Purification of Recombinant Cas9

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Objective

To purify recombinant Cas9 protein for genome editing experiments. Recombinant Cas9 will be used with synthesized crRNA:tracrRN to assemble ribonucleoproteins (RNPs) to target the nuclease activity of Cas9 to specific loci within the genome. Using RNPs as opposed to viral delivery of CRISPR gene editing components is advantageous as the expression of Cas9 is transient limiting off-target effects.

Experimental Methods & Results

Protein expression:

1. pMJ915 (Plasmid #69090 - https://www.addgene.org/69090/)[1] was transformed into Rosetta (DE3) Competent Cells (Novagen 70954) and plated on on LB-agar supplemented with 100 µg/mL ampicillin overnight.
2. The following day a colony was picked and inoculated in 300 mL of Terrific Broth (TB - Sigma T0918) supplemented with 100 µg/mL Ampicillin(Amp) and 34 µg/mL Chloramphenicol(Chloro). The starter culture was grown overnight at 37 °C with shaking at 200 rpm.
3. The next morning, 6 x baffled flasks containing ~1800 mL TB with 100 µg/mL Amp and 34 µg/mL Chlor was inoculated with ~20 mL overnight culture and grown at 37 °C until OD600 ~0.6 before reducing the temperature to ~15 °C and inducing at OD600 ~0.6 with 0.1 mM IPTG.
4. Cultures were grown for a further 20 hours before cells were harvested by centrifugation at 6000 rpm, 10 mins, 4 °C (JLA8.1000, Beckman).

Protein purification:

Ni-NTA Purification

1. Cell paste was resuspended in ~400 mL 20 mM HEPES pH 7.4, 500 mM NaCl, 1 mM TCEP, 1 mM PMSF, 1 mM benzamidine, 20 µg/mL benzonase.
2. The cell resuspension was then lysed by sonication and clarified by centrifugation 15000 rpm, 60 mins, 4 °C (JLA16.2500, Beckman).
3. Clarified lysate was bound in batch to ~5 mL Ni-NTA agarose resin (Qiagen) by rocking at 4 °C for 45 mins. The resin was washed extensively with 20 mM HEPES pH 7.4, 300 mM NaCl, 1 mM TCEP, 15 mM imidazole and the bound protein was eluted in 20 mM HEPES pH 7.4, 300 mM NaCl, 1 mM TCEP, 300 mM imidazole.
4. The His6-MBP affinity tag was removed by cleavage with TEV protease, while the protein was dialyzed overnight against 20 mM HEPES pH 7.4, 150 mM KCl, 1 mM TCEP, 10% glycerol using snakeskin (MWCO 10 000). The following morning a precipitate was observed after dialysis, which was removed by filtration using a 0.22 µm PES (Millipore) syringe filter.

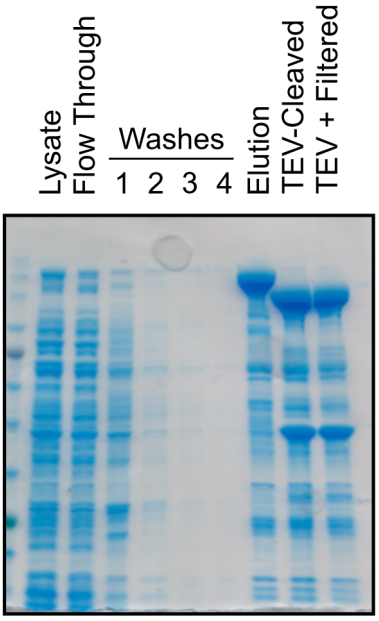

Figure 1: Nickel Purification of Cas9 Protein

Ion Exchange and Size Exclusion Chromatography

5. Cation exchange was performed by Mani Ravichandran. Briefly, TEV-cleaved samples were diluted in HEPES buffer without salt and loaded onto a primed MonoS column, followed by a column wash and elution with a KCl gradient.
6. Eluted protein was concentrated (spin concentrator MWCO 50,000) to 1ml and run on S200 16/60 in 20 mM HEPES pH 7.4, 150 mM KCl and 1 mM TCEP by Rachel Harding.
7. Samples were pooled and buffer exchanged using a spin concentrator (MWCO 100,000) into Cas9 storage buffer (300 mM NaCl, 10 mM Tris-HCl - pH 7.4 @ 25°C, 0.1 mM EDTA, 1 mM DTT, 50% Glycerol). Final yield was approximately ~300 µl at 5.9 mg/ml (~37 µM).
8. Concentrated samples were aliquoted in 5 µl aliquots, flash frozen, and stored at -80°C.

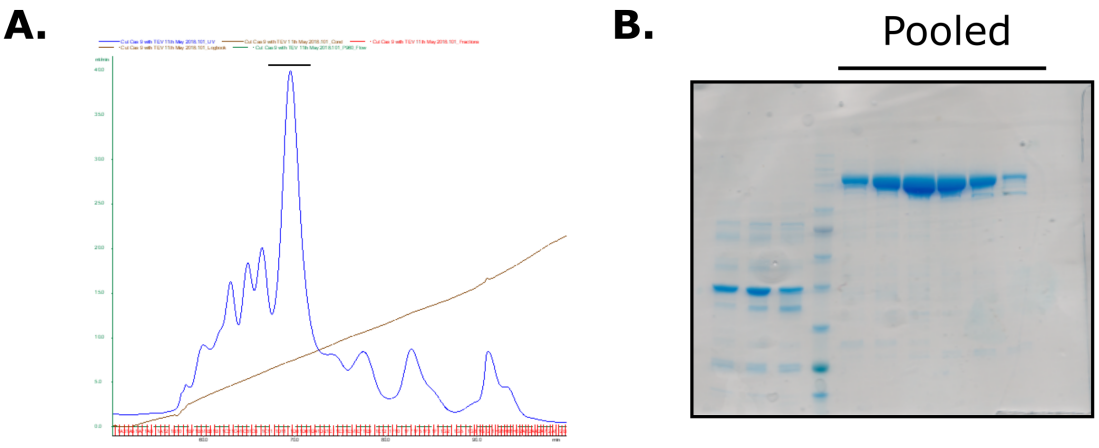

Figure 2: Cation Exchange Chromatography of Cas9. (A) Chromatogram (B) Fractions from major peakrun to the right of ladder, this corresponds to Cas9.

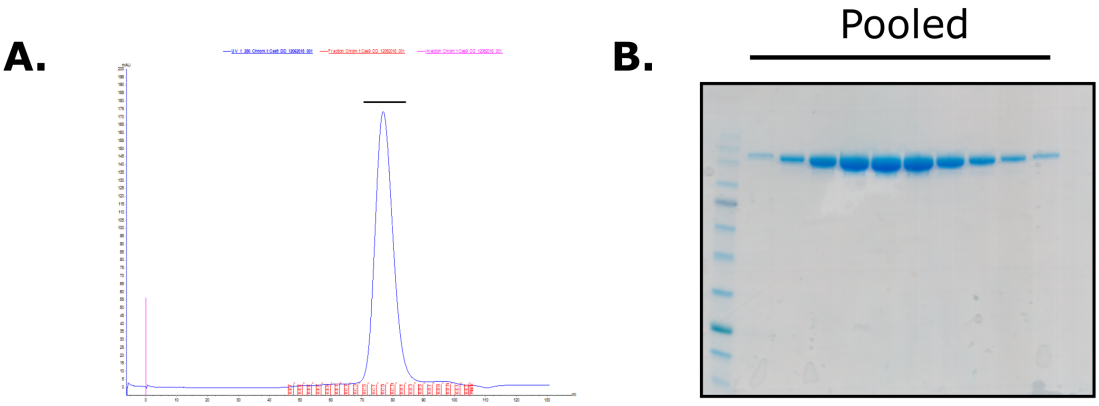

Figure 3: Size Exclusion Chromatography of Cas9 Sample

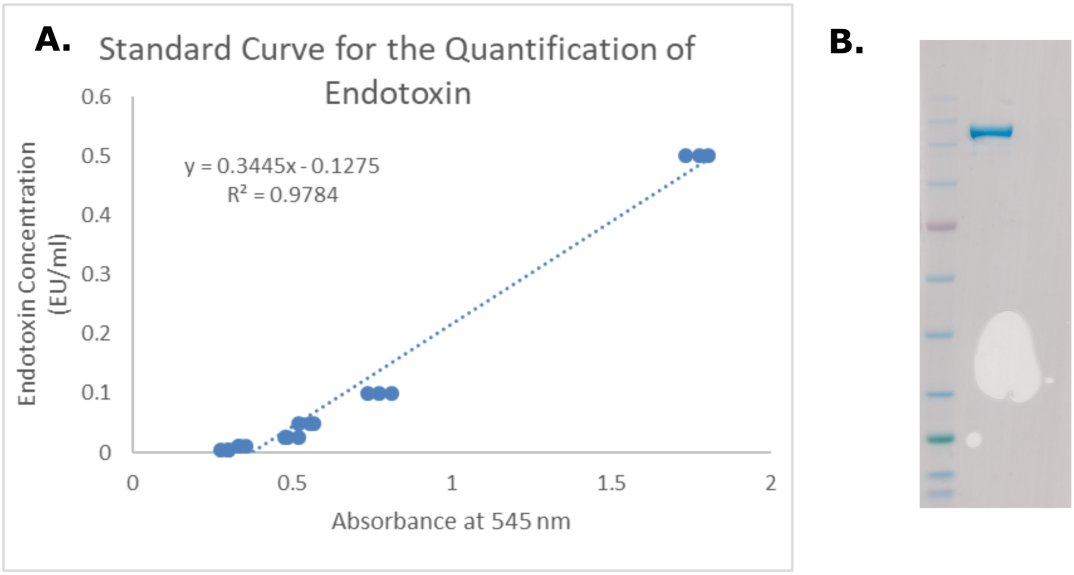

Figure 4: Quantification of Endotoxin Levels. (A) Standard curve (B) SDS-PAGE 1 µg of the purified Cas9 tested in this assay

Protein QC

Measuring Endotoxin Contamination

To ensure the recombinant protein is endotoxin-free for transfection/electroporation into mammalian cells we used the ToxinSensor Chromogenic LAL Endotoxin Assay Kit ((Cat# L00350) from GenScript. For this assay the manufacturer's protocol was followed as outlined and absorbance measurements were read in 96 well plate format on a CLARIOstar plate reader (BMF Labtech). To test our Cas9 protein it was diluted to 1 µM and and run in triplicate alongside a standard curve ranging from 0.01-0.5 EU/ml. We calculated the endotoxin concentration of the 1 µM sample to be 0.51 -/+ 0.01 EU/ml, which is acceptable for use on mammalian cell lines in a research setting. We also tested the endotoxin concentration at the working concentration for transfections (7.5 nM), in which the enotoxin level was below detection. Figure 4 shows the standard curve for this assay (A) and a SDS-PAGE of 1 µg of the purified Cas9 tested in this assay.

In vitro Activity Assay

Next, I set up a *in vitro* assay to test the activity of the purified Cas9 on DNA substrate. As a substrate for the reaction the coding sequence for NSD3-short was PCR amplified, purified by spin column, and diluted to ~30nM. The *in vitro* assay performed as outlined on the NEB website - https://international.neb.com/protocols/2014/05/01/in-vitro-digestion-of-dna-with-cas9-nuclease-s-pyogenes-m0386 (Accessed - August 1st, 2018). For the assembly of RNPs, I used separate tracrRNA and crRNA purchased from Integrated DNA Technologies (IDT). The tracrRNA and control crRNA came as part of the Alt-R® CRISPR-Cas9 Control Kit (IDT-192579151) and a custom NSD3-targeting crRNA was also purchased from IDT (/AlTR1/CCAGUGGACACUUCCGUUGUGUUUUAGAGCUAUGCU/AlTR2/). To anneal crRNA:tracrRNA, 1 ul of each RNA was added 98 ul of nuclease-free duplex buffer (IDT 11-01-02-02). The mixture was heated at 95°C for 5 min and cool on bench top.

1. Assembly the reaction at room temperature:

Component	30 µl reaction
Nuclease-free water	20 µl
10X Cas9 Nuclease Reaction Buffer	3 µl
1 µM crRNA:tracrRNA	0.9 µl
1 µM Cas9 Nuclease	0.9 µl

2. Pre-incubate for 10 minutes at 25°C
3. Add 3 µl of 30nM substrate DNA

4. Mix thoroughly and pulse-spin in a microfuge.
5. Incubate at 37°C for 15 minutes.
6. Add 1 ul of Proteinase K to each sample, Mix thoroughly and pulse-spin in a microfuge.
7. Incubate at room temperature for 10 minutes.
8. Samples were run on 1% agarose gel (TBA), stained with sybrGreen, and imaged.

10X Cas9 Nuclease Reaction Buffer:
-200 mM HEPES (pH 6.5 @ 25°C)
-1000 mM NaCl
-50 mM MgCl2
-1 mM EDTA

Importantly, only the RNP assembled with NSD3-targeting crRNA and not the non-targeting control was able to cleave the NSD3 PCR product, showing that our purified enzyme is both active and selective given the right guide (Figure 5).

Observations

Here we were able to produce a high-purity sample of Cas9, with minimal endotoxin contamination that is active in an *in vitro* digestion assay. This protein will used for gene editing experiments in the lab, beginning with generating NSD3 knock-out cell lines in relevant cancer models.

References

1. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. Lin S, Staahl BT, Alla RK, Doudna JA. Elife. 2014 Dec 15;3:e04766. doi: 10.7554/eLife.04766. 10.7554/eLife.04766 PubMed 25497837

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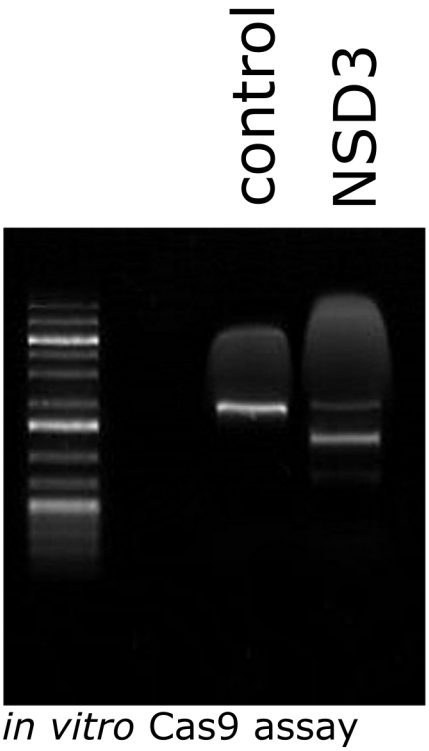

Figure 5: *In vitro* Cas9 Assay. NSD3 PCR product incubated with RNP assembled with either non-targeting or NSD3-targeting crRNA