Genomic DNA Extraction, Amplification and T7E1 Assay

T7 endonuclease cleaves double-stranded DNA at positions of mismatches. NHEJ repair of Cas9-induced breaks will leave a variety of different mutations, and there will almost always be some wild type sequence remaining. Thus, when you amplify the target region, denature and renature the products, there will be mismatches at the target site, if cleavage was effective.

Genomic DNA extraction:
Trypsinize the cells, and put half of the volume in a 1.5 ml microfuge tube and spin briefly at 4°.
Remove supernate and wash cells with 1 ml PBS.
Add 100 µl of Quick Extract
Vortex 15 sec
Heat at 65° for 6 min, then 2 min at 95° (programmed for you)
Measure DNA concentration with Nanodrop spectrophotometer
Dilute to 20 ng/µl with more Quick Extract

PCR amplification of target:
Mix: (9.0 µl H₂O 10.0 µl 5x Q5 Buffer 1.0 µl 10 mM dNTPs 2.5 µl Fwd primer (10 µM) 2.5 µl Rev primer (10 µM) 5.0 µl Genomic DNA 0.5 µl Q5 DNA polymerase (2u/µl) 50.0 µl)

PCR: 98° 30 sec 98° 10 sec 66° 30 sec 72° 20 sec 72° 2 min 4° hold
Run 3 µl of the PCR product on a 2% agarose gel.

Purification of PCR product:
Add 250 µl of Zymo DNA binding buffer to the PCR product
Load onto Zymo Clean and Concentrate spin column
Spin 30 sec in microfuge; discard flow through
Add 200 µl DNA Wash buffer; spin 30 sec; discard flow through
Repeat wash step
Place spin column in a fresh 1.5 ml tube
Add 20 µl of EB (elution buffer); spin 1 min in microfuge
Measure DNA concentration with Nanodrop spectrophotometer
T7 endonuclease assay:
Mix: 10 µl PCR product
   2 µl 10x NEB Buffer 2
   8 µl H₂O

Anneal:  95°  5 min
   95->85° at 2°/sec
   85->25° at 0.1°/sec
   4° hold
Remove 10 µl to serve as undigested sample.
To the remainder, add 1 µl T7 Endonuclease; incubate 15 min, 37°.
Run the digested and undigested samples in adjacent lanes on a 2% agarose gel.

Dana Carroll, June 24, 2016