HYBRID
LAB NETWORK

In vitro cleavage - 3h

In vitro cleavage of DNA using CRISPR/Cas9 – 3.00h

The objective of this step is to make the CRISPR/Cas9 reaction, run the in vitro digestion in a gel, interpret the results and discuss them.

SUMMARY OF SEQUENCES

- sgHL3 target site and sgRNA sequence
- sgHL4 target site and sgRNA sequence

In vitro cleavage of DNA using CRISPR/Cas9 - Needed Materials:

- Nuclease-free water;
- 10x Reaction Buffer;
- Synthetized sgRNA (sgHL3 or sgHL4);
- 500 ng/µL Cas9 protein;
- 100 ng/µL Target DNA (698bp fragment containing the target site);
- 20 mg/ml Proteinase K;
- RNase-free tubes and filter pipette tips;
- Micropipettes;
- 37°C / 65°C heat block/incubator.

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In vitro cleavage of DNA using CRISPR/Cas9 - Protocol:

a) Perform the DNA digestion reaction by assembling the following components:

Reagent	Volume (µL)
Nuclease-free water	5
10x BSA (10% BSA)	1.5
10x Reaction Buffer	1.5
sgRNA	5
Cas9 protein (500 ng/µL)	1
Target DNA (100 ng/µL)	1
Final Volume (µL)	15

b) Mix gently and incubate at 37°C for 60-90 min.

c) Add 0.5µL of Proteinase K (20 mg/ml) and incubate at 65°C for 15 min to stop the reaction and release the DNA from the Cas9 endonuclease.
Proteinase K degrades proteins and will destroy the Cas9 protein, thereby releasing the bound DNA and RNA fragments.

d) Add an appropriate volume of Loading Dye and run the samples on a 1% agarose gel alongside 3µL of GeneRuler 1 kb DNA Ladder (refer to step V. Agarose Gel Electrophoresis for full protocol).

NOTE: The DNA fragments will not migrate in the gel unless released from the Cas9 protein during the previous step.

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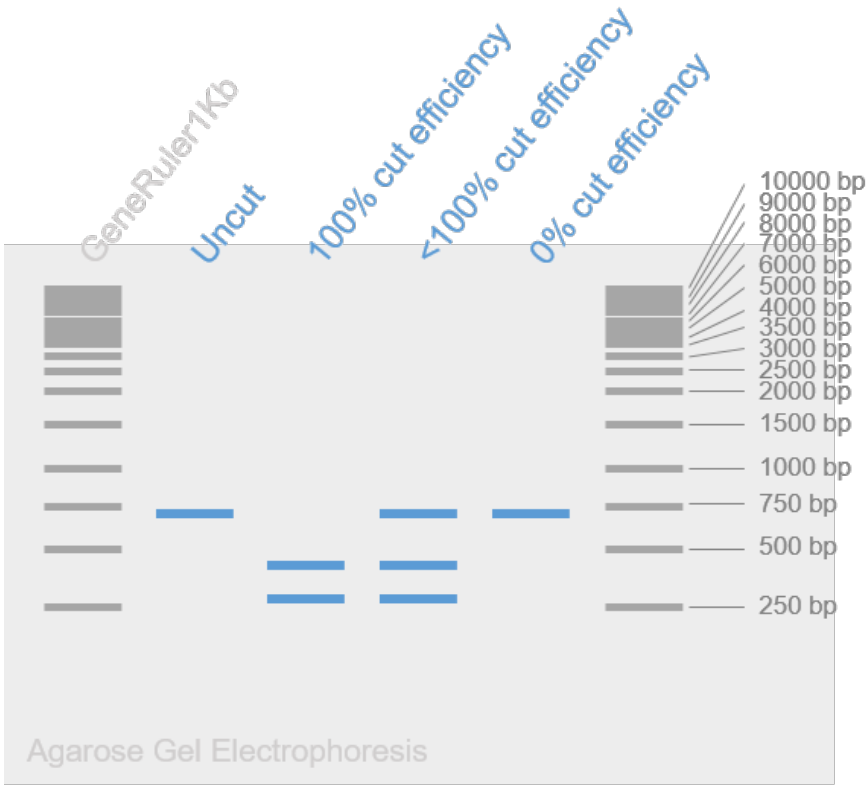

Expected Results:

- 100% cleavage efficiency: two bands from cleaved template
- <100% cleavage efficiency: three bands (two from cleaved template and one uncut)
- 0% cleavage efficiency: single band from uncut template.

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SUMMARY OF SEQUENCES:

sgHL3 target site and sgRNA sequence

DESIGN OF sgRNA (sgHL3)

Target DNA sequence (698 bp):
5′ –
CTTCTCCTCGACGTGCACGGACTGGACCTCGCCGAGCAGGATGATGTGGTCGCCCGCCGGGTAGCGCTCG
TGGACCGTGCACTCGACGACCGCGACCGCTCCGTCGAGCACGGTCGCTCCCCGCGCGGTACGGACGAAC
TCCCCGCCCGCGAACTTGTCCGCGGACTTGCGCGCGAAGCGCATGGCCAGGTCCGTGTGGTCCTCGCGCA
GCACGCTCACCGCGAACTCGCCGCAACTGTCGAACACCGGGAAGGAGTTGGCCGTACGAGCCAGGCAGA
CCAGTGCCAGTGGCGGCTCCATCGAGACGGACACGAACGAACTGGCGGTGAAACCGTGCGGGACTCCCC
CGCGGTCATGGGCGGTGACGAGCGCCACCCCGGCCGGCACCCGGGCCATGGC**GTCCCGGAGCATTCCCT
GGT**CGGCTGCCATCTTCGAACTCCCTAGGCGAGGCAGGTGGGCAGCTGCCCACCGTTGTAGGCGACCAGC
TCCGCGAAGATCGCCATGGCGTCACGCACGTGCCCGCCCAGCAGCTCCTCGTGGGCCCGGTGCAGGTTCG
CGACGAGGCGCTCCGCGTCCAGCAGCCCGGCGAAGGCGCCGAGGTCCGCCCGGCGCGCGGCCTGCAAC
GGTGTCAGCCGGCGGTCGACCGCATCGGCGGCGAGGCGCTGGACCCAGCGCAGATAGTCCCGGTTGGCG
TCGAT
– 3′

1. Select target site *(use of a target DNA selection program is recommended)*

sgRNA (sgHL3) target site: 5′ - **G**TCCCGGAGCATTCCCTGGT - 3′
*(At least one "**G**" is necessary for RNA transcription; if no "G" is present at the 5′ end of the target site sequence, add one "G")*

2. Append T7 RNA Polymerase promoter sequence to the 5′ end:

5′ - TTCTAATACGACTCACTATA**GTCCCGGAGCATTCCCTGGT** - 3′

3. Append Scaffold overlap sequence to the 3′ end:

5′ - TTCTAATACGACTCACTATA**GTCCCGGAGCATTCCCTGGT**GTTTTAGAGCTAGA - 3′

7 RNA polymerase
Target site
Scaffold overlap sequence

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SYNTHESIS OF sgRNA (sgHL3)

sgHL3 oligo:
5′ - TTCTAATACGACTCACTATA**G**TCCCGGAGCATTCCCTGGTGTTTTAGAGCTAGA - 3′

Scaffold oligo:
5′ – AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAA CTTGCTATT**TCTAGCTCTAAAAC** - 3′

The 14 nt sequence at the 3′ end (purple underlined) is complementary to the Scaffold overlap sequence at the 3′ end of the sgRNA oligo (purple)

1. Generate sgHL3 dsDNA template (120 bp):

Amplicon sequence that results from PCR amplification using sgHL3 oligo + Scaffold oligo (no dsDNA template is needed):

5′ – TTCTAATACGACTCACTATA**G**TCCCGGAGCATTCCCTGGTGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT - 3′

2. Transcribe RNA from dsDNA template using T7 RNA polymerase

*The T7 RNA polymerase starts transcription at the underlined **G** in the promoter sequence (see above) and transcribes RNA using the opposite strand as a template from 5′→3′, i.e. The first base in the transcript will be G.*

sgHL3 (100 bp):
5′ - **G**UCCCGGAGCAUUCCCUGGUGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGG CUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU - 3′

The resulting sgRNA contains the desired 20 nt sequence (orange) complementary to the target DNA sequence.

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IN VITRO CRSPR/CAS9 (sgHL3)

Target DNA sequence (698 bp):

5′ –
CTTCTCCTCGACGTGCACGGACTGGACCTCGCCGAGCAGGATGATGTGGTCGCCCGCCGGGTAGCGCTCG
TGGACCGTGCACTCGACGACCGCGACCGCTCCGTCGAGCACGGTCGCTCCCCGCGCGGTACGGACGAAC
TCCCCGCCCGCGAACTTGTCCGCGGACTTGCGCGCGAAGCGCATGGCCAGGTCCGTGTGGTCCTCGCGCA
GCACGCTCACCGCGAACTCGCCGCAACTGTCGAACACCGGGAAGGAGTTGGCCGTACGAGCCAGGCAGA
CCAGTGCCAGTGGCGGCTCCATCGAGACGGACACGAACGAACTGGCGGTGAAACCGTGCGGGACTCCCC
CGCGGTCATGGGCGGTGACGAGCGCCACCCCGGCCGGCACCCGGGCCATGGC**GTCCCGGAGCATTCCCT|**
GGTCGGCTGCCATCTTCGAACTCCCTAGGCGAGGCAGGTGGGCAGCTGCCCACCGTTGTAGG
CGACCAGCTCCGCGAAGATCGCCATGGCGTCACGCACGTGCCCGCCCAGCAGCTCCTCGTGGGCCCGGT
GCAGGTTCGCGACGAGGCGCTCCGCGTCCAGCAGCCCGGCGAAGGCGCCGAGGTCCGCCCGGCGCGCG
GCCTGCAACGGTGTCAGCCGGCGGTCGACCGCATCGGCGGCGAGGCGCTGGACCCAGCGCAGATAGTCC
CGGTTGGCGTCGAT
– 3′

Cas9 protein complexed with sgRNA binds to complementary DNA sequence on the target strand after recognition of the PAM site (5′-NGG-3′, in red). The Cas9 nuclease cuts 3 nt upstream of the PAM site (red line).

Cleaved sequence (416 + 282 bp):

5′ –
CTTCTCCTCGACGTGCACGGACTGGACCTCGCCGAGCAGGATGATGTGGTCGCCCGCCGGGTAGCGCTCG
TGGACCGTGCACTCGACGACCGCGACCGCTCCGTCGAGCACGGTCGCTCCCCGCGCGGTACGGACGAAC
TCCCCGCCCGCGAACTTGTCCGCGGACTTGCGCGCGAAGCGCATGGCCAGGTCCGTGTGGTCCTCGCGCA
GCACGCTCACCGCGAACTCGCCGCAACTGTCGAACACCGGGAAGGAGTTGGCCGTACGAGCCAGGCAGA
CCAGTGCCAGTGGCGGCTCCATCGAGACGGACACGAACGAACTGGCGGTGAAACCGTGCGGGACTCCCC
CGCGGTCATGGGCGGTGACGAGCGCCACCCCGGCCGGCACCCGGGCCATGGC**GTCCCGGAGCATTCCCT**
– 3′
+
5′ –
GGTCGGCTGCCATCTTCGAACTCCCTAGGCGAGGCAGGTGGGCAGCTGCCCACCGTTGTAGGCGACCAGC
TCCGCGAAGATCGCCATGGCGTCACGCACGTGCCCGCCCAGCAGCTCCTCGTGGGCCCGGTGCAGGTTCG
CGACGAGGCGCTCCGCGTCCAGCAGCCCGGCGAAGGCGCCGAGGTCCGCCCGGCGCGCGGCCTGCAAC
GGTGTCAGCCGGCGGTCGACCGCATCGGCGGCGAGGCGCTGGACCCAGCGCAGATAGTCCCGGTTGGCG
TCGAT – 3′

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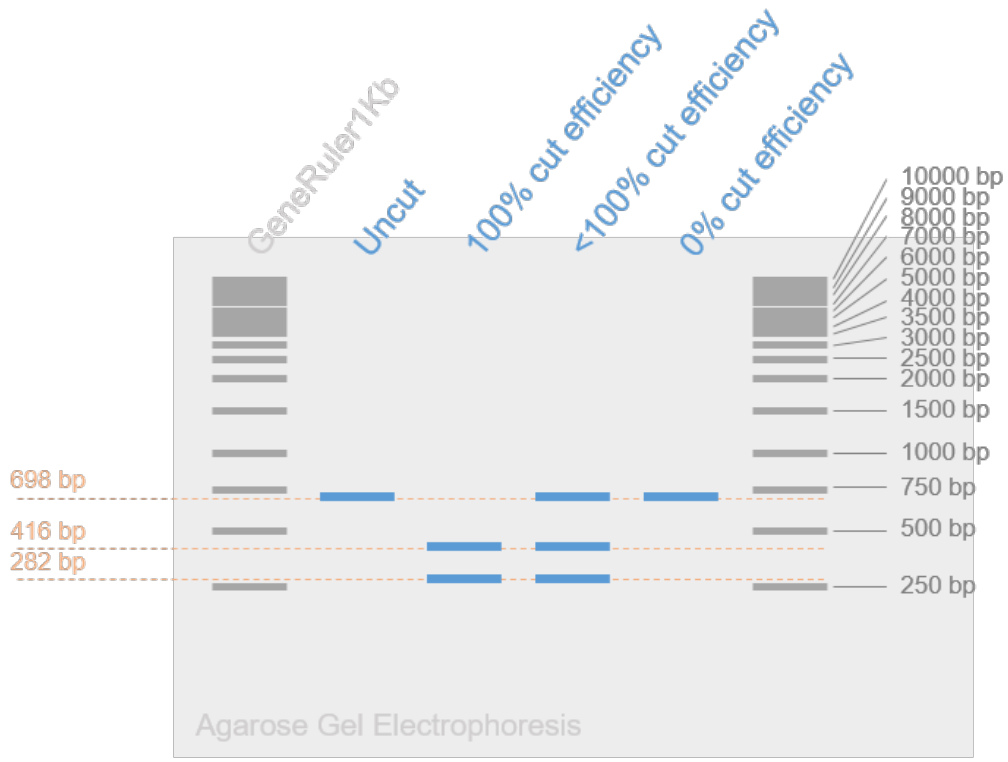

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SUMMARY OF SEQUENCES:

sgHL4 target site and sgRNA sequence

DESIGN OF sgRNA (sgHL4)

Target DNA sequence (698 bp):
5′ –
CTTCTCCTCGACGTGCACGGACTGGACCTCGCCGAGCAGGATGATGTGGTCGCCCGCCGGGTAGCGCTCG
TGGACCGTGCACTCGACGACCGCGACCGCTCCGTCGAGCACGGTCGCTCCCCGCGCGGTACGGACGAAC
TCCCCGCCCGCGAACTTGTCCGCGGACTTGCGCGCGAAGCGCATGGCCAGGTCCGTGTGGTCCTCGCGCA
GCACGCTCACCGCGAACTCGCCGCAACTGTCGAACACCGGGAAGGAGTTGGCCGTACGA**GCCAGGCAGA**
CCAGTGCCAGTGGCGGCTCCATCGAGACGGACACGAACGAACTGGCGGTGAAACCGTGCGGGACTCCCC
CGCGGTCATGGGCGGTGACGAGCGCCACCCCGGCCGGCACCCGGGCCATGGCGTCCCGGAGCATTCCCT
GGTCGGCTGCCATCTTCGAACTCCCTAGGCGAGGCAGGTGGGCAGCTGCCCACCGTTGTAGGCGACCAGC
TCCGCGAAGATCGCCATGGCGTCACGCACGTGCCCGCCCAGCAGCTCCTCGTGGGCCCGGTGCAGGTTCG
CGACGAGGCGCTCCGCGTCCAGCAGCCCGGCGAAGGCGCCGAGGTCCGCCCGGCGCGCGGCCTGCAAC
GGTGTCAGCCGGCGGTCGACCGCATCGGCGGCGAGGCGCTGGACCCAGCGCAGATAGTCCCGGTTGGCG
TCGAT
– 3′

1. Select target site *(use of a target DNA selection program is recommended)*

sgRNA (sgHL4) target site: 5′ - **G**CCAGGCAGACCAGTGCCAG - 3′
*(At least one "**G**" is necessary for RNA transcription; if no "G" is present at the 5′ end of the target site sequence, add one "G")*

2. Append T7 RNA Polymerase promoter sequence to the 5′ end:

5′ - TTCTAATACGACTCACTATA**GCCAGGCAGACCAGTGCCAG** - 3′

3. Append Scaffold overlap sequence to the 3′ end:

5′ - TTCTAATACGACTCACTATA**GCCAGGCAGACCAGTGCCAG**GTTTTAGAGCTAGA - 3′

7 RNA polymerase
Target site
Scaffold overlap sequence

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SYNTHESIS OF sgRNA (sgHL4)

sgHL4 oligo:
5′ - TTCTAATACGACTCACTATA**G**GCCAGGCAGACCAGTGCCAGGTTTTAGAGCTAGA - 3′

Scaffold oligo:
5′ – AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAA CTTGCTATT**TCTAGCTCTAAAAC** - 3′

The 14 nt sequence at the 3′ end (purple underlined) is complementary to the Scaffold overlap sequence at the 3′ end of the sgRNA oligo (purple)

1. Generate sgHL4 dsDNA template (120 bp):

Amplicon sequence that results from PCR amplification using sgRNA oligo + Scaffold oligo (no dsDNA template is needed):

5′ – TTCTAATACGACTCACTATA**G**GCCAGGCAGACCAGTGCCAGGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT - 3′

2. Transcribe RNA from dsDNA template using T7 RNA polymerase

*The T7 RNA polymerase starts transcription at the underlined **G** in the promoter sequence (see above) and transcribes RNA using the opposite strand as a template from 5′→3′, i.e. The first base in the transcript will be G.*

sgHL3 (100 bp):
5′ - **G**GCCAGGCAGACCAGUGCCAGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGG CUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU - 3′

The resulting sgRNA contains the desired 20 nt sequence (orange) complementary to the target DNA sequence.

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IN VITRO CRSPR/CAS9 (sgHL4)

Target DNA sequence (698 bp):

5′ –
CTTCTCCTCGACGTGCACGGACTGGACCTCGCCGAGCAGGATGATGTGGTCGCCCGCCGGGTAGCGCTCG
TGGACCGTGCACTCGACGACCGCGACCGCTCCGTCGAGCACGGTCGCTCCCCGCGCGGTACGGACGAAC
TCCCCGCCCGCGAACTTGTCCGCGGACTTGCGCGCGAAGCGCATGGCCAGGTCCGTGTGGTCCTCGCGCA
GCACGCTCACCGCGAACTCGCCGCAACTGTCGAACACCGGGAAGGAGTTGGCCGTACGA**GCCAGGCAGA**
CCAGTGC|CAGTGGCGGCTCCATCGAGACGGACACGAACGAACTGGCGGTGAAACCGTGCGGGACTCCCC
CGCGGTCATGGGCGGTGACGAGCGCCACCCCGGCCGGCACCCGGGCCATGGCGTCCCGGAGCATTCCCT
GGTCGGCTGCCATCTTCGAACTCCCTAGGCGAGGCAGGTGGGCAGCTGCCCACCGTTGTAGGCGACCAGC
TCCGCGAAGATCGCCATGGCGTCACGCACGTGCCCGCCCAGCAGCTCCTCGTGGGCCCGGTGCAGGTTCG
CGACGAGGCGCTCCGCGTCCAGCAGCCCGGCGAAGGCGCCGAGGTCCGCCCGGCGCGCGGCCTGCAAC
GGTGTCAGCCGGCGGTCGACCGCATCGGCGGCGAGGCGCTGGACCCAGCGCAGATAGTCCCGGTTGGCG
TCGAT
– 3′

Cas9 protein complexed with sgRNA binds to complementary DNA sequence on the target strand after recognition of the PAM site (5′-NGG-3′, in red). The Cas9 nuclease cuts 3 nt upstream of the PAM site (red line).

Cleaved sequence (285 + 413 bp):

5′ –
CTTCTCCTCGACGTGCACGGACTGGACCTCGCCGAGCAGGATGATGTGGTCGCCCGCCGGGTAGCGCTCG
TGGACCGTGCACTCGACGACCGCGACCGCTCCGTCGAGCACGGTCGCTCCCCGCGCGGTACGGACGAAC
TCCCCGCCCGCGAACTTGTCCGCGGACTTGCGCGCGAAGCGCATGGCCAGGTCCGTGTGGTCCTCGCGCA
GCACGCTCACCGCGAACTCGCCGCAACTGTCGAACACCGGGAAGGAGTTGGCCGTACGA**GCCAGGCAGA**
CCAGTGC – 3′
+
5′ –
CAGTGGCGGCTCCATCGAGACGGACACGAACGAACTGGCGGTGAAACCGTGCGGGACTCCCCCGCGGTC
ATGGGCGGTGACGAGCGCCACCCCGGCCGGCACCCGGGCCATGGCGTCCCGGAGCATTCCCTGGTCGGC
TGCCATCTTCGAACTCCCTAGGCGAGGCAGGTGGGCAGCTGCCCACCGTTGTAGGCGACCAGCTCCGCGA
AGATCGCCATGGCGTCACGCACGTGCCCGCCCAGCAGCTCCTCGTGGGCCCGGTGCAGGTTCGCGACGA
GGCGCTCCGCGTCCAGCAGCCCGGCGAAGGCGCCGAGGTCCGCCCGGCGCGCGGCCTGCAACGGTGTC
AGCCGGCGGTCGACCGCATCGGCGGCGAGGCGCTGGACCCAGCGCAGATAGTCCCGGTTGGCGTCGAT –
3′

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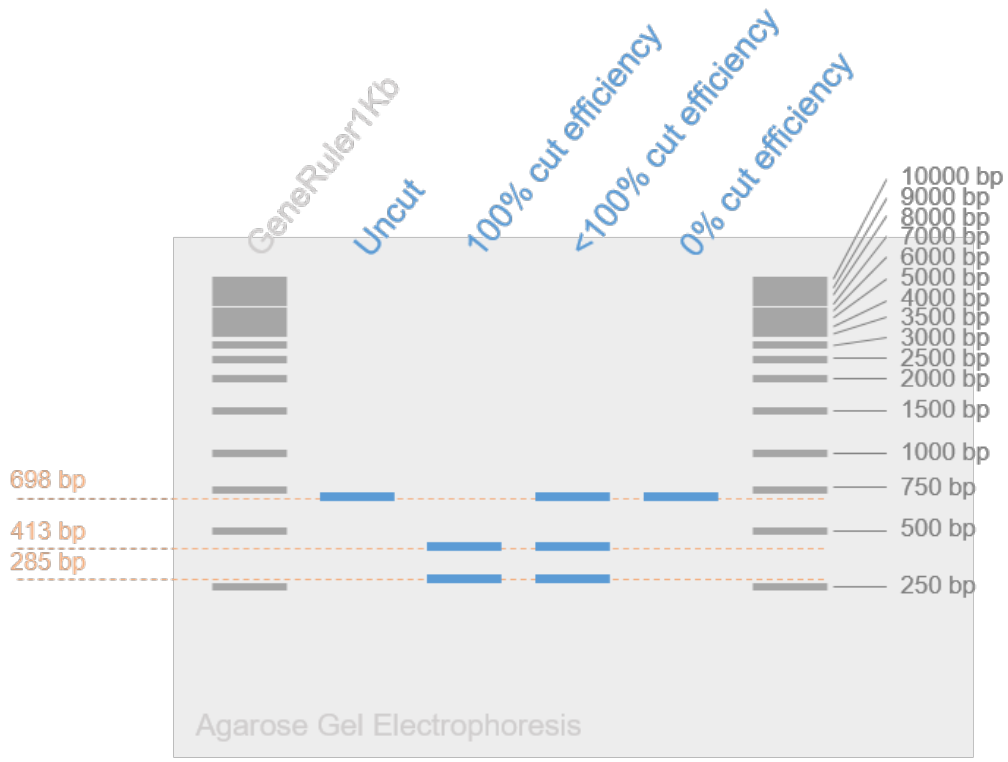

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