I. Introduction

The CRISPR/Cas9 system can be used to generate insertions or deletions (indels) in mammalian cells that can result in gene knockouts. In most cells, there are two copies of any given gene, and indels can be generated in either one or both alleles. The Guide-it Genotype Confirmation Kit is used after gene editing to determine if a given clone has mutations in one allele (monoallelic), both alleles (biallelic), or is unchanged (wild type). The protocol involves amplification of the target site and *in vitro* cleavage with Cas9 and the sgRNA used for the original CRISPR/Cas9 gene editing experiment. If indels are present at the target site, the original sgRNA/Cas9 complex will be unable to cleave the site, whereas wild-type alleles will be recognized and cleaved (Figure 1). This kit avoids the laborious process of screening a large number of clones for those with the desired genotype. Each kit contains sufficient reagents for 100 extraction, amplification, and cleavage reactions (i.e., one 96-well plate).

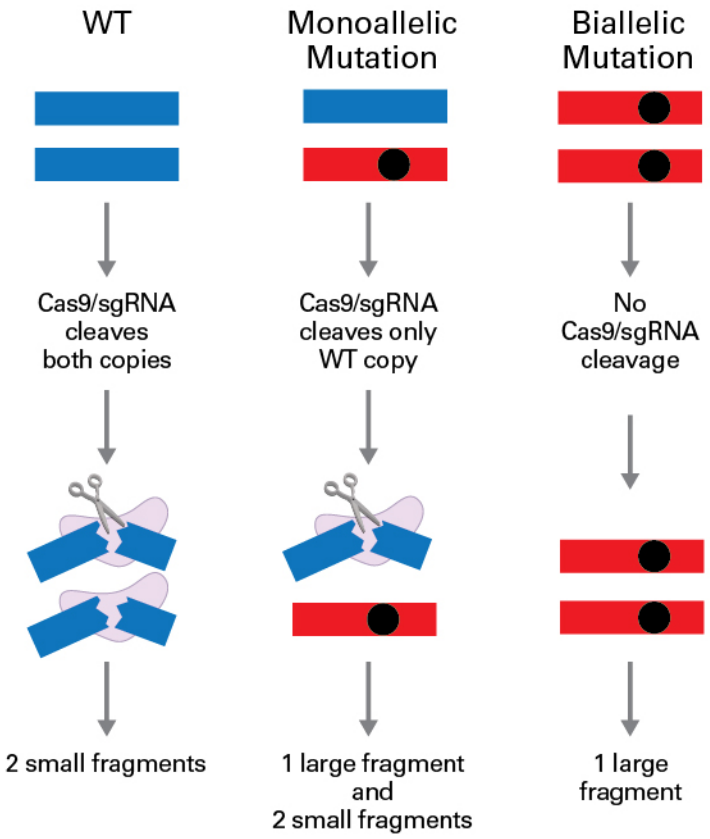

Figure 1. Principle of the Guide-it Genotype Confirmation Kit. If indels are present at the target site (black circle), the original Cas9-sgRNA complex will be unable to cleave the site; in contrast, wild-type alleles (blue) will be recognized and cleaved. The three different cleavage patterns can be distinguished by agarose gel electrophoresis.

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:

- Guide-it sgRNA *In Vitro* Transcription Kit (Cat. No. 632635)
- Target-sequence-specific PCR primers
- 200-µl PCR reaction tubes or plates

III. Protocol

A. *In Vitro* Transcription of the sgRNA

NOTE: The same sgRNA that was used for gene editing in your target cells should be used for the genotype confirmation reaction.

We recommend using the Guide-it sgRNA *In Vitro* Transcription Kit (Cat. No. 632635) for *in vitro* transcription of sgRNAs. Please refer to this product's user manual for a detailed protocol for sgRNA production. After production, sgRNAs should be diluted in RNase-free Water to a final concentration of 50 ng/µl before use in the genotype confirmation assay.

B. Target DNA Amplification from Modified Cells

1. Extraction of genomic DNA

- After CRISPR/Cas9 editing, isolate and grow single cells (i.e., clonal isolation); cells that are 50–90% confluent are preferred.

NOTE: Be sure to make a replica plate for other analyses or freezer stocks.

a) Individual sample method

In this method, cells are harvested from the wells after clonal isolation and prior to crude DNA extraction.

1. Harvest cells from individual wells and add an appropriate volume of Extraction Buffer 1 to the cell pellet depending on the plate type used (refer to Table I, next page); vortex briefly.
2. Incubate at 95°C for 10 min.
3. Add an appropriate volume of Extraction Buffer 2 (refer to Table I, next page); vortex briefly.
4. Dilute the lysate 1:9 with RNase-free Water (e.g., 5 µl lysate + 45 µl RNase-free Water)

NOTE: Depending on the amplification efficiency of the target, a range of dilutions (from undiluted to 1:50) might be required to achieve clear amplification results.

5. The lysate contains genomic DNA and will be used directly for PCR.

b) Plate method

In this method, crude DNA extracts are prepared directly from cells in the plate wells.

1. Remove the medium from the wells of the plate. Add an appropriate volume of Extraction Buffer 1 (refer to Table I below).
2. Incubate at 70°C for 10 min.
3. Vortex for 5 min.
4. Add an appropriate volume of Extraction Buffer 2 (refer to Table I below) and vortex for 30 sec.
5. Collect the lysate into a fresh tube.
6. Dilute the lysate 1:9 with RNase-free Water (e.g., 5 µl lysate + 45 µl RNase-free Water)

NOTE: Depending on the amplification efficiency of the target, a range of dilutions (from undiluted to 1:50) might be required to achieve clear amplification results.

7. The lysate contains genomic DNA and will be used directly for PCR.

Table I. Reference volumes for genomic DNA extraction.

Plate type (wells)	96	48	24	12
Extraction Buffer 1	36 µl	90 µl	180 µl	360 µl
Extraction Buffer 2	4 µl	10 µl	20 µl	40 µl

2. PCR amplification

Design primers to amplify the region targeted by CRISPR/Cas9. The optimal amplicon size is 600–800 bp, with the sgRNA target sequence located asymmetrically within the amplicon; each cleavage fragment should be at least 250 bp, and there should be >100 bp difference in size between the fragments after Cas9 cleavage (Figure 2). These guidelines ensure efficient amplification and good assay resolution.

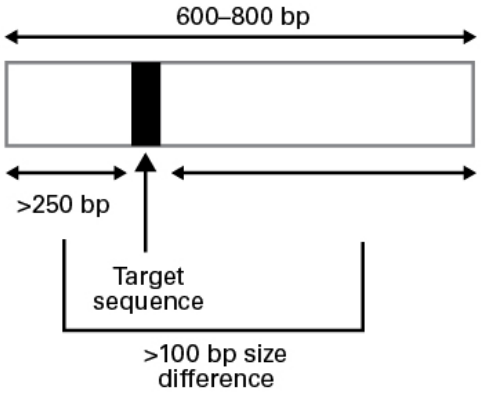

Figure 2. PCR amplicon design.

1. Combine the following components in a 200-µl PCR tube:

25 µl	2X Terra™ PCR Direct Buffer (with Mg^{2+},dNTP)
1.5 µl	Forward primer (10 µM)
1.5 µl	Reverse primer (10 µM)
1.0 µl	Terra PCR Direct Polymerase Mix (1.25 U/µl)
19.0 µl	RNase-Free Water
2.0 µl	Prepared lysate
50 µl	Total volume

NOTE: If you have multiple samples, make a master mix, excluding the lysate.

2. Perform PCR using the following reaction conditions:

98°C	2 min	
98°C	10 sec	30–40 cycles
60°C	15 sec	
68°C	1 min/kb	
4°C	hold	

NOTE: Adjustments to cycling conditions may be necessary depending on your target fragment.

3. Use 5 µl of the PCR product solution for agarose gel electrophoresis; run the sample on a 1.5–2% agarose gel.

NOTE: Make sure that PCR yields a strong, single band; 100–250 ng of PCR product (in a volume <5 µl) will be required for the subsequent Cas9 cleavage assay. Using this amount ensures that the bands remain easily visible after the cleavage assay.

C. Cas9 Cleavage Assay

1. Combine the following components in a 200-µl PCR tube:

1.0 µl	Target-specific sgRNA or Control sgRNA (50 ng/µl)
0.5 µl	Guide-it Recombinant Cas9 Nuclease (500 ng/µl)
1.5 µl	Total volume

NOTE: If you have multiple samples, make a master mix.

2. Mix well, very gently by pipetting, and incubate using a thermal cycler with the following conditions:

37°C	5 min
4°C	hold

3. Combine the following components in a 200-µl PCR tube in the order listed. If you have multiple samples, make a master mix.

Up to 5.0 µl	PCR reaction solution (100–250 ng) or Control Fragment
1.0 µl	15X Cas9 Reaction Buffer
1.0 µl	15X BSA
6.5 µl	RNase-Free Water
1.5 µl	Cas9/sgRNA mix (from Step 2 above)
15 µl	Total volume

NOTE: The volume of PCR sample used should not exceed 5 µl; carryover of PCR reaction buffer might inhibit Cas9 activity. The optimal range of DNA input is 100–250 ng. Always include a positive control (i.e., the wild-type target fragment and the target-specific sgRNA).

4. Mix well, very gently by pipetting, and incubate using a thermal cycler with the following conditions:

37°C	1 hr
80°C	5 min
4°C	hold

5. Add an appropriate volume of loading buffer, and run the entire sample on a 1.5–2% agarose gel along with an appropriate DNA marker.

NOTE: The sgRNA (~140 bases) may also be visible after agarose gel electrophoresis. If this interferes with assay interpretation, the reaction samples can be treated with RNase (e.g., 5 µg RNase for 30 min at 37°C) prior to loading on the gel.

6. Analyze the results. Refer to Figure 3 for assay interpretation.

NOTE: The control fragment included in this kit has a size of 614 bp; it is cleaved by the control sgRNA/Cas9 complex to form two fragments that are 350 bp and 264 bp, respectively.

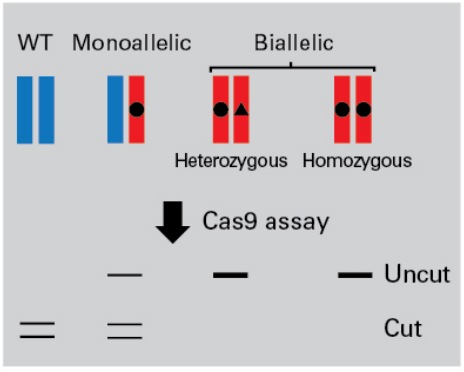

Figure 3. Interpretation of genotype confirmation assay results. In the case of a wild-type (WT) genotype, both alleles will be cleaved by the Cas9-sgRNA complex resulting in 2 small bands on an agarose gel. However, for mutant cells, different banding patterns will be present depending on the genotype. For monoallelic mutants, only the amplified WT allele will be cleaved, resulting in two small bands and one large uncut band. For biallelic mutants, neither amplified allele will be cleaved resulting in a single large band on the gel.

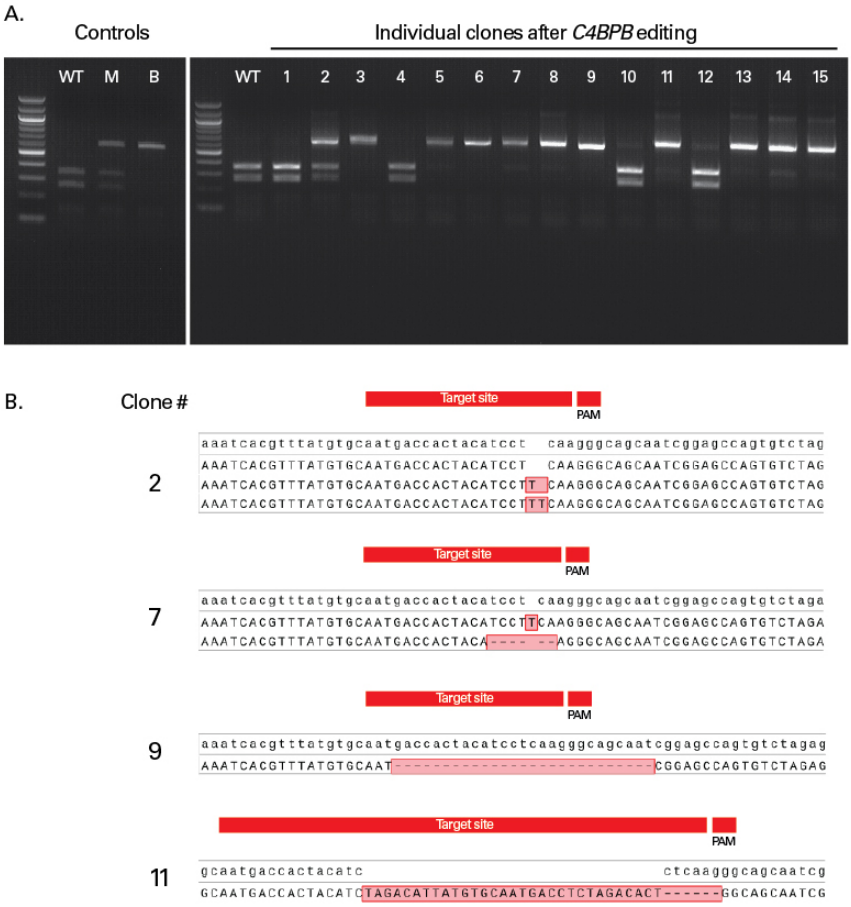

Figure 4. Determination of genotype at the *C4BPB* locus after CRISPR/Cas9 editing. In this experiment, HEK 293 cells were treated with Cas9 and a sgRNA targeting the *C4BPB* gene. Fifteen single-cell clones were generated, and the Guide-it Genotype Confirmation Kit was used to determine the genotype at the *C4BPB* target site (Panel A, right). Wild-type (WT), monoallelic (M), and biallelic (B) control reactions were included in the analysis (Panel A, left). The results indicated that clones 1, 4, 10, and 12 were wild-type; clone 2 was monoallelic; and clone 3, 5–9, 11, and 13–15 were biallelic. Select clones from the top panel were sequenced, and aligned sequences for representative clones are shown in Panel B, with the WT sequence in lowercase letters. For clones identified as biallelic in the genotype confirmation assay (Panel A), sequencing indicated that clone 7 is heterozygous and clones 9 and 11 are homozygous. For clone 2, three different alleles were detected (a mixture of WT and mutant alleles); it is possible that this result stems from copy number variation in the HEK 293 cell line.

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