T7 Endonuclease Assay

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Abstract

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Protocol

Genomic DNA primer design

Step 1.

Design genomic DNA primers that are approximatley 18 to 22 basepairs in length and have 45-55% GC content. For best results, use primers with a Tm greater than 55 C. Design primers to yield amplicon length between 400-500 bp. In addition, design primers so that the predicted cleavage site is not in the center of the amplicon and the detection reaction will yield two distinct product bands.

DNA extraction

Step 2.

Spin down cells transfected with CRISPR constructs at 200g for 5 min at 4°C.

DNA extraction

Step 3.

Carefully remove supernatant and wash once with 1XPBS.

DNA extraction

Step 4.

Extract genomic DNA using Epicentre QuickExtract.

DNA extraction

Step 5.

Dilute samples to 40ng/µl.

Amplification with AmliTaqGold 360 Master Mix (Applied Biosystems 4398881).

Step 6.

Vortex DNA.

Amplification with AmliTaqGold 360 Master Mix (Applied Biosystems 4398881).

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Step 7.

Set up the following components for PCR:

Component	Sample
gDNA (40ng/µl)	2.5µl
10µM F/R primer mix	1µl
AmpliTaq Gold 360 Master Mix	25µl
Water	21.5µl
Total	**50µl**

Amplification with AmliTaqGold 360 Master Mix (Applied Biosystems 4398881).

Step 8.

For best results, add 5 µl of 360 GC Enhancer per 50µl PCR reaction when amplifying GC rich loci.

Amplification with AmliTaqGold 360 Master Mix (Applied Biosystems 4398881).

Step 9.

Run PCR reaction with the following conditions:

Stage	Temp	Time	Cycles
Enzyme activation	95°C	10 min	1X
Denature	95°C	30 sec	40X
Anneal	55°C(Tm)	30 sec	
Extend	72°C	30 sec	
Final extension	72°	7 min	1X

Amplification with AmliTaqGold 360 Master Mix (Applied Biosystems 4398881).

Step 10.

Clean up PCR reaction (Qiagen MiniElute PCR purification Kit). If 50 ng is present, run 3 µl of PCR product on a 1.5-2% agarose gel. If a single band of expected size is present, proceed to the denaturing and annealing step.

Hybridization of PCR products

Step 11.

In a PCR tube put the following:

Make two tubes for each DNA sample - one where T7 Endonuclease I is added and one with no T7E1 enzyme.

200 ng DNA

2 uL 10X NEB buffer 2

To19 µl Nuclease-free Water

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Hybridization of PCR products

Step 12.

Hybridation conditions

Step	Temperature	Ramp Rate	Time
Denaturation	95°C		5 min
Annealing	95-85°C 85-25°C	-2°C/sec -0.1°C/sec	
Hold	4°C		Hold

Hybridization of PCR products

Step 13.

Add T7 Endonuclease I to one tube for each DNA sample. (Add water to the other tube.)

Component	20 µl reaction
Annealed PCR product	19 µl
T7 endonuclease I (M0302) OR water	1 µl

Hybridization of PCR products

Step 14.

Incubate for 15 minutes at 37 C.

Hybridization of PCR products

Step 15.

Run products on 2% agarose gel or 10-20% TBE polyacrylamide gel and a 1X TBE buffer.

If the sgRNA created indels in your cells, you should see two small fragments below the wildtype band of 500 bp.

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