Screen It™ CRISPR Cas9 Cleavage Detection Kit

Product Manual

Cat. G990

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Introduction

abm's Screen It™ CRISPR Cas9 Cleavage Detection Kit is a robust and precise RNA-dependent assay used to identify successful insertions and/or deletions (indels) of genomic DNA following a CRISPR-Cas9 experiment. CRISPR-Cas9 can introduce indels on either one or multiple copies of a given mammalian gene. The Screen It™ CRISPR Cas9 Cleavage Detection Kit can easily identify if a given clone is monoallelic (has mutations in one copy), biallelic (all copies mutated) or is unedited (Wild-Type), which is considered a major screening advantage over the standard T7E1 Surveyor Assay. This kit uses PCR to first amplify the sgRNA target site from Wild-Type or CRISPR-Edited cells; this amplicon is subsequently cleaved by a ribonucleoprotein (RNP) complex, which generates a unique pattern that can be easily resolved on agarose gel and interpreted by the user. Using this kit after a gene editing experiment reduces the time and effort spent from the arduous screening process and provides clear genotyping results.

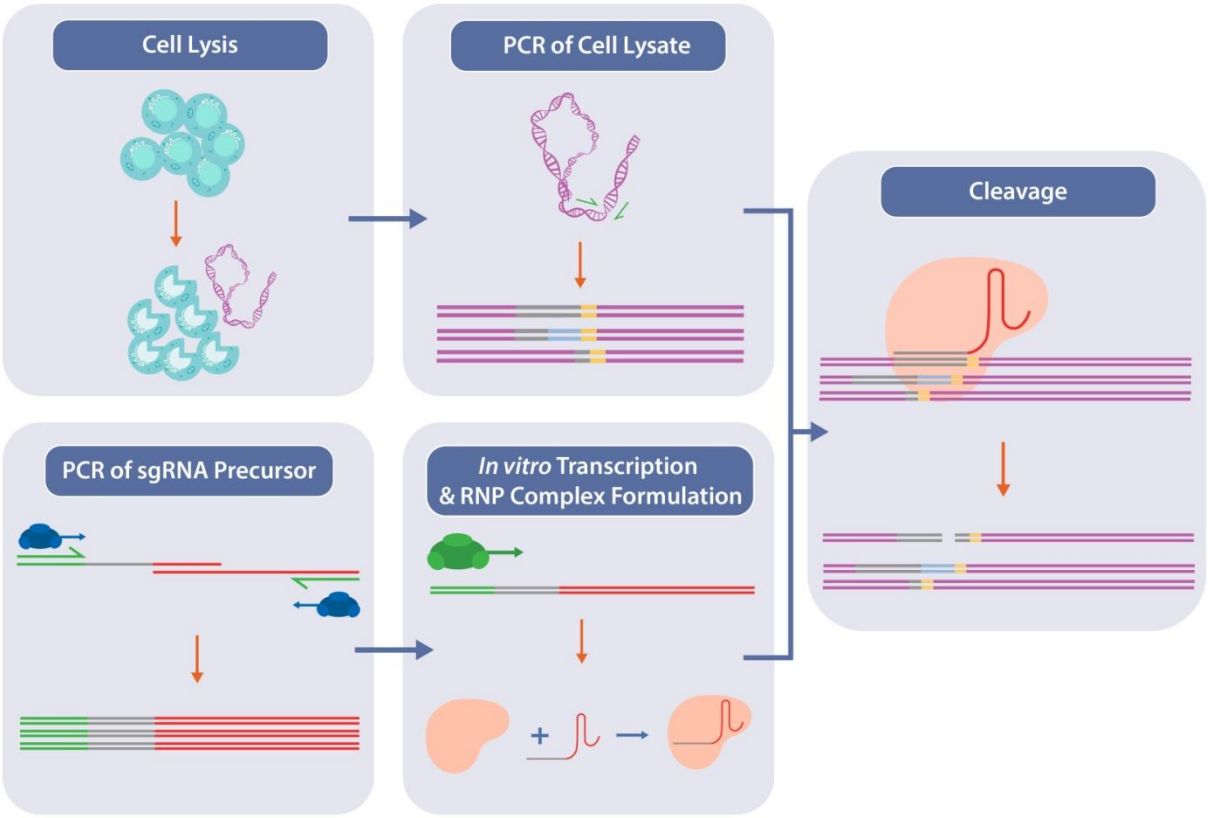

Figure 1. Overview of Screen It™ CRISPR Cas9 Cleavage Detection Kit. Wild-Type and CRISPR-Edited cells are initially lysed and the sgRNA target region is amplified using the cell lysates as a template. Subsequently, sgRNA precursor is PCR amplified and the PCR product is subjected to *in vitro* transcription. The synthesized sgRNA is fused with an spCas9 protein and the resulting RNP complex is used to cleave Wild-Type PCR fragments previously amplified. CRISPR-Edited PCR fragments would remain uncut showing a distinct difference between Wild-Type and CRISPR-Edited cells.

Components

Product Component	Quantity	Part No.
Cell Lysis Buffer	1.25 ml	P115
Protein Degrader	100 µl	P116
Scaffold Template and Primer Mix	100 µl	P990-1
2X sgRNA Synthesis Buffer	100 µl	P990-2
sgRNA Synthesis Enzyme Mix	50 µl	P990-3
sgRNA Control Oligo	20 µl	P990-4
Wild Type Control Primer and Template Mix	20 µl	P990-5
Monoallelic Control Primer and Template Mix	20 µl	P990-6
Biallelic Control Primer and Template Mix	20 µl	P990-7
RNP Degrader	100 µl	P990-8
spCas9 Nuclease Protein	250 µl	K143
10X Cas9 Reaction Buffer	1.25 ml	K000
MegaFi™ Pro Fidelity 2X PCR MasterMix	2 x 1.25 ml	P887-1

Additional Materials Required

Material	Required For	Notes
CRISPR-Edited Cell pellets	Part A	Pellets should contain 1 - 4 x 10^4 cells
Wild-Type Cell pellets	Part A	Pellets should contain 1 - 4 x 10^4 cells
Target-Specific Primers	Part B	Primer design parameters in Page 4
sgRNA Target-Specific Oligo	Part C	Oligo design parameters in Page 5
Nuclease-Free Water	Part A-D	

Storage

Store all components at -20°C in a non-frost-free freezer. sgRNA synthesized in Part C (sgRNA Target-Specific Oligo) can be stored in -20°C for short-term storage or -80°C for long-term storage. All components are stable for 1 year from the date of shipping when stored and handled properly.

Target-Specific Primer Design

Screen It™ determines genomic DNA editing through *in vitro* cleavage of the PCR-amplified sgRNA target region. PCR primers need to be designed and synthesized prior to the experiment. Primers should amplify the sgRNA target region and the spCas9 cut site (3 base pairs upstream of the Protospacer Adjacent Motif [PAM] sequence) should not be positioned in the middle of the PCR amplicon in order to show a distinct cleavage pattern (Fig 1). To run the same PCR as the controls, design primers to amplify 500 bp to 1000 bp of the sgRNA target region with annealing temperatures at 55°C.

***Note: abm** offers sgRNA PCR Primer Pair Design & Synthesis Service (Cat. No. **C336**).*

Prepare 10 µM primer mix and store at -20°C prior to use in Part B.

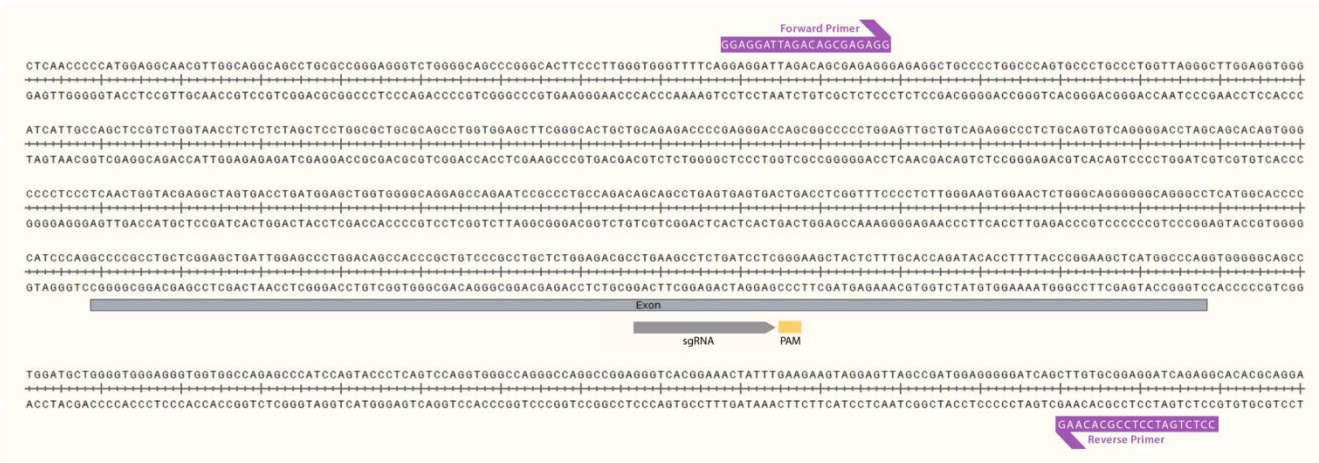

Figure 2. Sample primer design for amplifying the sgRNA target region. sgRNA target should not be positioned in the middle of the amplicon to show a discernible cleavage.

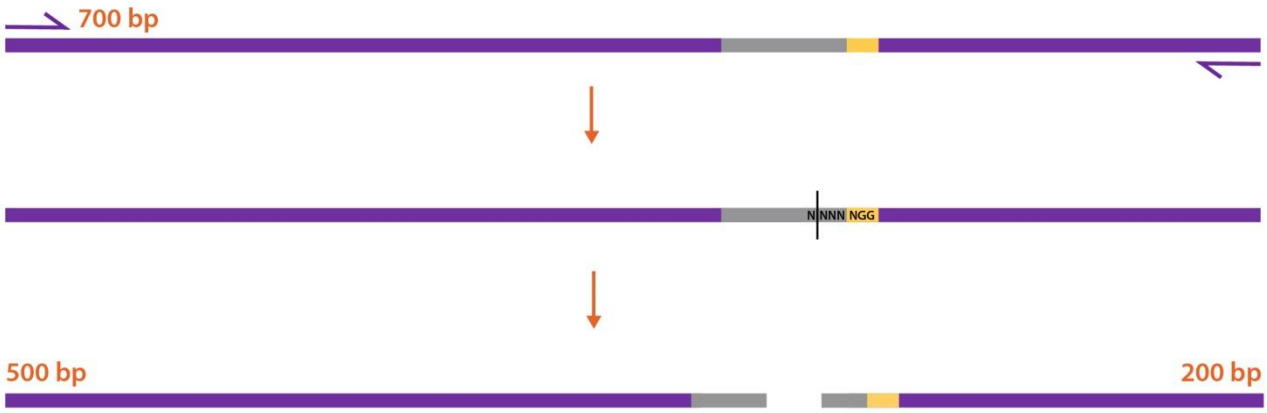

Figure 3. Sample 700 bp PCR product from Figure 2 showing sgRNA (gray) and PAM (yellow) being cleaved at the cut site showing 2 distinct bands 500 bp and 200 bp.

Target-Specific Oligo Design

The sgRNA *in vitro* transcription template is generated via oligo annealing and extension using the complementary sgRNA Scaffold Template and Primer Mix included in the kit. Input the specific sgRNA sequence used in your CRISPR Knock-out experiment between the **T7 Promoter** and the **Scaffold Overlap**:

TTCTAATACGACTCACTATAGGGNNNNNNNNNNNNNNNNNNNN**GTTTTAGAGCTAGAAATAGCAAG**

Resuspend the DNA oligo to a final concentration of 100 µM. Prepare 10 µM aliquot and store at -20°C prior to use in Part C.

Note: abm *offers sgRNA Target-Specific Oligo Design & Synthesis Service (Cat. No.* ***C337****).*

Protocol

Part A – Cell Lysis

Following your CRISPR/Cas9 editing experiment, isolate monoclones and grow selected cells in 96-well plates to reach 70–90% confluency to collect for lysis. Wild-Type and CRISPR-Edited cell lysates are used as the template for PCR amplification. Carry out a simple 2-step cell lysis using a combination of Cell Lysis Buffer (Part No. **P115**) and Protein Degrader (Part No. **P116**)

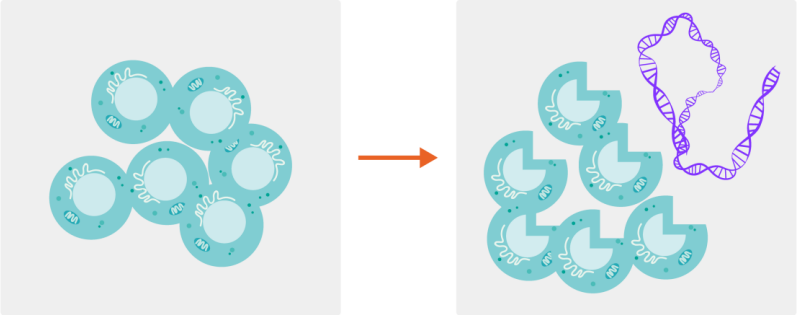

Figure 4. Genomic DNA is released from cells using a 2-step cell lysis. Lysates can be effectively used as PCR templates.

1. Pellet CRISPR-Edited and Wild-Type cells when confluency reaches 70-90% in a 96-well plate (approximately 1-4 × 10^4 cells) and discard supernatant.
2. In an eppendorf tube, mix the following components; adjust volumes accordingly depending on the number of cell pellets being tested.

Component	Volume per cell pellet
Protein Degrader	1 µl
Cell Lysis Buffer	10 µl
Nuclease-free Water	39 µl

3. Transfer 50 µl of the mixture into each cell pellet and gently resuspend cells.
4. Incubate at 68°C for 15 minutes followed by inactivation at 95°C for 10 minutes. The lysates can be used directly as PCR templates.

Part B – PCR

The Screen It™ Kit uses a robust polymerase, MegaFi™ Pro 2X PCR MasterMix (Part No. **P887-1**). MegaFi™ Pro boasts high fidelity and ultra-low error rates (over 2,000X less than Taq polymerase, representing the lowest error rate on the market), making it incredibly useful for analyzing CRISPR-editing. The MasterMix is especially formulated to work on impure templates, ultimately reducing preparation time. **abm** also offers MegaFi™ Pro 2X PCR MasterMix separately (Cat. No. **G887**).

The Screen It™ kit also features 3 controls: Wild-Type Control, Monoallelic Control, and Biallelic Control. These control primer and template mixes (Part No.'s **P990-5**, **P990-6**, **P990-7**) will demonstrate how each particular edit is represented in the assay.

1. Prepare the following mixes on ice:

Component	Wild Type Control	Monoallelic Control	Biallelic Control	Target-Specific[1]
Wild Type Control Primer and Template Mix	1 µl	-	-	-
Monoallelic Control Primer and Template Mix	-	1 µl	-	-
Biallelic Control Primer and Template Mix	-	-	1 µl	-
Target-Specific Primers (10 µM)	-	-	-	1 µl
Cell Lysate (Wild-Type cells or CRISPR-Edited cells)	-	-	-	1 µl
2X MegaFi™ Pro Fidelity PCR MasterMix	12.5 µl	12.5 µl	12.5 µl	12.5 µl
Nuclease-free Water	11.5 µl	11.5 µl	11.5 µl	10.5 µl
Total	**25 µl**	**25 µl**	**25 µl**	**25 µl**

[1] Target-Specific column is only for 1 reaction. Adjust the volumes accordingly depending on number of samples being tested.

2. Perform PCR amplification as follows:

Step	Temperature	Time	Cycle
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	15 sec	35
Annealing[2]	55°C for controls	15 sec	
Extension[2]	72°C	15 sec (for controls) 2-3 kb/min	
Final Extension	72°C	5 min	1

[2] Adjust Annealing Temperature and Extension Time for your Target-Specific Primers.

3. Run 5 µl of each product on a 2% agarose gel. Confirm correct size of Target-Specific amplicon (variable) and each Control amplicon (expected size is 503 bp). Keep the remaining PCR products on ice or at -20°C until Part D.
4. If non-specific amplifications occur, repeat the PCR using a Touchdown method (i.e. annealing temperature + 10°C in the first 5 cycles, then annealing temperature + 5°C in the following 5 cycles and keep the annealing temperature for the remaining 25 cycles).
   Note: Ensure that PCR results in a strong, single band; 100–250 ng/µl concentration. Continuing the protocol with multiple non-specific amplicons may impede the final interpretation of the screening results.

Part C – sgRNA Synthesis

sgRNA precursor DNA is initially amplified using a combination of MegaFi™ Pro Fidelity 2X PCR MasterMix, Scaffold Template and Primer Mix (Part No. **P990-1**), sgRNA Control Oligo (Part No. **P990-4**), and the Target-Specific sgRNA oligo previously synthesized.

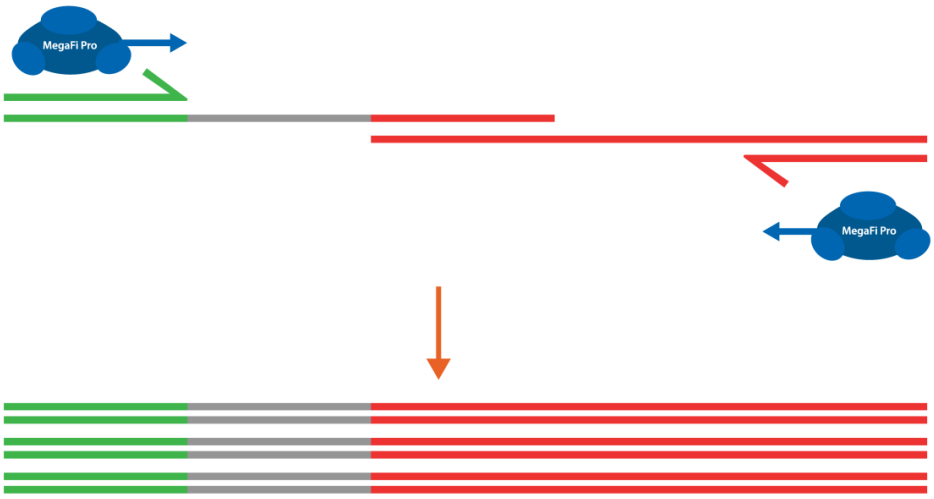

Figure 5. PCR amplification of Control/Target-Specific sgRNA oligo using MegaFi Pro Fidelity 2X PCR MasterMix forming a double-stranded sgRNA precursor DNA. Sequence would include T7 Promoter (Green), sgRNA (Grey), and Scaffold (Red).

1. Prepare the following reactions on ice:

Component	Control	Target-Specific
sgRNA Control Oligo	1 µl	-
Target-specific Oligo (10 µM)	-	1 µl
Scaffold Template and Primer Mix	1 µl	1 µl
MegaFi™ Pro Fidelity 2X PCR MasterMix	12.5 µl	12.5 µl
Nuclease-free Water	10.5 µl	10.5 µl
Total	**25 µl**	**25 µl**

2. Perform PCR Amplification as follows:

Step	Temperature	Time	Cycle
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	15 sec	35
Annealing	55°C	15 sec	
Extension	72°C	15 sec	
Final Extension	72°C	5 min	1

3. Run 5 µl of each product on a 2% agarose gel and expect 130 bp amplicons for both the control and target-specific sets.

sgRNA is directly synthesized from the PCR products using an sgRNA Synthesis Enzyme Mix (Part No. **P990-3**) and 2X sgRNA Synthesis Buffer (Part No. **P990-2**). The sgRNA Synthesis Enzyme Mix uses an optimized combination of T7 RNA Polymerase (Cat No. **E041**), RNaseOff Inhibitor (Cat. No. **G138** and Cat.No. **G591**), and Inorganic pyrophosphatase which transcribes a robust and stable sgRNA.

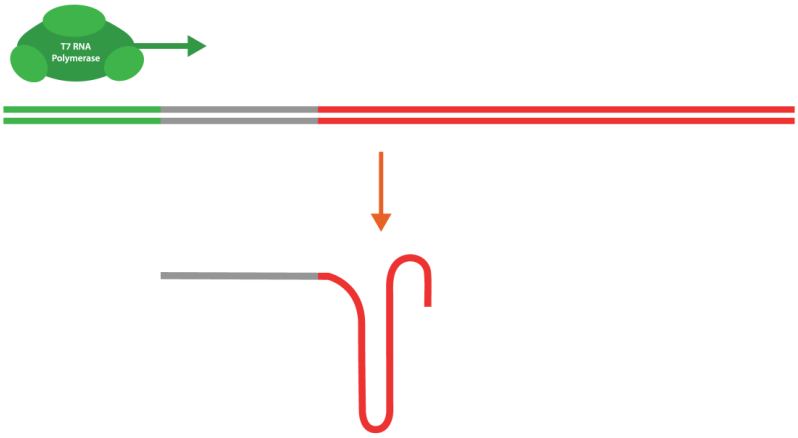

Figure 6. A single-stranded sgRNA is synthesized from the double-stranded DNA precursor.

4. After correct bands are confirmed, prepare the following mix on ice:

Component	Control sgRNA	Target-Specific sgRNA
sgRNA Control Oligo PCR Product	4 µl	-
Target-Specific Oligo PCR Product	-	4 µl
sgRNA Synthesis Enzyme Mix	4 µl	4 µl
2X sgRNA Synthesis Buffer	10 µl	10 µl
Nuclease-free Water	2 µl	2 µl
Total	**20 µl**	**20 µl**

5. Mix and centrifuge briefly. Incubate reactions at 37°C for 30 minutes to transcribe sgRNA.
6. Products can be used directly or stored at -80°C for long-term storage.

Part D – *In vitro* Cas9 Cleavage

spCas9 is initially fused with the sgRNA synthesized in Part C to form a ribonucleoprotein (RNP) complex. The complex will be used to introduce a double-stranded break on the PCR products amplified from Part B.

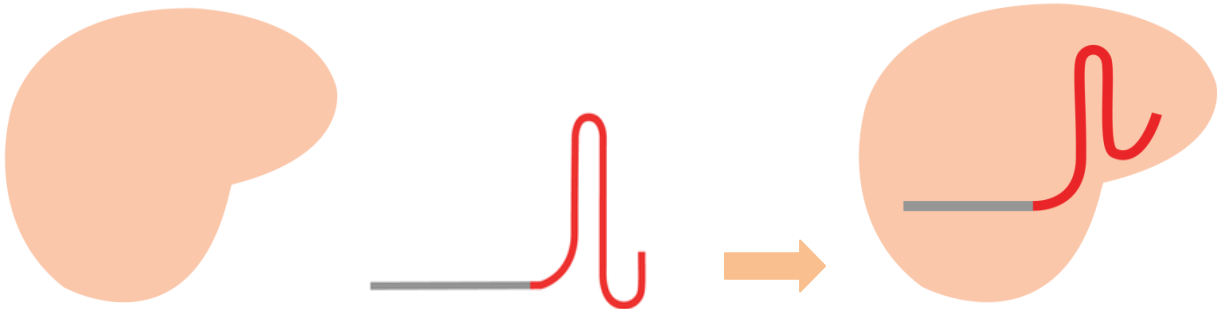

Figure 7. spCas9 Nuclease Protein (Part No. K143) is fused with sequence-specific sgRNA synthesized in Part C forming the Ribonucleoprotein (RNP) complex

1. Prepare the following reactions on ice:

Component	Control RNP Complex (Volume x 4)[3]	Target-Specific RNP Complex[4] (Volume x 1)
Control sgRNA	4 µl	-
Target-Specific sgRNA	-	1 µl
spCas9 Nuclease Protein	8 µl	2 µl
Nuclease-free Water	28 µl	7 µl
Total	**40 µl**	**10 µl**

[3] Single reaction volume is 10 µl. The column is to be used for the 3 controls.

[4] Target-Specific RNP complex mix recipe is for 1 reaction only. Adjust volumes accordingly depending on the number of pellets being tested.

2. Mix and centrifuge briefly. Incubate reactions at 37°C for 10 minutes to assemble RNP complexes.
3. Prepare the following reactions on ice:

Component	Wild Type Control	Monoallelic Control	Biallelic Control	Target Specific[5]
Wild Type Control PCR product from Part B	4 µl	-	-	-
Monoallelic Control PCR product from Part B	-	4 µl	-	-
Biallelic Control PCR product from Part B	-	-	4 µl	-
Target-Specific PCR product from Part B	-	-	-	4 µl
10X Cas9 Reaction Buffer	2 µl	2 µl	2 µl	2 µl
Control RNP Complex from Part D	10 µl	10 µl	10 µl	-
Target-Specific RNP Complex from Part D	-	-	-	10 µl
Nuclease-free Water	4 µl	4 µl	4 µl	4 µl
Total	**20 µl**	**20 µl**	**20 µl**	**20 µl**

[5] Volume is only for 1 reaction. Prepare separate reactions for every target-specific (Wild-Type/CRISPR-Edited) samples.

4. Mix and centrifuge briefly. Incubate at 37°C for 1 hour followed by 95°C for 5 minutes.
5. Add 1 µl of RNP Degrader into each tube and pipette to mix. Incubate at 68°C for 5 minutes.
6. Run the assay products on a 2% agarose gel and evaluate cleavage patterns.

Part E – Analysis of Cleavage Products

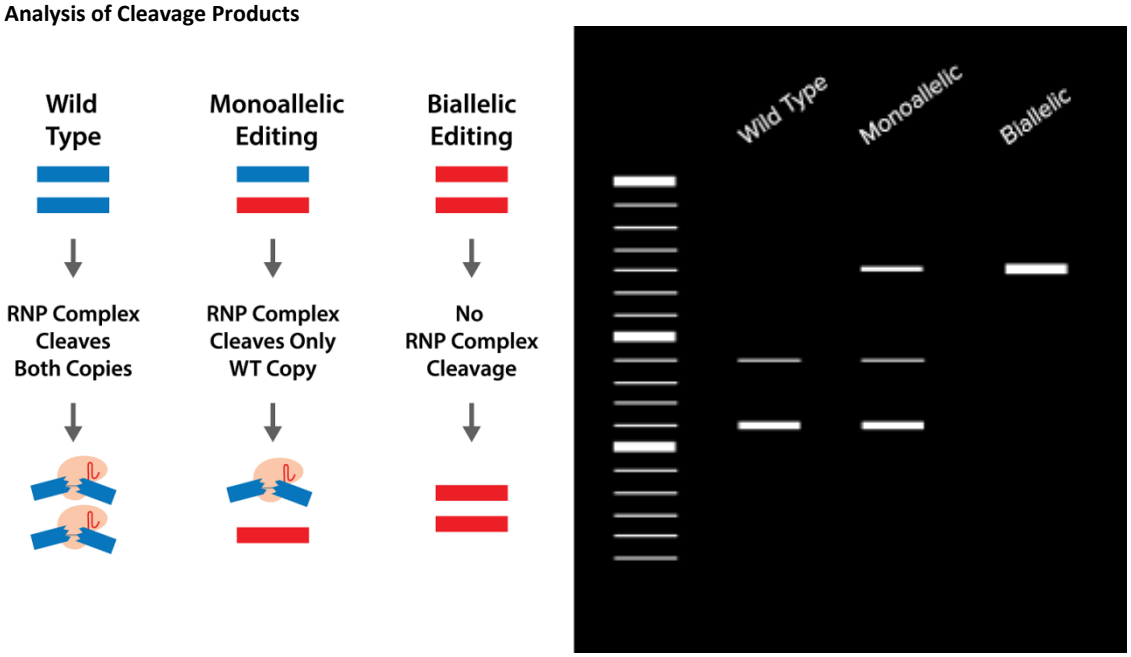

Figure 8. RNP complex introduces a double-stranded break on Wild-Type sample (Blue) but not on CRISPR-Edited samples (Red). Subsequent cleavage will show one cleaved band for Wild-Type (2 fragments), one uncleaved and one cleaved band for Monoallelic editing (3 fragments) and one uncleaved band for Biallelic editing (1 fragment).

Analyze controls: controls should yield the following cleavage patterns:

Sample	Expected Bands
Wild-Type Control	294 bp + 209 bp
Monoallelic Control	503 bp + 294 bp + 209 bp
Biallelic Control	503 bp

Target-specific samples - evaluate cleavage patterns based on PCR product length and sgRNA placement. spCas9 cleaves DNA 3 bases upstream of the PAM sequence.

- **Wild-Type & Unedited sample**: one cleavage event resulting in 2 bands.
- **Monoallelic edited sample**: a mixture of cleaved and uncleaved resulting in 3 bands.
- **Biallelic edited sample**: no cleavage resulting in 1 band.

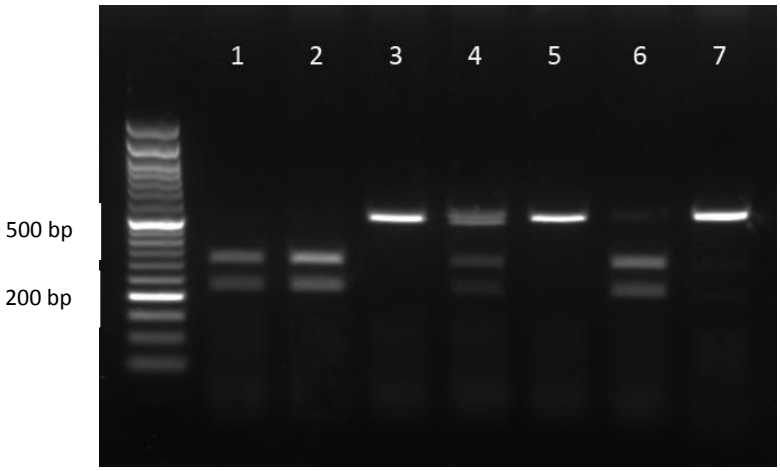

Figure 9. Sample gel electrophoresis of 8 clones subjected to *in vitro* Cas9 cleavage assay. Wild-Type sample (Lane 1) shows 2 bands, 300 bp and 210 bp. CRISPR-Edited samples (Lanes 2-7) shows the three possible outcomes. Lane 2 remains unedited (300 bp and 210 bp). Lanes 4, 6, and 7 shows monoallelic editing (510 bp, 300 bp, and 210 bp). Lanes 3 and 5 show biallelic editing (uncleaved 510 bp). Samples 3 and 5 can now be subjected to downstream applications such as Sanger sequencing to confirm if the edits are frameshift mutations which would eventually lead to knock-out of the gene.

This product is for research use only and is not intended for therapeutic or diagnostic applications. Please contact a technical service representative for more information.