Probing The Quality of Cas9 Ribonucleoprotein Complex Using Biochemistry and Biophysics

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Background

- Editas Medicine is developing CRISPR Cas9, an RNA-guided DNA nuclease, as a gene editing therapeutic. In *ex vivo* gene editing, isolated cells will be modified by direct delivery of the Cas9-gRNA ribonucleoprotein complex (the Cas9 RNP) (**Figure 1**).
- This approach requires a detailed characterization of the Cas9 RNP complex with regard to composition, stability, and potency among others.
- To assess the complexation quality of the RNP, differential scanning fluorimetry (DSF), a well-established biophysical assay, was used to measure the thermostability of RNPs (**Figure 2**).
- Two plate types were screened to optimize the DSF fluorescence signal fidelity. White coated plates otherwise optimal for fluorescence measurements showed spurious peaks whereas clear bottom plates showed only one peak indicating a clear melting transition which allows for accurate melting temperature (Tm) measurement (**Figure 3**). The higher the Tm the greater is the stability of the RNP complex.
- To characterize the RNP from two gRNA formats (full length and truncated) we examined the DSF profiles and biochemical cutting activity (**Figure 4**). Truncated gRNA (lengths < 80 nucleotides) which are easier to obtain synthetically show reduced efficacy and lower stability.
- Proper folding of this gRNA is likely required for optimal RNP complexation and nuclease activity. We investigated the effect of folded and misfolded or aggregated gRNA on RNP complexation by DSF (**Figure 5**). Misfolded gRNA destabilized the Cas9 protein reducing its Tm as compared to Apo Cas9. Finally, RNP complexes from misfolded gRNA were able to edit cells less efficiently than those from properly folded gRNA (**Figure 6**).
- These results indicate that gRNA format and folding influence RNP stability and activity. Understanding the impact of folding on efficient and inefficient gRNA will significantly help improve the fidelity of screening campaigns to identify the optimal gRNA to mediate a genomic edit.

Conclusions

- We demonstrate the characterization of the Cas9 RNP complex by an optimized differential scanning fluorimetry assay.
- We show that the folding of the gRNA affects RNP complexation and has an impact on the activity of the RNP.
- The combined use of DSF, biochemical cutting and cellular potency will help to understand the various determinants of RNP stability towards enabling its use as a therapeutic.

Ex vivo Gene Editing Workflow

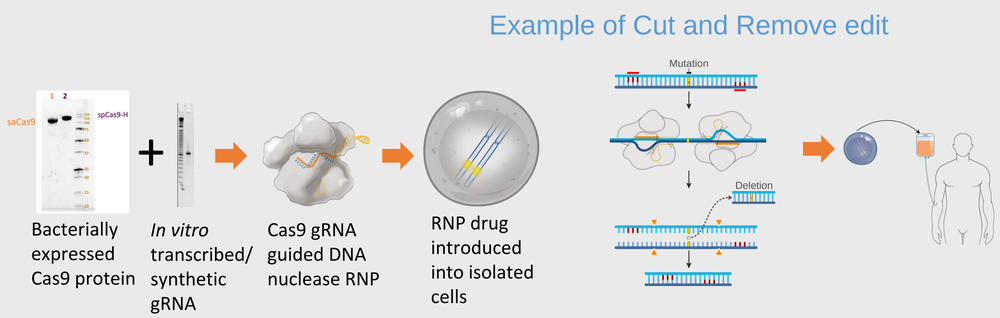

Figure 1: Purified Cas9 protein from bacteria are combined with *in vitro* transcribed or synthetic gRNA to form the Cas9 RNP. This RNP complex is then introduced into cells isolated from the patient to carry out the genomic edit. Edited cells are then introduced back into the patient.

Differential Scanning Fluorimetry (DSF)

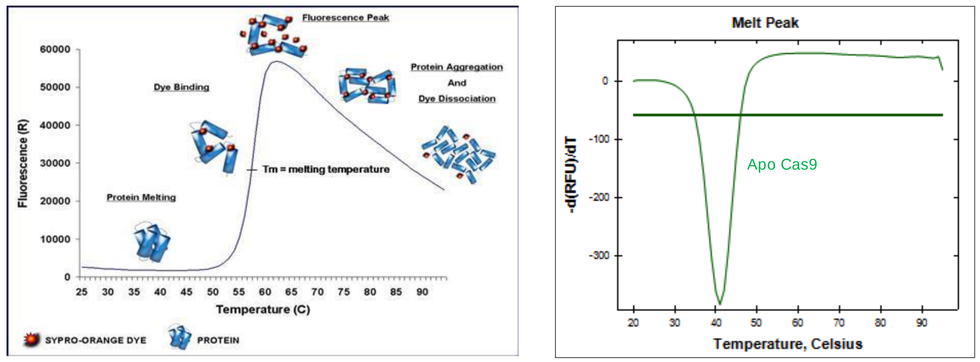

Figure 2: DSF works by monitoring the unfolding of a protein using sypro-orange dye. Unfolding of the protein exposes hydrophobic regions causing dye to bind and be less quenched than in free solution resulting in an increase in fluorescence (**left**). DSF profiles are analyzed as a second derivative of the fluorescence signal (**right**) where the trough indicates the melting point (Tm) of the complex being studied.

Assay Optimization: Clear Bottom Plates Give Optimal DSF Signal

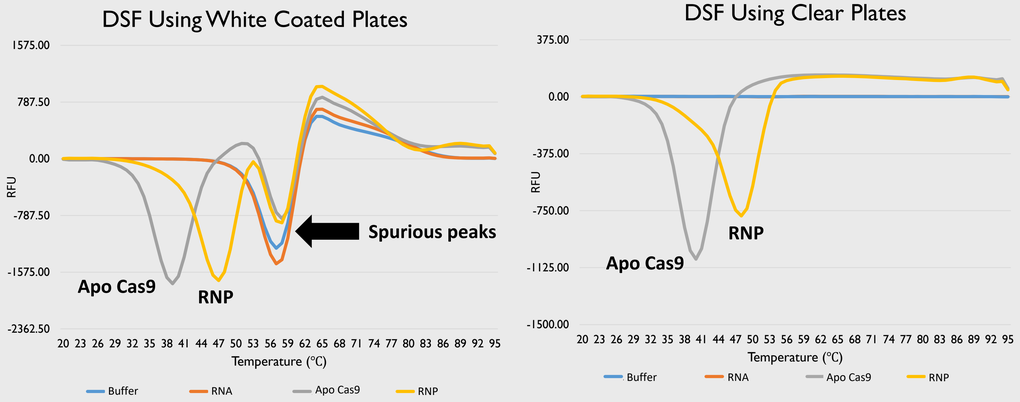

Figure 3: Comparison of DSF signal obtained using white coated plates (**left**) with clear bottom plates (**right**) across multiple CFX96 Real-Time PCR detection systems. The white coated plates irreproducible showed spurious peaks at 60 °C as compared to clear bottom plates that did not show this across multiple RT-PCR machines tested.

Truncations To gRNA Affect Biochemical Activity

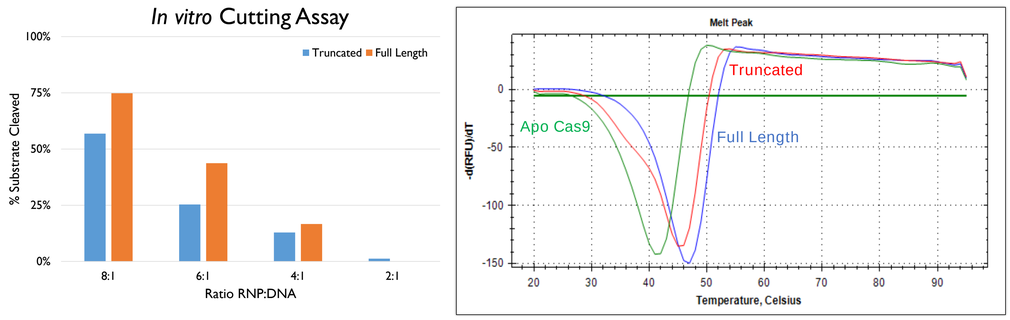

Figure 4: Comparison of *in vitro* biochemical cutting activity of truncated (blue) and full-length (orange) gRNA (**left**). DSF profile of truncated and full-length gRNA (**right**). Truncated gRNA complexes are less stable (lower Tm) and show less efficient biochemical cutting.

DSF Can Distinguish Folded and Misfolded gRNA RNP Complexes

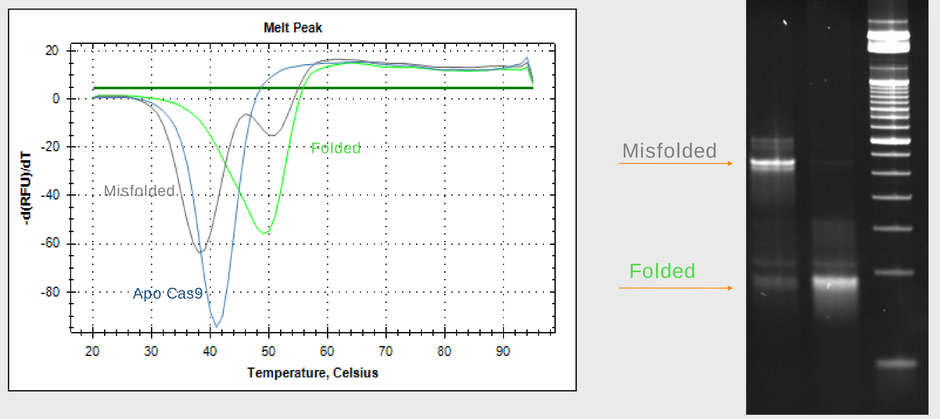

Figure 5: DSF profile (**left**) and native PAGE gel (**right**) of folded and misfolded or aggregated gRNA. Misfolded gRNA tend to destabilize the RNP complexes and show lower Tms.

RNP With Misfolded gRNA Cut Less Efficiently

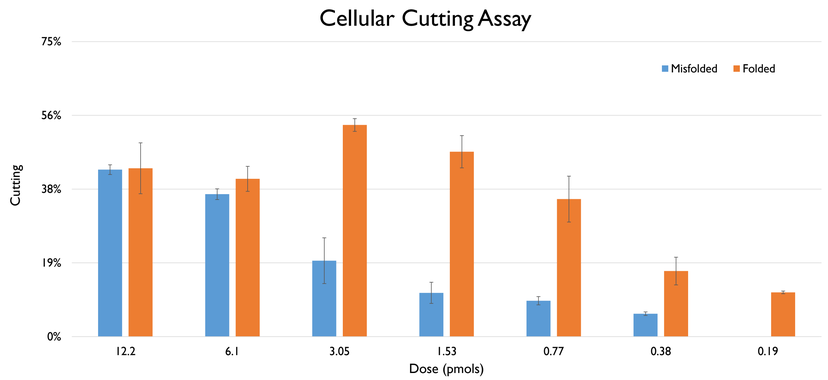

Figure 6: Cellular cutting efficiency of misfolded (blue) and properly folded (orange) gRNA. A higher amount of misfolded gRNA complex is required to get efficient cutting. The ability of improperly folded gRNA RNP complex to cut in cells suggests a possible ensemble of folded/unfolded states resulting in a reduced potency.