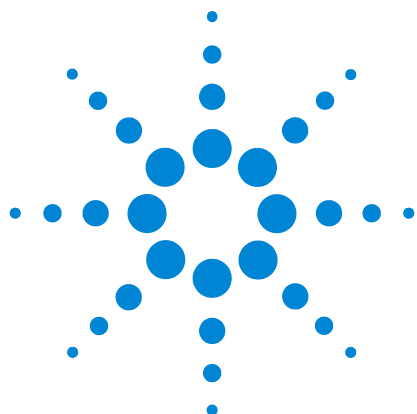




Agilent SureGuide Cas9 Nuclease Kit



Protocol

Revision B0, February 2015

5190-7714

5190-7715

5190-7716

5190-7718

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Agilent SureGuide Cas9 Programmable Nuclease Kit

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Kit Components and Storage Conditions

Component	SureGuide CRISPR/Cas Complete Kit, p/n 5190-7714	SureGuide Cas9 Programmable Nuclease Kit, 20 Reactions, p/n 5190-7715	SureGuide Cas9 Programmable Nuclease Kit, 100 Reactions, p/n 5190-7716	Storage Temperature
Cas9 Nuclease	40 µL	20 µL	100 µL	–20°C
10X Cas9 Digestion Buffer	2 × 40 µL	40 µL	200 µL	–20°C
RNase Free Water	2 × 1.5 mL	1.5 mL	2 × 1.5 mL	–20°C
Control DNA Target, 50 ng/µL	2 × 20 µL	20 µL	20 µL	–20°C
Control gRNA, 1 µM	2 × 10 µL	10 µL	10 µL	–20°C
T7 Promoter Forward Primer	25 µL	—	—	–20°C
Control Template	50 µL	—	—	–20°C
DTT	50 µL	—	—	–20°C
RNase Free DNase	50 µL	—	—	–20°C
T7 RNA Polymerase	50 µL	—	—	–20°C
100 mM rATP	50 µL	—	—	–20°C
100 mM rGTP	50 µL	—	—	–20°C
100 mM rUTP	50 µL	—	—	–20°C
100 mM rCTP	50 µL	—	—	–20°C
5X RNAMaxx Transcription Buffer	250 µL	—	—	–20°C
Yeast Pyrophosphatase	25 µL	—	—	–20°C
RNase Block	50 µL	—	—	–20°C
DEPC treated water	1 mL	—	—	–20°C
gRNA Binding Buffer	5 mL	—	—	Room temperature
5X gRNA Wash Buffer	7 mL	—	—	Room temperature
gRNA Elution Buffer	2.5 mL	—	—	Room temperature
RNA Binding Spin Cups and 2-mL Receiver Tubes	50 each	—	—	Room temperature
Tube, Micro 1.5-mL	50 each	—	—	Room temperature

Required and Optional Equipment and Reagents

Table 1 Required and optional equipment and reagents for the Cas9 nuclease protocol

Equipment or Reagent
Thermocycler or temperature blocks at 30°C and 65°C
Nuclease-free labware (e.g. reaction tubes)
(Optional) Agilent 2200 TapeStation system, p/n G2964AA or G2965AA
(Optional) Agilent Genomic DNA ScreenTape for the 2200 TapeStation system, p/n 5067-5365 or 5067-5366
(Optional) Agilent TapeStation DNA analysis platform and consumables

We recommend analyzing the Cas9 digestion control reaction using the Agilent Genomic DNA ScreenTape. The 2200 TapeStation analysis software can then be used to calculate the Cas9 cleavage efficiency.

Introduction to the Cas9 Nuclease

Cas9 (CRISPR associated protein 9) is an RNA-guided DNA nuclease associated with Type II bacterial CRISPR immunity systems. Cas9 is widely used to induce site-specific double strand breaks in DNA for multiple applications, many of which have yet to be developed. The guide RNA (gRNA) structure that binds and directs Cas9 is a hybrid of CRISPR RNA, containing homologous sequence to the cleavage target, and a trans-activating tracrRNA. A single guide RNA (sgRNA) chimera of the CRISPR and tracrRNAs can also direct DNA cleavage by Cas9 *in vitro* in lieu of the naturally occurring duplex¹. The kit includes a recombinant Cas9 nuclease, a 10X digestion buffer, a linear plasmid control target, and a corresponding gRNA. The guidelines and reaction conditions provided were optimized using linear DNA targets ranging from 3 kb–15 kb in reaction volumes of 10–20 µL. You are required to provide your experimental DNA targets and gRNAs in addition to optimizing the best reaction conditions for your unique application. We recommend the Agilent SureGuide gRNA Synthesis Kit (p/n 5190-7719) for synthesizing your gRNAs.

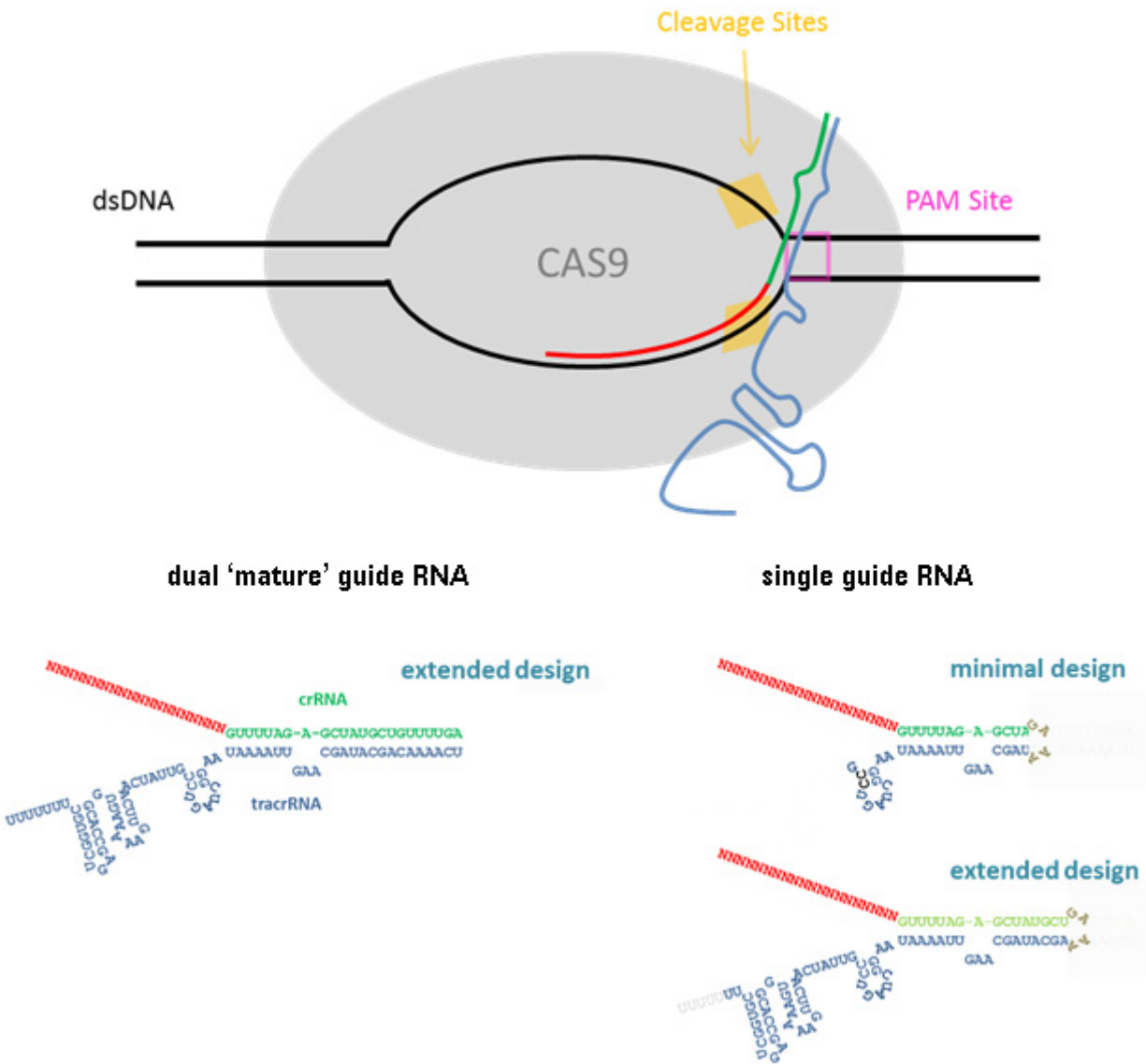


Figure 1 Depiction of gRNA directed Cas9 cleavage of double stranded DNA

Preprotocol Considerations

The reaction kinetics are determined by the total number of PAM sequences in the target DNA as well as relative concentrations of the three participant components: Target DNA, Cas9 and guide RNA. It may be necessary for you to empirically determine the optimal reaction conditions for your unique application.

Ratio of Cas9 to target

An excess of Cas9 results in aggregation of the target DNA and no observable cleavage. If too little Cas9 is used, it can result in low cleavage efficiencies. To determine the optimal amount of Cas9 to add to a given reaction, perform test digestions with a dilution series of Cas9. The protocol provided here is adequate for digestion of most plasmid DNA targets.

Guide RNA

A gRNA designed for use with the Cas9 Enzyme Kit should be compatible for use with the Type II Cas9 from *Streptococcus pyogenes*. gRNA may be synthesized by *in vitro* transcription. Not all guides perform equally well in Cas9 reactions. It may be helpful to design multiple guides for a given target area and screen them for optimal performance. It is possible to perform digests with multiple guides at once however, optimal guide design and combinations of guides that work well together must be empirically determined. Prepare working stocks of gRNAs at a concentration of 1 μ M in RNase-free TE, pH 7.0. Store gRNAs at -80°C .

The SureGuide gRNA Synthesis Kit (p/n 5190-7719) is available from Agilent.

Temperature

Cas9 is sensitive to temperature. We have observed significant loss of activity using incubations as low as 42°C . It is strongly recommended to confine Cas9 reactions to the $25\text{--}37^{\circ}\text{C}$ range, preferably 30°C .

Generation of blunt end fragments

Blunt end DNA fragments suitable for sub-cloning can be produced by digestion with Cas9. You can include more than one gRNA in the digest, to cut the DNA at more than one site, or perform multiple digests serially. The orientation of the guides should be designed such that the PAM sites end up on the DNA fragment of interest after cleavage (pink fragment in diagram below).



Procedures

Digest DNA with Cas9 nuclease

Use universal precautions for working with RNA throughout the procedure.

- 1 Thaw the kit components (Cas9 Nuclease, the 10X Cas9 Digestion Buffer, the RNase Free Water, the Control DNA Target, and the Control gRNA) as well as your experimental DNA targets and gRNAs. Store all reagents on ice until use.
- 2 Program a thermocycler with the following program and pre-warm the thermal block for immediate use once the reactions are ready.
 - 30 minutes at 30°C
 - 15 minutes at 65°C
 - Hold at 4°C

NOTE

Instead of a thermocycler, you can use heat blocks set to 30°C and 65°C. Transfer the reactions to ice immediately after the 65°C incubation.

- 3 On ice, prepare the reactions in reaction tubes suitable for a thermocycler (or suitable for a heat block if using heat blocks in [step 2](#)). The volumes of each reaction component are shown in [Table 2](#). Use the left column of for the control reaction with the Control DNA Target and Control gRNA. Use the right column for your experimental DNA targets and gRNAs. Keep the reactions chilled until transferred to a pre-warmed thermocycler or heat block. You can prepare a bulk master mix if multiple reactions will be performed.

Table 2 Component volumes per reaction

Component	Volumes for control reaction	Volumes for experimental reactions
Cas9 Nuclease	1 μ L	1 μ L
DNA Target (Control DNA Target or experimental DNA target)	2 μ L	X μ L
gRNA (Control gRNA or experimental gRNA)	1 μ L	X μ L
10X Cas9 Digestion Buffer	2 μ L	2 μ L
RNase Free Water	14 μ L	X μ L (sufficient for a total reaction volume of 20 μ L)
Total	20 μL	20 μL

- 4 Mix the contents of the reaction tubes, briefly spin, and then transfer them to the pre-warmed thermocycler.
- 5 After incubations are completed, analyze samples as desired. If using the Agilent 2200 TapeStation system, analyze 1 μ L of each reaction.

Interpretation of the Control Reaction Results

A Control DNA Target and corresponding Control gRNA are included in the Cas9 Programmable Nuclease Kit (enough for 10 control reactions). Digestion of the Control DNA Target (2,973 bp) with the Control gRNA results in two fragments of 1,800 bp and 1,173 bp. The expected cleavage efficiency is $\geq 90\%$ when using the procedure described above (“[Digest DNA with Cas9 nuclease](#)”). Digested samples can be analyzed by any convenient electrophoretic method for DNA fragments in this size range. [Figure 2A](#) shows an example of the control reaction analyzed on a standard 1% agarose gel stained with ethidium bromide. In this instance, use the gel to estimate the percentage of target cleaved.

If a more concrete value for the cleavage efficiency is desired, analyze samples with the Agilent 2200 TapeStation system using a Genomic DNA ScreenTape (or equivalent). To calculate the cleavage efficiency (% of target cleaved) you must first export results to an Excel file. The output file will list sizes and amounts for the fragments generated by the digest. The cleavage efficiency for each digest is calculated by dividing the sum of the amounts of the 1800 bp fragment and the 1173 bp fragments by the sum of the 3 most prominent fragments (2973, 1800, and 1173 bp). Note that the sizes of the products determined by the TapeStation are only accurate within 19% of the expected value and may thus vary from sample to sample. See [Figure 2](#) for examples of a TapeStation analysis of the kit control.

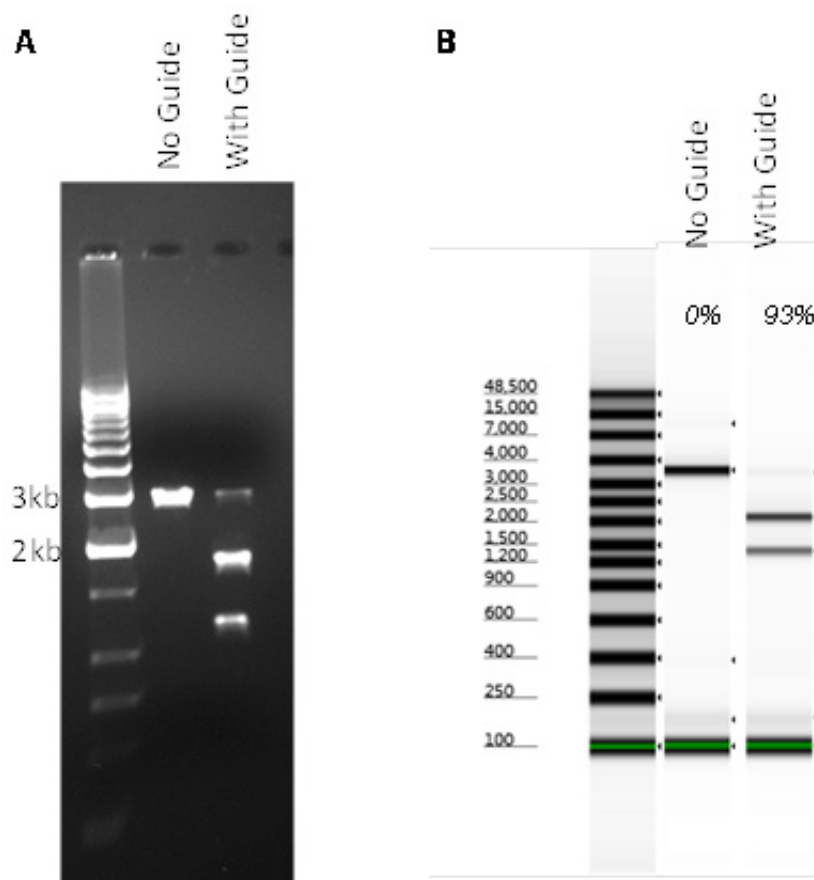


Figure 2 Analyses of Control Cas9 Digestion. A) 1% Agarose/TBE gel of two Cas9 digests using the provided Control DNA Target. The left lane has no gRNA added to the reaction, and no digestion is observed. The right lane shows Cas9 digestion in the presence of the Control gRNA. B) Gel image from an Agilent 2200 TapeStation confirming Cas9 digestion of the Control DNA Target.

Table 3 The table below illustrates how the calculation for % cleavage is performed using the % integrated area that is generated by the TapeStation software. In this example, the resulting cleavage efficiency was 93% (>90% is expected). Note that faint nonspecific bands are often present. Use only the % integrated area for the 3 relevant bands to perform the calculation.

DNA species	Size [bp]	% Integrated area	% Cleaved calculation
Marker	100	-	
Unknown (ignore)	186	20.17	
Cleavage product - 1	1,326	29.28	$\frac{(cp-1 + cp-2)}{(cp-1 + cp-2 + uncut)} \times 100:$ $\frac{(29.28 + 36.48)}{(29.28 + 36.48 + 4.9)} \times 100 = 93\%$
Cleavage product - 2	1,817	36.48	
Uncut	3,083	4.9	

Reference

Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821 (2012).

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In this Book

**This guide contains
information to use the
Agilent SureGuide Cas9
Nuclease Kit.**

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