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Product Information

CRISPR Integration Kit

Product Number **HSOLIGOINT**

TECHNICAL BULLETIN

CRISPR/Cas systems have evolved within bacterial and archaeal organisms as a defense against viruses and foreign plasmids. The function of the native CRISPR pathway is to target nucleases to invasive DNA, creating a potentially lethal double-strand break (DSB). Shortly after the function of this pathway was recognized, the type II CRISPR/Cas system from the bacterium *Streptococcus pyogenes* was repurposed to create a simple, yet powerful molecular tool that could be programmed to target nucleases to a specific genomic sequence in eukaryotes. To create such a tool, the endogenous bacterial CRISPR system was reduced to two principal components: the Cas nuclease (Cas9, specifically) and a guide RNA (gRNA) 1-7. When complexed together, the nuclease and gRNA require two specific DNA sequences to bind and cut: the Cas9 protein requires a short protospacer adjacent motif (PAM) and the gRNA requires 17-21 bases of RNA-to-DNA homology immediately upstream of the PAM (Fig. 1). After Cas9 binds the PAM, it attempts to align the gRNA to the genomic DNA and the gRNA-Cas9 complex creates a targeted DSB at this precise location in the genome.

CRISPR/Cas9 enables permanent, heritable modification of chromosomal DNA. Following targeted DSB formation, any resulting mutation is dependent on the repair mechanisms that are active within the cell. Generally, the cell will attempt to repair the DSB via one of two pathways, non-homologous end joining (NHEJ) or homology directed repair (HDR), the latter requiring a double stranded donor molecule. Both NHEJ and HDR can be co-opted to achieve mutagenesis, either by disrupting a coding sequence to functionally inactivate or knockout a gene (NHEJ), or by adding new DNA to knock-in specific sequence changes (HDR) (Bibikova et al., 2002).

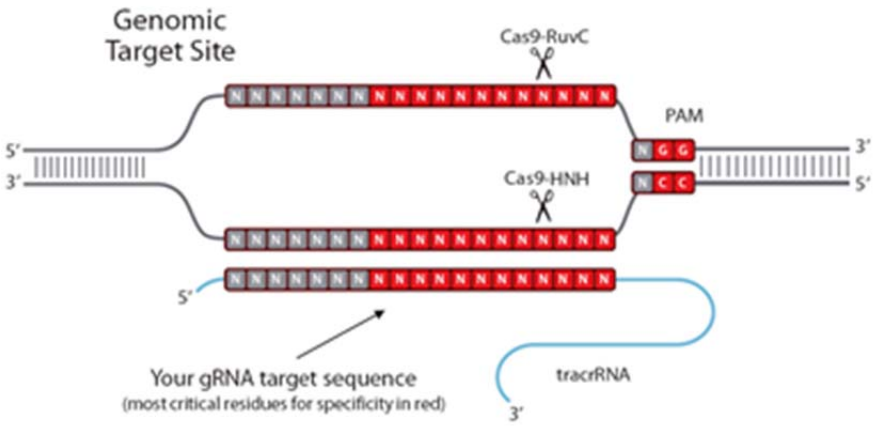

Figure 1. Schematic of a CRISPR/Cas-targeted double-strand break.

For specific sequence changes, DSBs have been shown to enhance targeted integration significantly (Rouet et al., 1994). To achieve this, a DNA 'donor' or repair template containing the desired sequence must be delivered to the cell(s) of interest along with the CRISPR. Traditional sequence change methods relied on lengthy donor plasmids for repair, but relatively short sequence changes can be achieved with comparable efficiency using a short single-stranded oligonucleotide (ssODN) donor (Chen et al., 2011). Use of ssODNs can reduce both the time and cost of many targeted gene editing experiments.

Even with the aid of CRISPR, in most cell lines the efficiency of HDR-directed knock in gene edits is generally much lower (<10% of modified alleles) than knock out. Cell lines vary greatly in their capacity to perform HDR-directed integration (Fig 2.) Variation in HDR rates may affect the number of clones to be screened or the detectability of cell modification phenotype by orders of magnitude. For this reason, it is strongly recommended that researchers determine the predicted HDR-efficiency of the target cell line prior to beginning a CRISPR gene editing experiment.

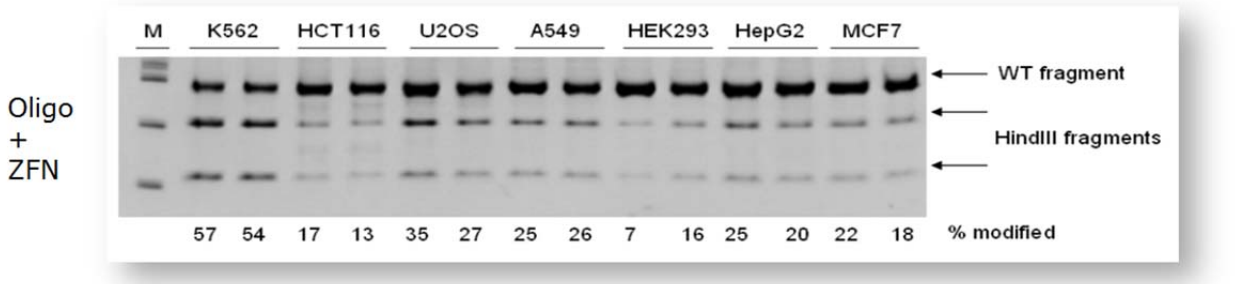

Figure 2. Cell line-specific integration frequencies at the human AAVS1 locus. F Chen et al, *Nature Methods*, 2011.

Intended use

The Sigma-Aldrich CRISPR Integration Kit provides the essential gene editing reagents necessary to integrate a BstNI restriction site to the human KRAS locus. This kit is intended to serve as a tool to evaluate cell lines for ability to integrate new sequences at CRISPR-effected double strand breaks with the aid of short oligonucleotide donor. Integration efficiencies obtained with this kit enable rational design of genome editing experiments in a wide range of cell lines.

Kit components

Component	Product Number	Quantity
1: gRNA plasmid - KRAS	HSINTKRAS-1VL	10 reactions = 50 µg @ 1 µg/µL
2: Cas9 GFP plasmid	INTCAS9-1VL	10 reactions = 50 µg @ 1 µg/µL
3: BstNI Donor Oligo	HSINTBSTD-1VL	10 reactions = 50 µL @ 100 µM
4: Forward PCR Primer	HSINTPCRF-1VL	10 reactions = 50 µL @ 20-25 µM
5: Reverse PCR Primer	HSINTPCRR-1VL	10 reactions = 50 µL @ 20-25 µM
6: Genomic DNA control	HSINTCTRL-1VL	20 reactions = 250 ng @ 10 ng/µL

Reagents and Equipment Recommended but Not Provided:

- Lonza Amaxa Nucleofector™ 2b Thermal Cycler
- Cell Line Nucleofector® kit-V (Lonza Product Number VCA-1003)
- Hanks' Balanced Salt Solution (Sigma Product Number H6648)
- GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma Product Number G1N70)
- JumpStart™ Taq ReadyMix™ (Sigma Product Number P2893)
- Water, PCR Reagent (Sigma Product Number W1754)
- CEL-I Digest (Surveyor® Mutation Detection Kit) (IDT Product Number 706020)
    - Note: EnGen® Mutation Detection Kit may also be used (NEB Product Number E3321S)
- GenElute™ PCR Clean-Up Kit (Sigma Product Number NA1020)
- BstNI (NEB Product Number R0168S)
- 10× CutSmart®-Buffer (NEB Product Number B7204S)
- Gel Loading Buffer (Sigma Product Number G2526)
- 10% Mini-PROTEAN® TBE Precast Gels, 15-well comb, 15 µL (Bio-Rad Product Number 4565036)
- Ethidium Bromide Solution 10 mg/mL (Sigma Product Number E1510)
- Tris-Borate-EDTA Buffer, 5x concentrate (Sigma Product Number T3913)
- Centrifuge Tubes
- PCR Reaction Tubes

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage and Stability

All components can be stored at –20 °C for up to 12 months. For storage long-term, store at –80 °C.

Procedures

I. Transfection

The Sigma-Aldrich CRISPR Integration kit is compatible with a variety of standard transfection protocols. The following are guidelines for application by nucleofection which are also intended to serve as a starting point for optimization of alternative protocols.

Day 0

- Lonza Amaxa Nucleofector™ 2b Thermal Cycler
- Nucleofector® kit-V (Lonza Product Number VCA-1003)
- Nucleofector Program T-016

Per nucleofector manufacturer's instructions, prepare 1 million K562 cells per reaction (1 million cells /well of 6-well plate).

Day 1

1. Prepare 10 µL sample for nucleofection. To a new tube, add 4 µL Cas9-GFP (1 µg/µL), 3 µL KRAS gRNA plasmid (1 µg/µL), and 3 µL Donor Oligo (100 µM).
2. Aliquot 8 µL of the 0.5 µg/µL pmaxGFP® Vector (supplied in kit, Lonza Product Number VCA-1003) for the transfection control.
3. Count cells and transfer 1 million cells per sample to a new tube.
4. Centrifuge cells at low rpm (e.g. 750 rpm, Beckman GS-6R Centrifuge / GH-3.8 Swinging Bucket Rotor) for 5 minutes, then slowly aspirate off the media.

5. Wash cells twice with 10-15 mL of Hanks' Balanced Salt Solution (Sigma Product Number H6648).
6. Centrifuge cells at low rpm for 5 minutes, then slowly aspirate buffer.
7. Re-suspend cells in 100 µL/sample of supplemented nucleofection solution V (supplied in kit, Lonza Product Number VCA-1003).
8. Aliquot 100 µL of cell suspension to each reaction tube, mix with plasmid DNAs, and proceed immediately to Amaxa Nucleofector program T-016.
9. After Amaxa electroporation, use disposable pipet to add pre-warmed media to cuvette and transfer to 6-well plate (1 well for each reaction).
10. Incubate all plates at 37 °C, 48 hours or appropriate recovery time.

II. Harvest and Testing

Days 3-7
Isolate genomic DNA 48 hours post-transfection by harvesting cells.

11. Transfer 1 mL of cells to a new 1.5 mL tube (for sample and negative control).
12. Pellet cells by centrifugation at 1000 RCF for 5 minutes. Remove supernatant.
13. Harvest genomic DNA using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma Product Number G1N70).
14. Prepare PCR reaction mixtures for genomic DNA amplification. Include reactions for Negative and Positive Control DNA.

Reagent	**Volume**
JumpStart Taq ReadyMix (Sigma Product Number P2893)	25 µL
Water, PCR Reagent (Sigma Product Number W1754)	21 µL
Forward PCR Primer (25mM)	1 µL
Reverse PCR Primer (25mM)	1 µL
Genomic DNA (from step 13)	2 µL
Total Volume	**50 µL**

Note: PCR annealing times/temperatures may vary by thermal cycler.

15. Amplify DNA using the following PCR program.

Step	Temperature	Time	
1	98 °C	2 minutes	
2	98 °C	30 seconds	35 cycles
3	62 °C	30 seconds	
4	72 °C	40 seconds	
5	72 °C	3 minutes	
6	4 °C	Hold	

16. Divide each PCR reaction into two new PCR tubes, 20 µL each, for the CEL-I Assay (step 17) and the RFLP Assay (step 20).

II. Mutation Detection Assay (CEL-I Assay)

To measure cleavage efficiency in the cell, we recommend using the CEL-I or Surveyor® assay, or similar techniques, which can detect and quantify NHEJ-errors. In this type of assay, the target region is amplified by PCR using genomic DNA from the transfected cell pool as template. With active nuclease, the genomic DNA will be a mixture of wild-type and NHEJ-error products (insertions or deletions at the target site). The PCR product is denatured under high temperatures, then allowed to anneal such that wild-type and mutant products hybridize. A double- strand DNA is formed in the tube with mismatches around the cleavage site, which are available for cleavage by CEL-I or T7E1 (see Figure 4 below).

CEL-I Digest

17. Reanneal 20 µL of the PCR reaction for all samples and controls in a thermal cycler following the conditions below.

CEL-I Anneal

Step	Temperature	Time
1	99 °C	3 minutes
2	-1 °C/seconds to 60 °C	
3	4 °C	Hold

18. Using CEL-I Digest (Surveyor® Mutation Detection Kit, IDT Product Number 706025), add 1 µL of Enhancer and 1 µL of Nuclease S to each 20 µL aliquot of PCR reaction. Alternatively, a master mix of enhancer and nuclease can be prepared just prior to digest. Incubate, 42 °C, 30 minutes. Note: EnGen® Mutation Detection Kit alternatively may be used (NEB Product Number E3321S)
19. Resolve digested products by PAGE. See Steps 23-26.

RFLP (BstNI) Digest

20. Purify PCR products with GenElute PCR Clean-Up Kit (Sigma Product Number NA1020) and measure DNA concentration.
21. Perform restriction digest as follows:

Component	Volume
Purified PCR product	x µL (300-400 ng)
10× CutSmart®-Buffer (NEB Product Number B7204S)	2.0 µL
BstNI (NEB Product Number R0168S)	0.5 µL
Water, PCR Reagent (Sigma Product Number W1754)	to volume
Total volume	20 µL

Incubate at 60 °C for 30 minutes.

PAGE Resolution

Note: PAGE is strongly recommended for optimal resolution. It is possible that a well-designed RFLP assay with sufficient band size difference may be separated by 2% TBE Agarose gel.

22. Following digests, add 6 µL Gel Loading Buffer (Sigma Product Number G2526) to each sample from both CEL-I and RFLP assays.
23. Resolve by non-denaturing 10% TBE PAGE (10% Mini-PROTEAN® TBE Precast Gels, 15-well comb, 15 µL, Bio-Rad Product Number 4565036), at 220 V for 30 minutes.
24. Stain gels in 100 mL 1x Tris-Borate-EDTA Buffer (Sigma Product Number T3913) with 3 µL of Ethidium Bromide Solution 10 mg/mL (Sigma Product Number E1510) for 3 minutes and destain with 1× TBE.
25. Visualize with UV illuminator.

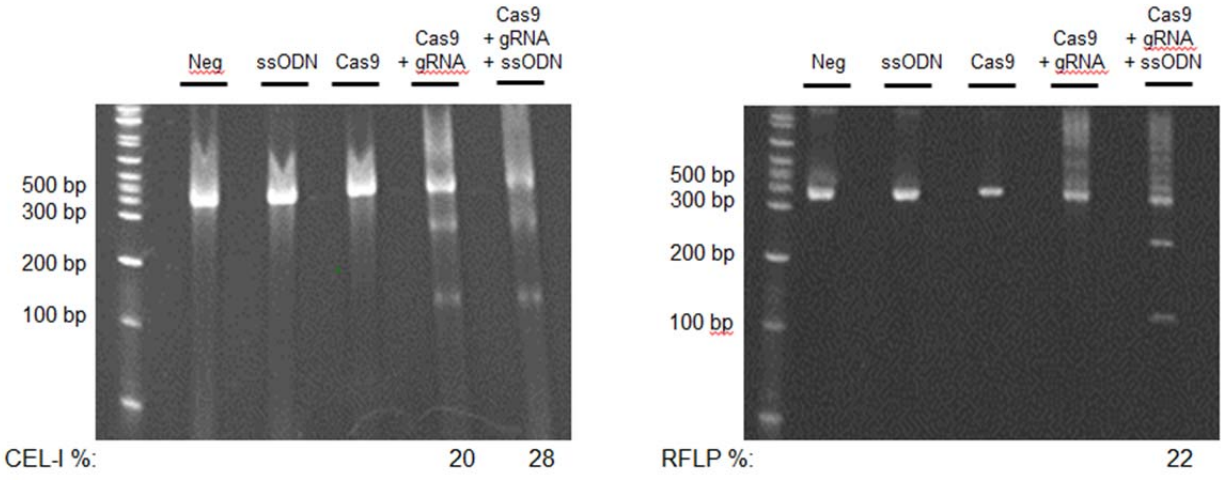

Figure 3. CEL-I and BstNI cleavage at Kras target site in K562 cell line using CRISPR Integration Assay.

Cited References

- Bibikova, Marina, et al. "Targeted chromosomal cleavage and mutagenesis in Drosophila using zinc-finger nucleases." *Genetics* 161.3 (2002): 1169-1175.
- Chen, Fuqiang, et al. "High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases." *Nature Methods* 8.9 (2011): 753-755.
- Rouet, Philippe, Fatima Smih, and Maria Jasin. "Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells." *Proceedings of the National Academy of Sciences* 91.13 (1994): 6064-6068.

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