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:			Final conc.
	29.0 µl	H_2O	
	10.0 µl	5x Q5 Buffer	1x
	1.0 µl	10 mM dNTPs	200 µM
	2.5 µl	Fwd primer (10 µM)	0.5 µM
	2.5 µl	Rev primer (10 µM)	0.5 µM
	5.0 µl	Genomic DNA	100 ng
	0.5 µl	Q5 DNA polymerase (2u/µl)	1 unit
	50.0 µl		

PCR:			
	98˚	30 sec	
	98˚	10 sec	30x
	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

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T7 endonuclease assay:

Mix: 10 μl PCR product
2 μl 10x NEB Buffer 2
8 μl H_2O

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.

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