Tribioscience

CRISPR Cas9 Cleavage Detection Kit

Catalog	Unit
TBS6097	100 Reactions

Description

CRISPR Cas9 Cleavage Detection Kit is a robust and precise RNA-dependent assay used to identify successful insertions and/or deletions in genomic DNA following CRISPR/Cas9 experiments. CRISPR/Cas9 can introduce insertion or deletion mutations (indels) on either one or both copies of a given mammalian gene. The Kit can easily identify if a given clone is monoallelic (has mutations in one copy), biallelic (both 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 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 gene editing saves time and energy from the arduous screening process and provides clear genotyping results.

Component

Product Component	Quantity
Cell Lysis Buffer	1.25 ml
Protein Degrader	100 µl
Scaffold Template and Primer Mix	100 µl
2X sgRNA Synthesis Buffer	100 µl
sgRNA Synthesis Enzyme Mix	50 µl
sgRNA Control Oligo	20 µl
Wild-Type Control Primer and Template Mix	20 µl
Monoallelic Control Primer and Template Mix	20 µl
Biallelic Control Primer and Template Mix	20 µl
RNP Degrader	100 µl
spCas9 Nuclease Protein	250 µl
10X Cas9 Reaction Buffer	1.25 ml
Pro Fidelity 2X PCR MasterMix	2 x 1.25 ml

Additional Materials Required
- Target-Specific Primers
- CRISPR-Edited Cells
- Nuclease-Free H_2O
- sgRNA Target-Specific Oligo
- Wild-Type Cells

Store at -20°C.

Target-Specific Primer Design

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 (2-3 bases upstream of PAM) should not be in the middle of the PCR amplicon in order to show a distinct cleavage pattern. Prepare 10 µM Primer mix and store at -20°C prior to use in Part B.

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 your sgRNA sequence used in the CRISPR Knock-out in between the T7 Promoter and the Scaffold Overlap: TTCTAATACGACTCACTATAGGGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG. Resuspend the DNA oligo at 100 µM and store at -20°C prior to use in Part C.

Protocol

Part A – Cell Lysis

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 a new eppendorf tube, mix the following:

Product 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

1. Prepare the following mixes on ice.

Product 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
MegaFi Pro Fidelity 2X 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

[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 control	15 sec	
Extension[2]	72°C	15 sec (for control) 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. If non-specific amplifications appear, repeat the PCR using touchdown method.

Part C – sgRNA Synthesis

1. Prepare the following reactions on ice.

Product 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
Pro Fidelity 2X PCR MasterMix	12.5 µl	12.5 µl
Nuclease-free Water	10.5 µl	10.5 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.
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

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

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

[3] Control RNP complex is multiplied by 4 (3 controls + 1 to account for pipetting error)

[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 the RNP complexes.
3. Prepare the following reactions on ice:

Component	Wild-Type Control	Monoallelic Control	Biallelic Control	Target-Specific
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

4. Mix and centrifuge briefly. Incubate at 37°C for 1 hour.
5. Inactivate reactions by adding 1 µl of RNP Degrader into each tube. Incubate at 68°C for 15 minutes.
6. Run the assay products on a 2% agarose gel and assess the cleavage patterns.

Part E – Analysis of Cleavage Products

Analyze the cleavage of the controls. Controls should show 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

For Target-Specific samples, assess cleavage patterns based on PCR product length and sgRNA placement. spCas9 cleaves DNA 3 bases upstream of the PAM sequence. Wild-Type cell samples should fully cleave showing 2 bands. For CRISPR-edited cells:

1 band - Biallelic editing
2 bands - Unedited WildType cells
3 bands - Monoallelic editing

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