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

**Fig. 3a** Schematic drawing of Cas9-gRNA-DNA target with cutting site of each domain.

**Fig. 3b** 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).

**Fig. 3c** 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. 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.

**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.

**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.

**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.

**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