E) CRISPR Cas9 – *In vitro* cleavage of target DNA

Materials:
Purified Amplicon → your original DNA template you wish to cut
Target-specific sgRNA (50ng/µl) → The guide RNA created in the last step
Cas9 Nuclease (500ng/µl)
10% BSA
Nuclease-free water

10X Cas9 Nuclease Reaction Buffer

10X Cas9 Nuclease Reaction Buffer (1 mL)*	**Vi (µL)**	**Cf**
HEPES 1M	200	200 mM
NaCl 4M	250	1 M
MgCl2 2M	25	50 mM
EDTA 0.5M pH 8.0	2	1 mM
Nuclease-free water	523	-

* https://sfvideo.blob.core.windows.net/sitefinity/docs/default-source/protocol/alt-r-crispr-cas9-protocol-in-vitro-cleavage-of-target-dna-with-rnp-complex.pdf?sfvrsn=88c43107_24

In vitro Digestion Reaction

Perform the in vitro digestion reaction by assembling the following components;

	Vol (µL)	**Cf**
Purified Amplicon	5	(100–250 ng)
10x NEBuffer 3	1.5	1X
Cas9 protein (500 ng/ µL)	0.5	250 ng
Target-specific sgRNA (50ng/µl)	1	50 ng
10% BSA	1.5	1%
Nuclease-free water	5.5	-
Total volume	**15**	-

Incubate the reaction at 37 °C for 1 h.
Add 1 µl of RNase (4 µg/µl) and incubate at 37 °C for 15 min.
Add 0.5 µl of Proteinase K (5 µg/µl) and incubate at 55 °C for 10 min.

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Homemade 10x NEBuffer 3 (1 mL)	**Vi (µL)**	**Cf**
Tris HCl pH8 1M	500	0.5 M
NaCl 4M	250	1 M
$MgCl_2$ 2M	50	100 mM
DTT 0.1 M	100	10 mM
Nuclease-free water	100	-

Add an appropriate volume of loading buffer and run the samples on a 1.5-2% agarose gel alongside a negative control (uncut Amplicon).

Anticipated results:
a) 100 % cleavage efficiency: two bands from cleaved template,
b) <100 % cleavage efficiency: three bands (two from cleaved template and one uncut),
c) ~0 % cleavage efficiency: single band from uncut template.

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