I. Introduction

The **Guide-it Mutation Detection Kit** (Cat. Nos. 631443 and 631448) contains all the reagents needed for PCR-based identification of insertions or deletions generated in the genome of cells treated with engineered nucleases such as zinc-finger nucleases (ZFNs), transcription-activator-like effector nucleases (TALENs), or CRISPR/Cas9. The first step is the amplification of the putative target sequence directly from cells or crude lysates (this kit uses Terra™ PCR Direct Polymerase Mix and Buffer, so there is no need to extract genomic DNA from your cell population prior to amplification of your target sequence). The amplicon is then melted and hybridized to form the mismatched targets for cleavage by the Guide-it Resolvase. Cleavage efficiency can then be determined by analyzing samples on an agarose gel, followed by densitometric analysis. Sufficient material is provided for 100 amplification and cleavage reactions.

II. General Considerations

A. Storage

Store all components at -20°C upon receipt.

B. Additional Materials Required

The following materials are required but not supplied:

- **Target sequence-specific PCR primers** with $T_m \geq 60°C$. Avoid using primers that contain inosine. The optimum amplicon size range is 400–800 bp. This ensures efficient amplification and good resolution with the Guide-it Resolvase.
- **PCR-grade water.** Avoid using autoclaved H_2O; the recycled steam in some autoclaves can introduce contaminants that may interfere with PCR.
- **PCR reaction tubes or plate**
- **Aerosol-resistant pipette tips**, preferably equipped with hydrophobic filters.
- **High-resolution agarose**

III. Protocol: Mutation Detection

This protocol describes how to prepare samples for amplification (Section C) and hybridization/cleavage analysis (Section D) using a concentrated cell lysate (Section B). If you wish, you may use direct amplification from cells (Section A) to quickly prescreen screen multiple samples for effective genomic modification before proceeding to Section B to obtain a more accurate assessment of the cleavage efficiency of the samples that were modified.

NOTE: Best results are observed if samples are processed and analyzed on the same day.

A. Rapid Screening of Modified Cells by Direct Amplification

NOTE: Do not perform direct amplification on cells treated with polybrene (i.e., those that have undergone lentiviral or retroviral transduction). We recommend isolation of gDNA first.

1. Cells can be collected by gently scraping a small sample from the culture surface using a 10 µl pipette tip and then adding the scraped cells directly into the PCR reaction mix (Section C).

   NOTE: Using too many cells can inhibit the PCR reaction or limit the amount of amplicon produced, making it difficult to analyze.

2. Once modification is confirmed, the entire culture can then be analyzed using the sample preparation method outlined in Section B.

B. Sample Preparation for Amplification from Concentrated Lysate

We recommend this method of sample preparation when performing a quantitative assessment of modified cells or when modification efficiency is low (<10%).

NOTE: Do not perform direct amplification on cells treated with polybrene (i.e., those that have undergone lentiviral or retroviral transduction). We recommend isolation of gDNA first.

1. Harvest 5 x 10^5–1 x 10^6 cells for analysis and add 180 µl of Extraction Buffer 1. Resuspend well by gently pipetting using a wide-bore pipette tip (sample will be viscous).
2. Incubate at 95°C for 10 min.
3. Add 20 µl of Extraction Buffer 2.
4. Mix by gently pipetting until homogeneous, using a wide-bore pipette tip (sample may be viscous).
5. Add 2 µl of the lysate from Step 4 to 14 µl of PCR-grade water.
6. Vortex briefly to mix.

NOTE: depending upon the efficiency of amplification, a range of dilutions (1:2–1:16) may be required to achieve a clear, single amplicon.

C. Amplification of Mutated Region

Primer Design Guidelines

- The optimum amplicon size range is 400–800 bp. This ensures efficient amplification and good resolution with the Guide-it Resolvase.
- We recommend primer placement such that cleavage will result in clear separation of the cleavage products (i.e., a fragment size difference greater than 100 bp).
- Gene-specific PCR primers should have a $T_m \geq 60°C$. Avoid using primers that contain inosine.

PCR Reaction Setup

1. Thaw and mix the reagents. Before adding them to the reaction tubes, make sure each reagent is homogeneous by vortexing at low speed or pipetting up and down until it is thoroughly mixed.
2. Prepare a master mix for all reactions plus one by combining all the reagents shown in Table I in a PCR tube on ice.

Table 1. Guide-it PCR Reaction Volumes

Reagent	Amount	Final Concentration
2X Terra PCR Direct Buffer (with Mg^{2+} and dNTPs)[a]	25 µl	1X[b]
Forward Primer	15 pmol	0.3 µM
Reverse Primer	15 pmol	0.3 µM
Sample (cells or diluted lysate)	<5 µl	
Terra PCR Direct Polymerase Mix	1 µl	1.25 U
PCR-grade water	to 50 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 it is homogeneous. This may require slightly warming the tube and mixing until homogeneous.

[b] The final concentration of Mg^{2+} is 2 mM and the final concentration of each dNTP is 400 µM.

3. Mix the contents of each tube by tapping the bottom of the tube, then centrifuge briefly.
   - **3-Step PCR** (for amplification of standard targets < 2 kb):

     98°C 2 min*
     98°C 10 sec
     60°C 15 sec
     68°C 1 min/kb
     ] 35 cycles

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

   - **2-Step PCR** (for amplification of targets that are GC-rich or ≥ 2 kb):

     98°C 2 min*
     98°C 10 sec
     68°C 1 min/kb
     ] 35 cycles

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

4. Analyze 5–10 µl of the PCR product on a 2% agarose gel to ensure that a single amplicon was obtained. If not, optimize PCR conditions, including redesign of primers.

D. Cleavage of Mismatched Heteroduplexes

We recommend performing the DNA hybridization in this protocol using a final PCR product that has a concentration of >20 ng/µl (estimated using a quantitative marker on gel), and contains little to no background, including primer dimers. Gel purification of the amplicon is only necessary if a single clean band cannot be amplified. In this case, we recommend using the Nucleospin Gel & PCR Clean-up Kit (Cat. No. 740609.50).

1. Mix 10 µl of your PCR product (from Section C) and 5 µl of PCR-grade water in a fresh PCR tube.

2. Add 15 µl of positive control DNA to a fresh PCR tube. Prepare duplicate tubes for controls either with or without Resolvase.

3. Perform DNA hybridization as follows:

95°C	5 min
95°C–85°C	2°C/sec*
85°C–25°C	0.1°C/sec*
Cool to 4°C	

   * Decrease temperature at this rate.

4. Add 1 µl of Guide-it Resolvase, and incubate at 37°C for 15 min.

5. Run entire reaction on a gel.

6. Determine the cleavage efficiency using a gel imaging system and gel analysis software to determine the relative proportions of the bands produced.

7. Expected results (shown in Figure 1):
   - The positive control containing the Resolvase will generate bands of 470 bp, 290 bp, and 180 bp. The control without Resolvase will generate a single band of 470 bp.

- In most cases where the cleavage efficiency is <100%, three bands should be observed—one band containing the uncut amplicon, and two bands resulting from cleavage of the amplicon.
- If smearing is observed, reduce the *amount* of amplicon used in the cleavage reaction by dilution in PCR-grade water, but make sure that the total amplicon *volume* is maintained at 10 µl.

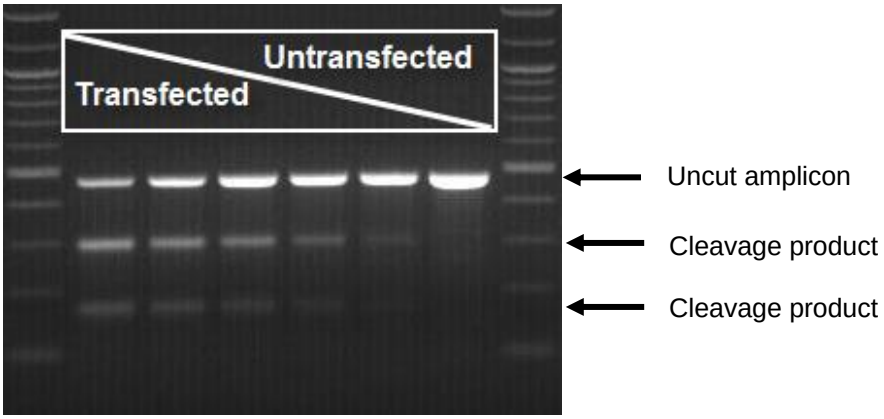

Figure 1. Analysis of cleavage products. 293T cells were transfected with plasmids encoding Cas9 and a sgRNA specific for the AAVS1 locus using Xfect™ Transfection Reagent. Transfected cells were mixed at varying ratios with untransfected cells and an amplicon containing the targeted AAVS1 locus was generated and subsequently cleaved using the Guide-it Resolvase according to this protocol. Cells which had undergone cleavage and subsequent NHEJ-based repair were easily discernable using this assay.

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