PROTOCOL

Dharmacon™ T7E1, TIDE, and NGS analysis protocol for Edit-R™ gene editing experiments

Product description

Analyzing efficiency of Dharmacon™ Edit-R™ CRISPR-guided gene editing requires PCR-amplification of an amplicon spanning the target edit site. PCR replication of amplicons using predesigned primers can be subjected to mismatch detection assays (T7E1 or TIDE) or NGS methodology to verify and quantitate efficiency of gene editing. Where indicated in PCR reaction assembly (Step 4), appropriate primers should be used for downstream editing assay of choice. Primers designed for T7E1/TIDE analysis will generally amplify a longer stretch of genomic DNA (gDNA), while those designed for NGS will generally span a shorter region.

The following protocol is an example of PCR conditions, thermocycler settings, and editing assays, using genomic gDNA from direct cell lysis.For cell lysis, cells were treated for gene editing in a 96-well format.

1. Lyse cells directly in culture wells in 100 µL of 1x Phusion High-Fidelity buffer with additives.

Reagent	Volume	Final concentration
5x Phusion HF Buffer	20 µL	1x
Proteinase K (~ 20 mg/mL)	5 µL	~ 1 mg/mL
RNase A (10 mg/mL)	5 µL	0.5 mg/mL
Water, nuclease free	70 µL	N/A

2. Seal the 96-well plate with a plate seal to minimize evaporation and cross contamination. Incubate for 15–30 minutes at 56 °C, followed by deactivation for 5 minutes at 96 °C. Briefly centrifuge plate to collect liquid at bottom of wells.
3. Set up 50 µL PCR for each sample to be analyzed.
   Note: Other PCR reagents can be used if PCR optimization is performed for mismatch detection conditions and compatibility with direct cell lysis or purified gDNA.

Reagent	Volume	Final concentration
5x Phusion HF buffer	10 µL	1x
Forward primer (50 µM)	0.5 µL	500 nM
Reverse primer (50 µM)	0.5 µL	500 nM
dNTPs (10 mM)	1 µL	200 µM each
Phusion Hot Start II DNA Polymerase	1 µL	0.04 U/µL
Water, nuclease free	32 µL	N/A
Cell lysate	5 µL	N/A

4. Thermal cycling condition.

Reagent	Temperature	Time	Cycle(s)
Initial denaturation	98 °C	3 min	1
Denature	98 °C	10 s	10
Touchdown annealing	72 °C–1 °C/cycle	15 s	
Extension	72 °C	30 s	
Denature	98 °C	10 s	25
Annealing	62 °C	15 s	
Extension	72 °C	30 s	
Final extension	72 °C	10 min	1

5. Depending on primers used for PCR and application, proceed to either NGS or T7E1/TIDE analysis.

For NGS analysis

A. PCR product (~5 µL) can be run on 2% agarose gel to confirm amplification. Products will range from 200-300 bp in length, and present as a single band.

B. Following conformation via electrophoresis, PCR products should be purified (DNA-binding columns or gel purification) and adjusted to concentration required for analysis.

C. Submit PCR products for NGS. Since amplicons range from 200–300 bp, 2x150 bp sequencing is the minimum read length to ensure complete coverage.
Note: Primers do not contain adapters, and so adapter ligation is necessary (most NGS providers can accommodate this service).

For T7E1 and TIDE analysis

A. PCR product generated using T7E1/TIDE primers can be used for both analyses.

B. PCR product (~5 µL) can be run on 2% agarose gel to confirm amplification. Products will range from 500-1000 bp in length, and present as a single band.

C. Split remaining 45 µL PCR reaction into two aliquots for T7E1 and TIDE analysis.

T7E1 analysis

A. Heat PCR samples to 95°C for 10 minutes and then slowly (> 15 minutes) cool to room temperature (~ 25°C)

B. Set up 15 µL reactions for T7EI mismatch detection assay. Be sure to include at least one sample without the T7EI enzyme (No enzyme negative control) and/or Untreated (no gene editing negative control).
Note: Other mismatch detection assays can be used following the manufacturer's recommended protocol.

Reagent	Volume	Final concentration
PCR from gDNA (300-500 ng/ reaction)	10 µL	variable
Water, nuclease free	3 µL	N/A
NEBuffer 2 (10x)	1.5 µL	1x
T7 Endonuclease I (10 U/µL)	0.5 µL	0.33 U/µL

C. Incubate for 25 minutes at 37°C

D. Since the T7E1 enzyme cannot be heat inactivated, immediately run entire reaction volume with appropriate gel loading buffer on 2% agarose gel. Compare samples with expected gene editing to the No enzyme and/or Untreated negative controls.

For TIDE analysis

A. Column or gel purify PCR reaction and adjust to concentration required for DNA sequencing service.

B. Dilute T7E1/TIDE primer(s) to required concentration for DNA sequencing. The same primer(s) used for PCR amplification should be used for sequencing.

C. Submit samples to Sanger sequencing provider using one or both of the primers used for PCR amplification. Using both forward and reverse primers will ensure complete amplicon coverage, but might be redundant in some cases.

D. Analyze sequencing data for editing efficiency with TIDE algorithm (TIDE (nki.nl))

Materials

- T7 Endonuclease I, 10 U/µL (NEB, Cat #M0302S)
- Thermo Scientific Proteinase K,~ 20 mg/mL (Cat #EO0492)
- Thermo Scientific RNase A, 10 mg/mL (Cat #EN0531)
- Phusion Hot Start II DNA Polymerase (2 U/µL) (Cat #F549L)
- Thermo Scientific 5x Phusion HF Buffer (Cat #F-518L)
- NEBuffer 2 (NEB, Cat #B7002S)
- Appropriate DNA purification kit
- dNTP Mix set (10 mM each A,C,G,T) (Cytiva, Cat #28406564)

PCR Troubleshooting

This protocol, when used with predesigned primers, will generate single species PCR products in most cases. However, there are situations where cell density and primer design can influence yield and quality.

1. Gel analysis exhibits nonspecific amplification or smears
   a. might be due to excess lysate concentration (high cell density). Consider lowering lysate input to reduce off-target amplification.
2. No PCR product
   a. Low gDNA concentration in lysates. Possible in cases with low cell density. Increasing lysate input and / or number of PCR cycles can help.
   b. High GC content in primers (>65%). Consider repeating PCR experiment with additives that can destabilize secondary structure such as DMSO (1–10% final concentration) or glycerol (5–10% final concentration).
   Note that these additions might influence T7E1 activity, so purification of PCR products might be necessary.

For more information

To find the contact information in your country for your technology of interest, please visit us at **horizondiscovery.com/contact-us**

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