Webinar: Cas9/sgRNA ribonucleoprotein (RNP) delivery to improve your KO and KI gene editing experiments

Q1: What is a gesicle? Please explain more.

A1: Gesicles are cell-derived nanovesicles in which you can pack the Cas9/sgRNA RNP complex. Gesicles are produced in HEK293T cells following overexpression of specific glycoproteins. Gesicle fusion with the target cells is mediated via VSV-G, present on the surface of the gesicles. The tropism of gesicles is similar to VSV-G pseudotyped lentivirus, meaning it´s very broad mammalian tropism. Gesicles can be used to deliver RNP complexes to dividing and non-dividing cells.

Q2: Do the gesicles work in mouse ES cells and do you now if this effects germ line transmission?

A2: We do not work with mESCs in house, and I am not aware of any publication showing the use of gesicle technology in mouse ES cells so far. But we have tested gesicle-mediated gene knockout in human iPS cells. Gesicles work very well in hiPSCs and we did not observe any effect on pluripotency.

Q3: Why are Gesicles not working KBM7 cells?

A3: I do not know the exact reason why gesicle-mediated gene editing did not work in KBM-7 cells. It could be due either to inefficient delivery or due to inefficient gene editing (accessibility of the target site). KBM-7 cells are a chronic myelogenous leukemia (CML) cell line. Plasmid transfection did not work either, so delivery seems to be rather tricky. If VSV-G pseudotyped lentivirus does not work either, this would indicate that the surface receptors for docking of VSV-G are not well expressed in this cell type.

Q4: What is the role of the PAM sequence?

A4: The PAM sequence needs to be present in the genome of your target cells to be recognized by the Cas9/sgRNA complex. The PAM sequence for wt Cas9 from *Streptococcus pyogenes* is "NGG", where N can be any base. This is a very frequent sequence in the human genome which occurs every ~40 bases.

Q5: How stable is the complex of sgRNA and Cas at 4° and to which concentration can the Cas9 be diluted and still be working?

A5: Cas9/sgRNA RNP complexes are very stable. We recommend forming the RNP complex via incubation of the Cas9 protein and the sgRNA at for 5 min 37°C. The ssDNA repair template will be less stable. For KI experiments it is best to assemble the RNP complex first, then put the complex on ice and add the HDR template just before electroporation.

The optimal concentration of Cas9, sgRNA, and HDR template needs to be determined empirically for your specific target cells. I would recommend to perform some dose-response experiments first to check for gene editing efficiency and toxicity when using different amounts of the RNP complex.

Q6: Does one need to get specific primers for ssDNA production or can plasmid specific primers used?

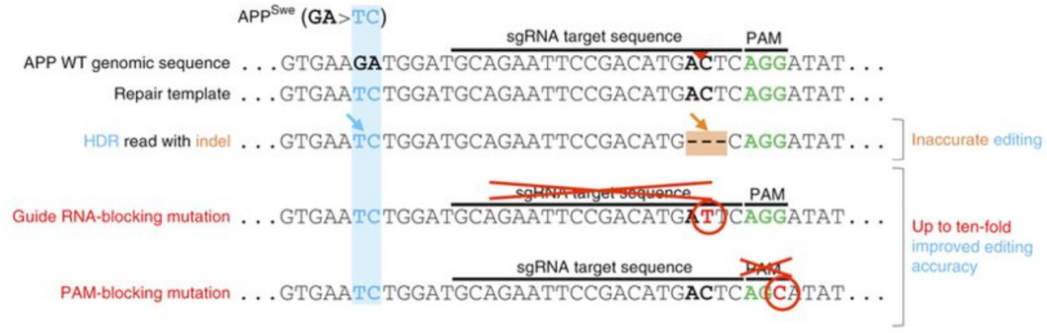
A6: One of the primers for amplification must contain a 5´-phosphorylation, so you cannot use existing plasmid specific primers that you may already have in the lab. I would recommend amplifying only the desired bases and homology arms, without plasmid-derived bases.

Q7: I would like to know more how to design HDR template. For replacing a mutated sequence with a normal sequence how do you design your HDR sequence. Obviously, we should shouldn´t have PAM sequence in ssDNA. How do you design it then? Point mutation in PAM sequence?

A7: You may indeed consider introducing a silent mutation that disrupts the PAM site or the sgRNA recognition sequence into the HDR template. This reduces the likelihood of subsequent cleavage (and error-prone repair):

Please see the reference/figure below for further details:

Kwart et al. *Nature Protocols* ***12***, 329-354 (2017)



You can find some additional tips here:

https://blog.benchling.com/2016/04/21/how-to-design-homologous-recombination-template-for-crispr/

Q8: Is gesicles a nanocapsule?

A8: Gesicles are cell-derived nanovesicles. They will bud from the cellular membrane of the producer cells.

Q9: Are there any successful stories on using sgRNA produced using Guide-it sgRNA IVT kit, together with recombinant Cas9, introduced into zebrafish by microinjection?

A9: Our Guide-it Recombinant Cas9 (Electroporation-Ready) (#632640/#632641) has been successfully used for microinjection into mouse one-cell embryos as well as silk worm. I am not aware of any publications showing use of our recombinant Cas9 for microinjection into zebrafish, but this should be feasible, too.

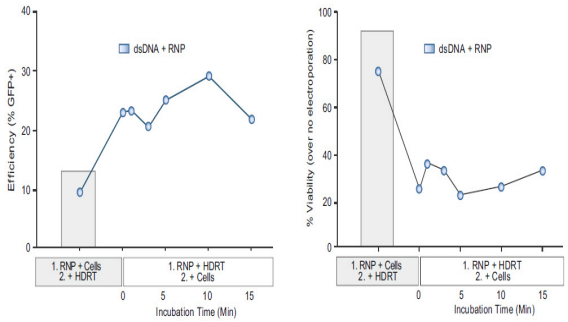
Microinjection of mouse one-cell embryos:
Generation of Pax6-IRES-EGFP knock-in mouse via the cloning-free CRISPR/Cas9 system to reliably visualize neurodevelopmental dynamics.
Inoue Y et al., Neurosci Res. 2018 Jan 31. pii: S0168-0102(17)30718-6.
https://www.ncbi.nlm.nih.gov/pubmed/29391173

Injection into newly laid eggs of Bombyx mori (silk worm):
Translucent larval integument and flaccid paralysis caused by genome editing in a gene governing molybdenum cofactor biosynthesis in Bombyx mori
Fujii T et al, Insect Biochemistry and Molecular Biology 99 (2018) 11-16
https://doi.org/10.1016/j.ibmb.2018.04.008

Q10: When using RNP electroporation for T cells, what is the percentage of viable T cells?

A10: Please have a look at the paper below. The authors did an extensive analysis regarding amounts of Cas9, sgRNA, and long ssDNA HDR templates used and the related viability of primary human T-cells:

Reprogramming human T cell function and specificity with non-viral genome targeting
Roth TL et al., Nature 559 :405–409 (2018)
https://www.nature.com/articles/s41586-018-0326-5



According to Roth et al., co-incubation of RNP and HDR template prior to application provides highest efficiency. But there is a tradeoff between efficiency and viability.

Q11: I have difficulties producing sgRNA in lab. The sgRNAs produced in lab seems to be inefficient. What can be the reason?

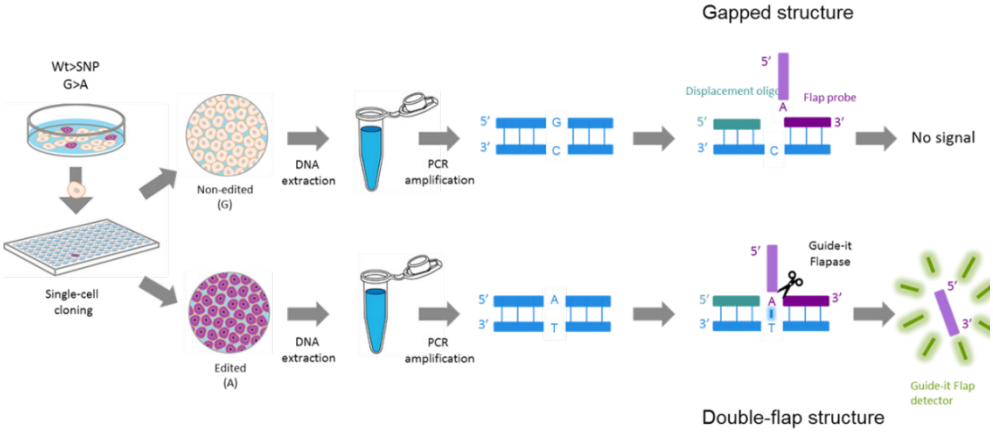
A11: I would recommend designing 4-5 sgRNAs per target. You may then screen these guides in vitro, to check their efficiency. This in vitro screen will help you to get rid of inefficient sgRNA. You can use our Guide-it™ sgRNA Screening Kit, #632639, for this purpose. The principle is explained in the TechNote below: Screening for effective guides

Q12: After ssDNA production how can the ssDNA HDR template be maintained as single strand because it may anneal to itself. We normally use high temperatures such as in PCR.

A12: After long ssDNA production, you just need to freeze the HDR template. Depending on the GC content and the specific sequence of the HDR template, secondary structures may occur. In house, we did not encounter any issues (self-annealing) due to secondary structures.

Q13: May you better explain how the screening kit works please?

A13: The principle is based on designing two oligos surrounding the SNP you are studying. The Displacement oligo will anneal just upstream of the SNP and the Flap probe oligo downstream of the SNP, including the interrogated base. If the SNP is present, the Flap probe oligo will form a double-flap structure which is recognized by Guide-it Flapase. The Flapase will cut off a 12 nt fixed sequence from the Flap probe oligo which will emit a fluorescent signal in the presence of the SNP. If the SNP is not present, a gapped structure will be formed. This structure is not recognized by Flapase, so no fluorescent signal will occur.



Video showing SNP Screening Kit Principle

SNP detection webinar

Q14: Are gesicles better when we have more than 2 alleles for knockout?

A14: It´s always more difficult to knockout several alleles, e.g. when working with polyploid cell lines compared to diploid cell lines. I do not believe that gesicles will show better efficiency for such context compared to other methods (plasmid or viral delivery). However, gesicles (RNP delivery) will ensure better specificity compared to DNA delivery.

Q15: Which method would you recommend for primary cells difficult to expand after gene editing, both for SNP modification and long knockin?

A15: Please note that HDR is limited to proliferating cells. If your primary cells do not divide at all, you cannot use HDR for knockins/SNP repair. The base editing technology (using Cas9 nickase fused to Cytidine deaminase or variant) could be an alternative for the insertion of SNPs in this case. If the cells are difficult to expand, the single cell cloning step will unfortunately be very tricky, too.

Q16: How to produce a guide RNA?

A16: We offer a kit based on in vitro transcription using T7 RNA polymerase (Cat# 632635):

Guide-it™ sgRNA In Vitro Transcription Kit

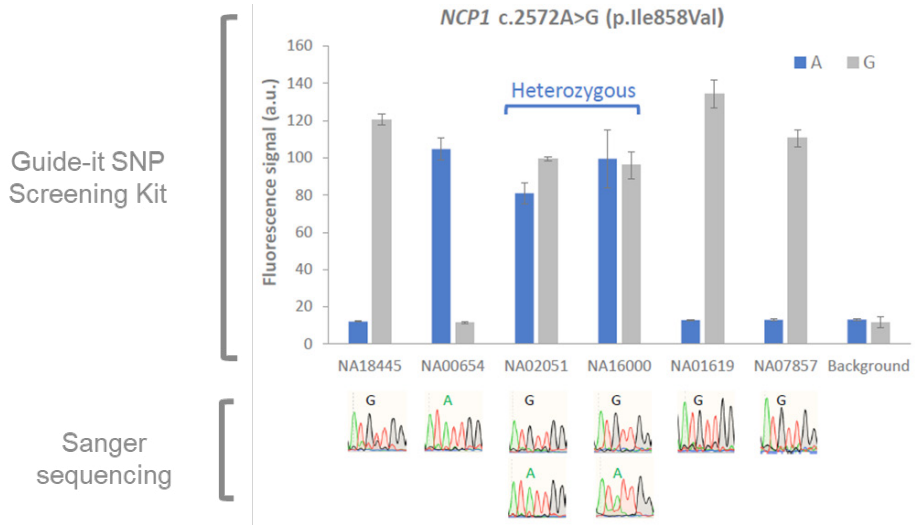
Guide-it sgRNA In Vitro Transcription and Screening Systems User Manual

Q17: How can we edit the two alleles of a gene and how can we be sure that the two alleles of the gene are edited?

A17: Downstream of CRISPR/Cas9 gene editing, you will get a mixed population with cells carrying various indels. Some cells will still be wt, some cells will carry monoalleic mutations, some will carry bi-allelic mutations (which can be homozygous or heterozygous). That´s why the single cell cloning step is very important - it will allow to screen and identify the clones that carry the desired mutation. We provide two different tools:

Our Guide-it™ Genotype Confirmation Kit, #632611, can be used for identification of monoallelic and biallelic indels downstream of CRISPR/Cas9 gene editing: TechNote monoallelic-versus-biallelic-mutants

Our Guide-it™ SNP Screening Kit, #632652/632653, can be used for genotyping of single nucleotide substitutions by designing two different Flap probe oligos, e.g. one for the WT base and one for the SNP:



Q18: Can the SNP screening kit be used only for SNPs?

A18: The current version is only suitable for detection of single nucleotide substitutions. Our R&D is also developing a kit for detection of longer insertions.

Q19: I electroporate in suspension leukemic cell lines. After the electroporation most of cells die. Do you have any suggestion?

A19: Please have a look at the paper below as well as my answer to Q10. The authors did an extensive analysis regarding amounts of Cas9, sgRNA, and long ssDNA HDR templates used and the related viability of primary human T-cells. You may do similar tests in your leukemic cell lines to optimize the amounts of Cas9, sgRNA and HDR template.

Reprogramming human T cell function and specificity with non-viral genome targeting
Roth TL et al., Nature 559 :405–409 (2018)
https://www.nature.com/articles/s41586-018-0326-5