Biophysical Characterization and Direct Delivery of *S. pyogenes* Cas9 Ribonucleoprotein Complexes

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Introduction

Several groups have demonstrated efficient genome editing in various mammalian cells by cationic lipid mediated delivery of purified Cas9 protein complexed with in-vitro translated or chemically synthesized guide-RNA (gRNA). Such “direct delivery” of the Cas9 ribonucleoprotein (RNP) complex allows for efficient gene-editing while minimizing off-target activity owing to the rapid turnover of the Cas9 protein in cells. Efficiency of gene-editing mediated by RNP delivery varies by locus, depends on the length of guide-RNA and on the amount and ratio of Cas9 protein and gRNA delivered.

The Cas9 complex with gRNA has been well characterized structurally and biophysically revealing a large contact area and a high affinity. Thermal melt curves are a useful property to detect the binding and stability of complexes. We have used the large increase in the melting temperature from apo-Cas9 to the Cas9 complexed with sgRNA to characterize the affinity of Cas9 for sgRNA. Multiple sgRNAs with differing lengths and base composition were complexed with Cas9. These biophysically characterized complexes were then transfected into 293T cells and the efficiency of indel generated was measured. We have found that subtle differences in the sgRNA length and base composition affect the binding and formation of RNP complex. Correlating binding affinity with efficiency of genome editing informs the design of an optimal composition of RNPs for cationic lipid mediated direct delivery.

Thermal Stability Assay

Fig.1a

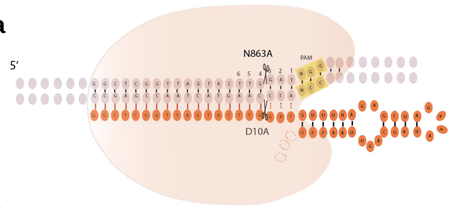
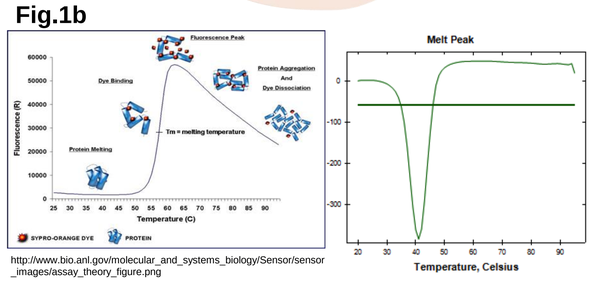


Fig.1b

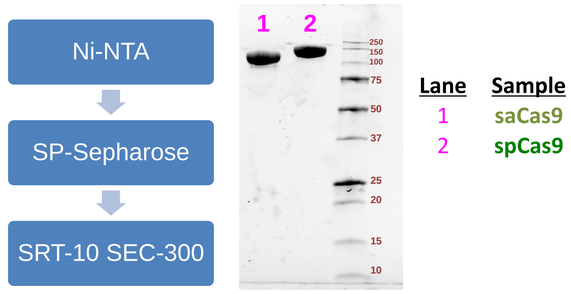


http://www.bio.anl.gov/molecular_and_systems_biology/Sensor/sensor_images/assay_theory_figure.png

Figure 1(a) Schematic drawing of Cas9-gRNA-DNA target with cutting site of each domain **(b)** On left is a schematic of acquired signal with state of protein and fluorescent dye. At low temp, the dye is free in solution and quenched by water. As the temperature increases, the protein unfolds and the dye binds hydrophobic residues resulting in signal. As the protein further denatures and aggregates the signal is once again quenched. On right is the inverse differential of the change in signal over time to elucidate the mid-point of denaturation (Tm).

Purification of Cas9

Fig.2



Lane	Sample
1	saCas9
2	spCas9

Figure 2 Purification scheme and gel of recombinant *S. pyogenes* and *S. aureus* Cas9 protein.

Thermal Stability of Cas9 Orthologs

Fig.3a

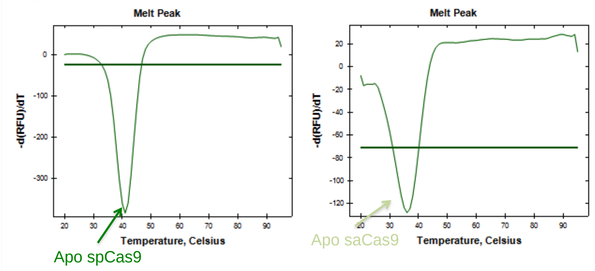


Fig.3b

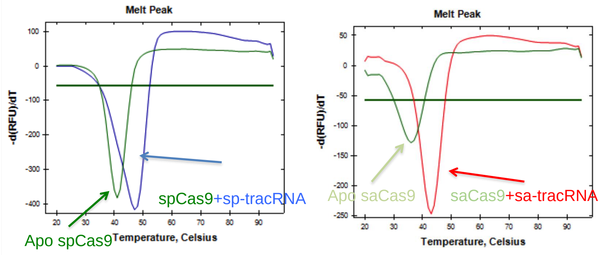


Fig.3c

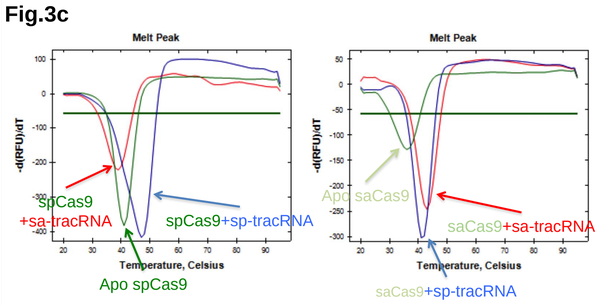


Figure 3(a) The thermal stability of apo Cas9 orthologs were measured to be spCas9 is unfolding at 41˚ C and the less stable saCas9 at 36˚ C. **(b)** When Cas9 is incubated in equal molar amounts of tracrRNA a shift is observed. **(c)** When each Cas9 is incubated with orthogonal tracrRNA, we do not observe an equivalent shift in thermal stability.

RNP Thermal Stability

Fig.4

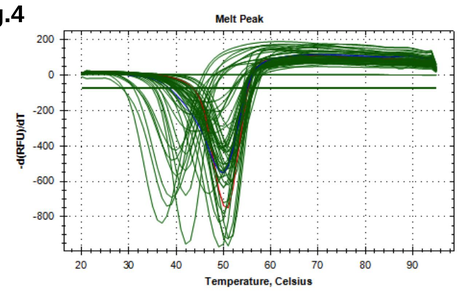


Figure 4 Thermal stability measurement of 43 spRNPs along with spCas9+sp-tracrRNA and apo spCas9

Table 1

ID	Tm	ID	Tm	ID	Tm	ID	Tm
A01	51	B01	49/43	C01	50	D01	45
A03	51	B03	50	C03	50	D03	47
A05	47/52	B05	50	C05	50	D05	52
A07	47/52	B07	50	C07	50	D07	51
A09	49	B09	42	C09	52	D09	52
A11	49	B11	50	C11	52	D11	50
A13	51	B13	51	C13	50	D13	50
A15	51	B15	51	C15	50	D15	52
A17	50	B17	51	C17	52	Cas9	42
A19	49	B19	49	tracr	50		
A21	50	B21	50	C21	47		
A23	50	B23	50	C23	44		

Table 1 Corresponding Tm for spRNPs in figure above. We observe that the majority of spRNPs show a single peak with a Tm greater than apo spCas9 with the majority of the shift due to the tracrRNA.

Indel Formation in Hek293FT Cells

Table 2

ID	Tm	Indel %	ID	Tm	Indel %	ID	Tm	Indel %
A01	51	0	B01	49/43	8.3	C01	50	17
A03	51	0	B03	50	2.6	C03	50	20.5
A05	47/52	0	B05	50	7.2	C05	50	16
A07	47/52	13.2	B07	50	ND	C07	50	26.1
A09	49	12.5	B09	42	9.6	C09	52	0
A11	49	7.7	B11	50	0	C11	52	0
A13	51	28.8	B13	51	4.8	C13	50	0
A15	51	16.7	B15	51	0	C15	50	0
A17	50	7.9	B17	51	4.2	C17	52	5.15
A19	49	0	B19	49	0	tracr	50	0
A21	50	0	B21	50	3.1	Cas9	42	0
A23	50	19.8	B23	50	7.4			

Table 2 Comparing the Tm to indel percentage as determined by T7E1, we see that RNP formation is not directly correlated to efficient indel formation in cells.

Thermal Stability as a measure of RNA Quality

Table 3

ID	Tm	Indel %
Batch 1	50	26.14
Batch 2	44	11.64
Batch 3	50	27.33
Batch 4	47	11.43
Batch 5	51	22.74

Table 3 We formed RNP consisting of spCas9 and 5 different lots of sgRNA with the same sequence. We observed that the most efficient indel formation occurred with the larger thermal shift. This suggests that Tm for a particular RNP is dependent on sgRNA quality, which leads to proper RNP formation and may lead to efficient indel formation.

Thermal Stability and Off-target Effects

Table 4

VEGF Target	Tm	VEGF site	Off-target Sites
GGGTGGGGGGAGTTTGCTCC	50	Site 1	21
GACCCCCTCCACCCCGCCTC	50	Site 2	151
GGTGAGTGAGTGTGTGCGTG	50	Site 3	59

Table 4 Thermal-stability of RNP with guides of known off—targets as previously determined by Guide-Seq show that RNA-protein stability is not the determining factor.

Methods:

RNP was formed by mixing equal-molar amounts of RNA and protein in H150 pH7.5 buffer then incubated at RT. A portion of this mixture was used for the thermal stability assay by incubating with 5x Sypro Orange and running a linear gradient from 20˚ C to 95˚ C on a Bio-Rad CFX384 Real-Time System C1000 Touch Thermal Cycler with a 1˚ C increase in temperature every 10”. The remaining material was transfected into HEK293FT cells via Lipofectamine2000. Indel formation was measured by a T7E1 assay.

Conclusions:

- A fluorescent thermal stability assay is a reliable method for determining RNP integrity
- This method can also be used for screening of novel tracrRNA like molecules
- While an increase in thermal stability is not directly correlated with indel formation it is highly correlated with RNP formation
- For a majority of guides tested thermal shifts were in a range 5-6 ˚ C