Clontech Laboratories, Inc.

Guide-it™ Indel Identification Kit User Manual

Cat. No. 631444
(120114)

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I. Introduction

A. Summary

Engineered nucleases, including clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 or RNA-guided endonucleases (RGENs)/sgRNA, transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and meganucleases, are used for precise gene editing. These nucleases are targeted to a specific genomic location where they create double-strand breaks (DSBs). One way a cell can repair a DSB is via the endogenous nonhomologous end joining (NHEJ) DNA repair pathway. However, NHEJ-based repair is error prone; thus, a variety of different insertions and deletions (indels) are often introduced at the target site.

The Guide-it Indel Identification Kit provides a complete workflow for identifying the variety of indels created via genome editing in a cell population; the kit contains all of the components needed to amplify, clone, and prepare modified target sites for DNA sequence analysis. This kit uses Terra™ PCR Direct to amplify a genomic DNA fragment (~500 to 700 bp) containing the target site directly from crude cell lysate. The resulting amplified fragments are cloned directly, without restriction digestion, into a pre-linearized pUC19 vector using the In-Fusion® cloning system. After transformation into an optimized *E. coli* strain, colony PCR is used to amplify the target region from the plasmid. DNA sequence analysis is then used to identify indels generated at the targeted genomic site.

B. Protocol Overview

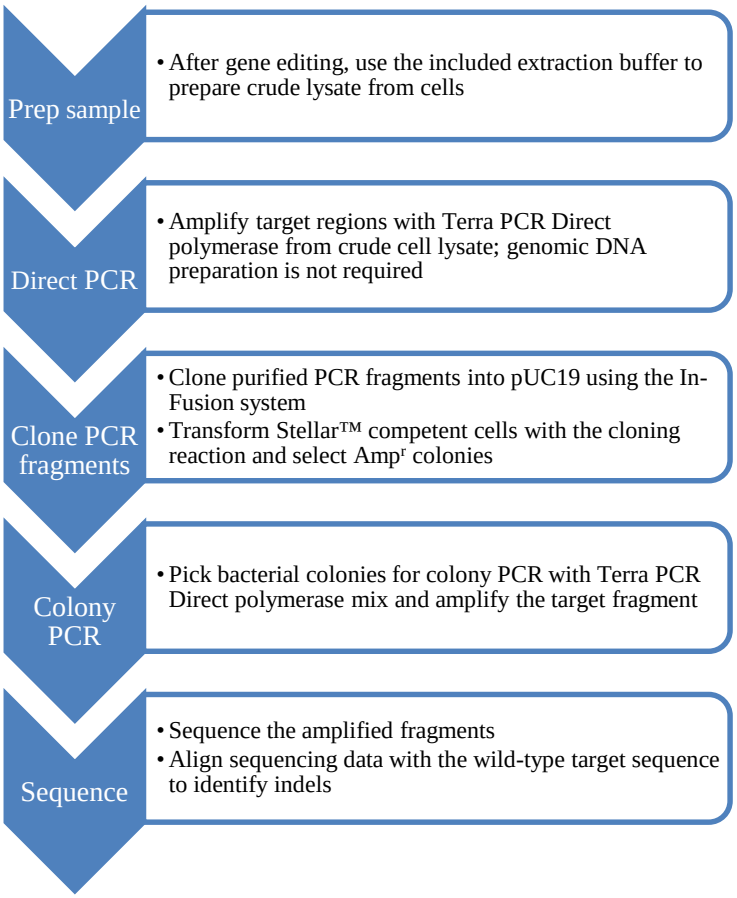

Figure 1. Protocol overview

II. List of Components

- **Guide-it Indel Identification Components (Cat. No. 631445; not sold separately)**
  - 110 µl Terra PCR Direct Polymerase Mix
  - 3 x 1 ml 2X Terra PCR Direct Buffer (with Mg^{2+}, dNTP)
  - 400 µl Extraction Buffer 1
  - 40 µl Extraction Buffer 2
  - 10 µl pUC19 Cloning Vector, linearized (50 ng/µl)
  - 200 µl Colony PCR Forward Primer (15 µM)
  - 200 µl Colony PCR Reverse Primer (15 µM)
  - 6 x 1 ml PCR-Grade Water

- **In-Fusion HD Cloning Kit (Cat. No. 639648; not sold separately)**
  - 20 µl 5X In-Fusion HD Enzyme Premix
  - 5 µl pUC19 Control Vector, linearized (50 ng/µl)
  - 10 µl 2 kb Control Insert (40 ng/µl)

- **NucleoSpin Gel and PCR Clean-Up Kit (Cat. No. 740609.10; not sold separately)**

- **Stellar Competent Cells (Cat. No. 636763)**
  - 10 tubes Stellar Competent Cells (100 µl/tube)
  - 10 tubes SOC Medium (1 ml/tube)
  - 10 µl pUC19 Vector (0.1 ng/µl)

NOTE: Terra PCR Direct Polymerase Mix (Cat. No. 639270) and the In-Fusion HD Cloning Plus Kit (Cat. No. 638909) can also be purchased separately.

III. Additional Materials Required

The following materials are required but not supplied:

- Genomic DNA-specific PCR primers with a $T_m \geq 60°C$ (see Section IV)
- PCR reaction tubes or plates
- Micropipette tips (with hydrophobic filters)
- Tabletop centrifuge
- Ampicillin (100 mg/ml stock)
- LB (Luria-Bertani) medium (pH7.0)
- LB/Ampicillin plates

IV. Primer Design for In-Fusion Cloning

The In-Fusion cloning system will allow you to join the PCR fragments with the provided pUC19 cloning vector as long as they share 15 bases of homology at each end. Therefore, PCR primers must be designed to generate PCR products containing ends that are homologous to those of the vector (Figure 2).

1. The 5’ end of each primer **must** contain 15 bases that are homologous to the 15 bases at the ends of the pre-linearized pUC19 fragment **(forward primer must contain** 5’-CGGTACCCGGGGATC; **reverse primer must contain** 5’-CGACTCTAGAGGATC**)**.
2. The 3’ end of each primer must contain sequence that is specific to the target sequence. The 3’ portion of each primer should:
    - Be specific to the genomic targeting site; the primers should amplify a 500–700 bp long genomic fragment.
    - Be between 18–25 bases in length and have a GC-content between 40–60%.
    - Have a melting temperature (T_m) of 58–65°C. The T_m difference between the forward and reverse primers should be ≤4°C to ensure successful amplification.

      NOTE: The T_m should be calculated based upon the 3’ (genome region-specific) end of the primer, and NOT the entire primer. If the calculated T_m is too low, increase the length of the genome-specific portion of the primer until you reach a T_m of 58–65°C.
    - Not contain runs of identical nucleotides. The last five nucleotides at the 3’ end of each primer should contain no more than two guanine (G) or cytosine (C) residues.
3. Avoid complementarity within each primer to prevent hairpin structures, and between primer pairs to avoid primer dimers.
4. The primer sites should be chosen so that a 500–700 bp amplicon containing the edited site is generated by PCR.
5. You can perform a BLAST search to determine if the 3’ portion of each primer is unique and specific to the target genomic site (www.ncbi.nlm.nih.gov/BLAST/).

Forward 5’-CGGTACCCGGGGATC XXX XXX XXX XXX XXX XXX…-3’
Reverse 5’-CGACTCTAGAGGATC YYY YYY YYY YYY YYY YYY…-3’

Gene-specific region (≤25 nt)

Figure 2. Structure of primers needed for In-Fusion cloning

V. Indel Identification

Read the entire protocol before beginning.

A. Protocol: Preparing Crude Lysate for PCR

1. Perform engineered nuclease-based genome editing in your desired target cells. Two to five days after gene editing, prepare crude genomic extracts:
    a. Harvest the cells from flasks, perform a cell count, and add 2 x 10^5 cells to a 0.5-ml microfuge tube.
    b. Pellet the cells by low-speed centrifugation (~1200 rpm) at 4°C; remove the supernatant.
    c. Resuspend the cell pellet in 40 µl of Extraction Buffer 1 by pipetting and vortex well.
    d. Incubate at 95°C for 10 minutes.
    e. Add 4 µl of Extraction Buffer 2 and incubate on ice for 2 minutes.
    f. Add 320 µl of PCR-grade water to dilute the sample.
2. Determine the DNA concentration using a NanoDrop spectrophotometer or an equivalent system. A total of 50–75 ng will be used for PCR (below).
3. Store the diluted crude lysate at 4°C.

NOTE: The lysate can be stored at 4°C for up to 12 months. Do not freeze the crude lysate.

B. Protocol: PCR Amplification of the Genomic Target

NOTE: Keep all reagents and solutions on ice.

1. Prepare Terra PCR Direct reactions by combining the following reagents:

Table 1. Recommended Reagent Volumes for Genome Amplification

Reagent	Amount	Final concentration
2X Terra PCR Direct Buffer (with Mg^{2+}, dNTP)[a]	25 µl	1X
Genome-targeting forward primer (15 µM) (designed following the guidelines in Section IV)	1 µl	0.3 µM
Genome-targeting reverse primer (15 µM) (designed following the guidelines in Section IV)	1 µl	0.3 µM
Diluted crude lysate (prepared above)	5 µl	50–75 ng DNA[b]
Terra PCR Direct Polymerase Mix	1 µl	1.25 U
PCR-Grade Water	17 µl	
Total volume per reaction	50 µl	

[a] A precipitate may be visible in the buffer. Mix the buffer until no precipitate is visible and the buffer is homogeneous. This may require slight warming of the tube and mixing until homogeneous.

[b] Determine the DNA concentration in the crude lysate using spectrophotometry.

2. Mix the contents of each tube by tapping the bottom of the tube, pipetting up and down, or gently vortexing. Then centrifuge briefly.
3. Program your thermal cycler with the following cycling conditions:

98°C	2 min*	
98°C	10 sec	28 cycles
68°C	1 min	

* The initial denaturation step must be performed at 98°C for 2 min in order to denature the hot-start antibody

4. Load entire PCR reaction (50 µl) on 1.5% agarose/ethidium bromide gel. Identify the desired PCR fragment and excise from the gel.
5. Purify the DNA fragment using the included NucleoSpin Gel and PCR Clean-Up Kit. A complete protocol can be found in the product user manual, which is available for download at www.clontech.com/manuals. Elute the purified PCR product in **15 µl** of Elution Buffer.

NOTE: The purified PCR product can be stored at –20°C for up to 12 months.

C. Protocol: Cloning PCR Products

1. Set up the In-Fusion cloning reaction mix:

2 µl	5X In-Fusion HD Enzyme Premix
1 µl	Linearized pUC19 Vector
7 µl	Purified PCR Fragment from step B.5 above (~100 ng)
10 µl	Total Volume

2. Vortex briefly to mix the reaction.
3. Incubate the reaction for **at 50 °C for 15 min**, then place on ice.
4. Continue to the Transformation Protocol.

NOTE: You can store the cloning reactions at –20°C until you are ready**.**

D. Protocol: Transformation

The following protocol has been optimized for transformation of the included Stellar Competent Cells.

1. Follow the protocol provided with the Stellar Competent Cells to transform 50 µl of cells with 2.5 µl of the In-Fusion cloning reaction mixture.

   IMPORTANT: DO NOT add more than 5 µl of the reaction to 50 µl of competent cells. More is not better. Using too much of the reaction mixture inhibits the transformation.

2. Add 940 µl of SOC medium to the transformation reaction and incubate with shaking (160–225 rpm) for 30 min at 37°C.
3. Spread 200 µl and 500 µl of the bacterial suspension onto respective LB/Amp plates [i.e., LB agar + Ampicillin (50–100 µg/ml)].
4. Incubate the plates overnight at 37°C.

E. Protocol: Colony PCR and Sequencing

To obtain good representation of the variety of indels in the cell population, it is recommended that at least 8 colonies are amplified and sequenced.

1. Prepare the Terra PCR Direct reactions by combining the following reagents:

Table 2. Recommended Reagent Volumes for Colony PCR

Reagent	Amount 1 reaction	Amount 9 reactions
2X Terra PCR Direct Buffer (with Mg^{2+}, dNTP)	25 µl	225 µl
Colony PCR Forward Primer (15 µM)*	1 µl	9 µl
Colony PCR Reverse Primer (15 µM)*	1 µl	9 µl
Terra PCR Direct Polymerase Mix	1 µl	9 µl
PCR-Grade Water	22 µl	198 µl
Total volume per reaction	50 µl	450 µl

*: Colony PCR Forward Primer: ACGTTGTAAAACGACGGCCAGTGA; Colony PCR Reverse Primer: CAATTTCACACAGGAAACAGCTATGACC

2. On ice, aliquot 50 µl of the master mix into nine individual PCR tubes.
3. Pick and inoculate eight tubes with an entire colony from the LB/Amp plate from Step D.4 above (one colony per tube). Resuspend the colony by pipetting well. Reserve one tube for the negative control (no colony).
4. Program your thermal cycler with the following cycling conditions:

98°C	2 min	
98°C	10 sec	20 cycles
68°C	1 min	
68°C	1 min	

5. Analyze 5 µl of the PCR reaction on a 1.5–2% agarose/ethidium bromide gel. Confirm the presence of a PCR product of the expected size.
6. Purify the remaining 45 µl of the PCR reaction using the included NucleoSpin Gel and PCR Clean-Up Kit. A complete protocol can be found in the product user manual, which is available for download at www.clontech.com/manuals. Elute the purified PCR product in **30 µl** of Elution Buffer.
7. Use 5 µl of the purified PCR product for DNA sequencing. Use the provided "Colony PCR Forward Primer" for sequencing.

NOTE: If you DO NOT column purify the PCR product before sequencing, please contact your sequencing provider/facility to determine if further purification of the PCR product is necessary.

F. Analysis of Sequencing Results

After receiving the sequencing data, align the sequence with the wild-type genomic DNA sequence using your software of choice. This alignment will not only help to determine the mutation frequency but also the nature of the mutations, such as single nucleotide deletion or substitutions upstream of the protospacer adjacent motif (PAM; the DNA sequence immediately following the Cas9-recognition site), or large deletions or insertions upstream of the PAM site. An example alignment is shown in Figure 3.

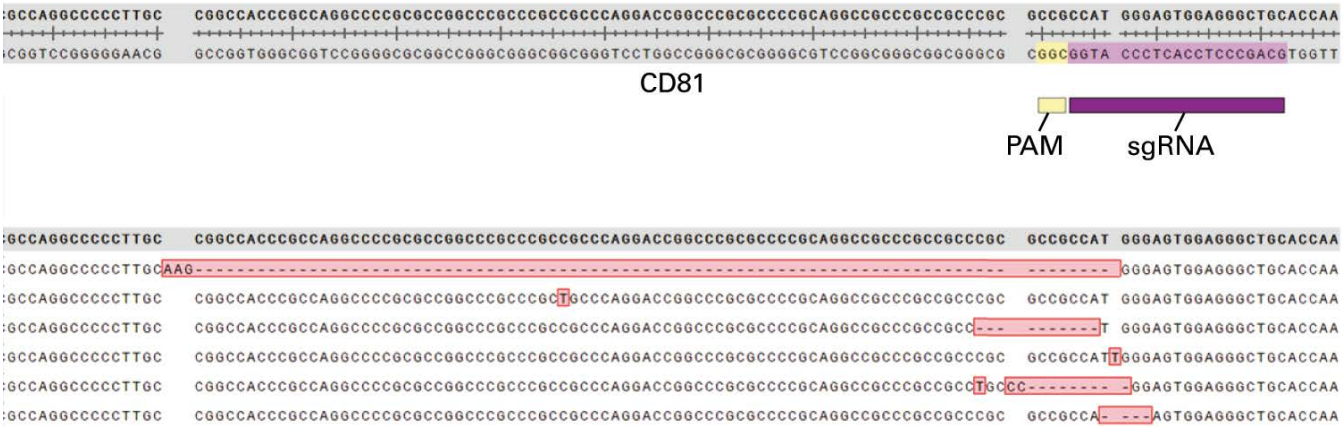

Figure 3. Sequence alignment of clones obtained from CRISPR/Cas9 targeting of CD81. HeLa cells were transfected with plasmids encoding Cas9 and an sgRNA targeting the CD81 gene. The Guide-it Indel Identification Kit was used to prepare modified CD81 target sites for DNA sequence analysis. The sequencing data was aligned with the wild-type sequence. The different clones show a broad range of indels in the CD81 gene.

Appendix A. Troubleshooting Guide

Table 3. Troubleshooting Guide

Protocol Step	Problem	Possible Explanation	Solution
PCR Amplification of the Genomic Target (Section V.B)	Amplification products are nonspecific; multiple bands or smear is obtained	Primers are annealing non-specifically; this often happens when the template contains a high GC content (>70%)	Increase the annealing stringency; use the same temperature for primer annealing and extension.
	No PCR product is obtained or band is diffuse	Primers are not optimal	Redesign your primers. Extend the gene-specific region to 25 nt.
		Too much template	Reduce the amount of template sample used.
Cloning PCR Products (Section V.C)	Low transformation efficiency	Transformed with too much of the In-Fusion reaction	Do not add more than 5 µl of the In-Fusion reaction to 50 µl of competent cells.
		Insufficient amount of insert fragment	Increase the amount of insert fragment.
Colony PCR (Section V.E)	High background obtained	Transformed clones were overgrown	Optimize the transformation time.

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