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sgRNA Synthesis & CRISPR-Cas9 Synthesis V.1

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A protein roadblock forms when a protein binds DNA and hinders translocation of other DNA binding proteins. These roadblocks can have significant effects on gene expression and regulation as well as DNA binding. Experimental methods for studying the effects of such roadblocks often target endogenous sites or introduce non-variable specific sites into DNAs to create binding sites for artificially introduced protein roadblocks. In this work, we describe a method to create programmable roadblocks using dCas9, a cleavage deficient mutant of the CRISPR effector nuclease Cas9. The programmability allows us to custom design target sites in a synthetic gene intended for *in vitro* studies. These target sites can be coded with multivalency—in our case, internal restriction sites which can be used in validation studies to verify complete binding of the roadblock. We provide full protocols and sequences and demonstrate how to use the internal restriction sites to verify complete binding of the roadblock. We also provide example results of the effect of DNA roadblocks on the translocation of the restriction endonuclease NdeI, which searches for its cognate site using one dimensional diffusion along DNA.

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Step 1:

- Nuclease-free water
- Q5 Master Mix (New England Biolabs)
- Forward and Reverse primers (10 uM) (See below)
- synthetic template DNA (See below)
- Sterile microcentrifuge tubes and PCR tubes
- PCR thermocycler

The sequence of the synthetic template DNA used:

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CCAACCTTAAT CGCCTTGCGC CGCCAGCTGG CGTAATAGCG AAGAGGCCCG CACCGATCGC
CCTTCCCAAC GTTGCAGCAG CCTGAATGGC GAATGGCGCT TTGCCTGGTA TCCTGCACCA
GGAGCGCTGC CGGAAAGCTG GCTGGAGTGC ATCTTCCTG AGGCCGATAC TGTCGTCGTC
CCCTCAAACCT GGCAGATGCA CGGTTACGAT GCGCCCATCT ACACCAACGT GACCTATCCC
ATTACGGTCA ATCCGCCGTT TGTTCCCAACC TAGAATCCGA CGGGTTGTTA CTCGCTCACA
TTTAATGTTG ATGAAAGCTG GCTACAGGAA GGCCAGACGC GAATTATTTT TGATGGCGTT
CCTATTGGTT AAAAAATGGATATCGATCCACGGATCCAGG TGCGAATTTT AACAAAATAT
TTACGGTTAC AATTTGGGTA TTTGCTTATA CAATCTTCCT GTTTTTGGGG CTTTTCTGAT
TATCAACCGG GGTACATATG AATTCCATGC TAGTTTTACG ATTACCGTTC TTCGTTTCTC
TTGTTTGCTC CAGACTCTCA GGCAATGACC TGATAGCCTT TGATAGTCTC TCAAAAATAG
GGATATCGATCCACGGATCC TGGTCAGCTA GAACGGTTGA ATATCATATT GATGGTGATT
TGACTGTCTC CGGCCTTTCT CACCCTTTTG AATCTTTACC TACACATTAC TCAGGCATTG
CAGGGAAAAT ATATGAGGGT TCTAAAATT TTTATCCTTG CGTTGAAATA AAGGCTTCTC
CCGCAAAAGT ATTACAGGGT CATAATGTTT TTGGTACAAC CGATTTAGCT TTATGCTCTG
AGGCTTTATT GCTTAATTTT GCTAATTCTT TGCCTTGCCT GTATGATTTA TTGGATGTTA
ATGCTACTAC TATTAGTAGA ATTGATGCCA CCTTTTCAGC TCGCGCCCCA AATGAAAATA
TAGCTAAACA GGTTATTGAG CGAAATGTAT CTAATGGTCA
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The forward primer sequence: 5'-CGCGTTGGCCGATTC-3'

The reverse primer sequence: 5'-AGGGTGAGAAAGGCC-3'.

Step 6:

- PCR product from Step 5
- Qiagen PCR clean up kit (with spin columns and all reagents)
- Microcentrifuge tube for storage of purified DNA

Step 8:

- Purified DNA from Step 7
- Water or EB buffer from PCR cleanup kit
- Nanodrop spectrophotometer

Step 14:

- Sterile nuclease-free microfuge tubes
- Nuclease-free water
- EnGen 2X sgRNA Reaction Mix, *S. pyogenes*
- Target-specific DNA oligo (1 μ M) (see below)
 - Create by adding 1 μ L oligo into 99 μ L RNase free water
- DTT (0.1 M)
- EnGen sgRNA Enzyme Mix

The sequence of the target specific DNA oligo:

TTCTAATACGACTCACTATAGGATATCGATCCACGGATCCGTTTTAGAGCTAGA

Step 20:

- RNA product from Synthesis protocol above
- Monarch RNA Cleanup Kit (50 μ g)
- \geq 95% Ethanol
- Microcentrifuge tube for storage of purified RNA

Step 22:

- Sterile nuclease-free microcentrifuge tubes
- Nuclease-free Water
- NEBuffer 3.1
- 500 nM sgRNA
 - Dilute product from sgRNA synthesis with nuclease-free water
- 1 μ M dCas9, *S. pyogenes*
- Substrate DNA
- Glycerol

PCR Preparation

- 1 Gather the materials used in Step 1 (See Materials section). Thaw components before use. Keep all on ice.
- 2 Dilute the primers for (See Materials) to 10 μM before using.

PCR reaction

- 3 Combine the components from Step 1 into PCR tube(s) in order, for the desired final volume. Use Table 1 for reference.

A	B	C	D	E	F
Component	Stock Concentration	4x Amount	2x Amount	1x Amount	Final Concentration
Nuclease-free Water	-	78 μL	39 μL	19 μL	-
Q5 Master Mix	2x	100 μL	50 μL	25 μL	1x
Forward primer (DnnnnF)	10 μM	10 μL	5 μL	2.5 μL	500 nM
Reverse primer (BnnnnR)	10 μM	10 μL	5 μL	2.5 μL	500 nM
Template DNA	4 $\mu\text{g/mL}$	2 μL	1 μL	1 μL	0.2 - 0.4 $\mu\text{g/mL}$
Total Volume		200 μL	100 μL	50 μL	

Table 1: Q5 Master Mix

- 4 Perform PCR using Table 2 for parameters

A	B	C
Step	Temperature ($^{\circ}\text{C}$)	Time (Seconds)
Denaturation	98	30
Melt	98	10
Anneal	60	30
Extend	72	30
Final Extend	72	120
Infinite Hold	4	-

5 Advance to purification steps

Purification of PCR Product

6 Gather the materials used in Step 6 (See Materials section).

7 Perform Qiagen PCR purification kit by following the printed protocol inside the kit. Make sure pure ethanol (200 Proof) has been added to the correct buffer.

For 1x volume (50 uL total), you must use a blank centrifuge tube with water to counterbalance the spin column.

For 2x volume (100 uL total), use two spin columns and add 50 uL to each column. This will produce the same concentration as 1x protocol, but twice the volume (2x the amount of DNA).

For 4x volume (200 uL total), use two spin columns and add 100 uL (two PCR tubes) to each column. This will produce twice the concentration of the 1x protocol, and twice the volume (4x the amount of DNA).

Store on ice after purification.

Determination of DNA Concentration

8 Gather the materials used in Step 8 (See Materials section).

9 Use a nanodrop or similar UV absorbance instrument for measurements.

10 Blank with Buffer EB or dd H₂O, depending on how you elute in your PCR purification kit.

11 Collect concentrations 2 times each.

- 12 Take the average of your 2 readings for each DNA sample.
- 13 Convert ng/uL to nM using the formula below. Record both ng/uL and nM numbers.

$$\text{Concentration [nM]} = 1.62 \times \text{Concentration [ng/uL]}$$

Preparation of sgRNA

- 14 Gather the materials used in Step 14 (See Materials section).
- 15 Thaw EnGen 2X sgRNA Reaction Mix, *S. pyogenes*, and the customer-supplied target-specific oligo (1 μM). Mix and pulse each component in microfuge prior to use for 10 seconds. Store enzyme mix on ice.
- 16 Assemble the reaction at room temperature in the order listed below. Add the enzyme last to each reaction.

A	B
Reagent	Amount
Nuclease-free Water	2 μL
EnGen 2X sgRNA Reaction Mix, <i>S. pyogenes</i>	10 μL
Target-specific DNA oligo (1 μM)	5 μL
DTT (0.1 M)	1 μL
EnGen sgRNA Enzyme Mix	2 μL
Total Volume	20 μL

Table 4: EnGen 2X sgRNA Reaction Mix

- 17 Mix thoroughly and pulse-spin in a microfuge for 10 seconds. Incubate at 37°C for 30 minutes.

- 18 Transfer reaction to ice.
- 19 For DNase treatment, bring volume to 50 μL by adding 30 μL of nuclease-free water. Add 2 μL of DNase I, mix and incubate at 37°C for 15 minutes.
- 20 Gather the materials used in Step 20 (See Materials section).
- 21 Use Monarch RNA Cleanup Kit (50 μg) or similar RNA purification kit. From here, follow the protocol printed inside of the kit. Ensure that ethanol has been added to the correct buffer.

Activation of dCas9 and Binding to DNA

- 22 Gather the materials used in Step 22 (See Materials section).
- 23 Thaw NEBuffer 3.1, 500 nM sgRNA, and 1 μM dCas9. Mix and pulse each component in microfuge prior to use. Store tubes on ice.
- 24 Assemble the reaction below, in order, at room temperature. The final concentration of both the dCas9 and sgRNA should be 60 nM.

A	B
Reagent	Amount
Nuclease-free Water	19.14 μL
NEBuffer 3.1	3 μL
500 nM sgRNA	3.24 μL
1 μM dCas9, S. pyogenes	1.62 μL
Total Volume	27 μL

Table 5: dCas9 System Reaction Mix

- 25 Mix thoroughly by pipetting mixture up and down gently. Pulse-spin in a microfuge. Incubate at

25°C for 10 minutes.

- 26 Add enough DNA to reaction tube so that the final concentration of DNA is 3 nM. The ratio of dCas9:sgRNA:DNA should be 20:20:1.
 - Ex. Add 1.12 µL of 75 nM stock DNA to reaction tube
- 27 Mix thoroughly by pipetting mixture up and down gently. Pulse-spin in a microfuge. Incubate at 37°C for 15 minutes.
- 28 Transfer reaction to ice.
- 29 Add enough glycerol to tube so that it is equal to total reaction volume. Mix thoroughly by pipetting mixture up and down.
 - Ex. Add 28.12 µL glycerol to 28.12 µL reaction mixture
- 30 Roadblocks are now bound to programmed sites on DNA. Store in freezer.