Assessing Cas9-gRNA ribonucleoprotein complex formation for development of ex-vivo therapeutics

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Abstract

Editas Medicine is working to develop therapies to correct the underlying cause of genetic diseases using the CRISPR-Cas9 system.

CRISPR-Cas9 is an RNA-guided DNA endonuclease where an ~100 nucleotide guide RNA (gRNA) is complexed with the ~135 kDa Cas9 protein to target it to the site of the genetic mutation. In the case of *ex vivo* gene editing therapies, isolated patient cells will be modified by direct delivery of the Cas9 ribonucleoprotein complex(RNP).

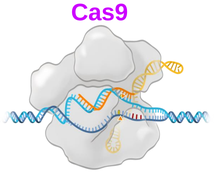
These approaches will require a detailed characterization of the RNP complex and its constituent Cas9 protein and gRNA.

Here we highlight the effect of gRNA folding behavior for the proper formation of functional RNP complexes. We use differential scanning fluorimetry (DSF), a fluorescence-based assay that monitors the thermal unfolding transition of the Cas9 RNP complex as a qualitative method to characterize RNP complexes.

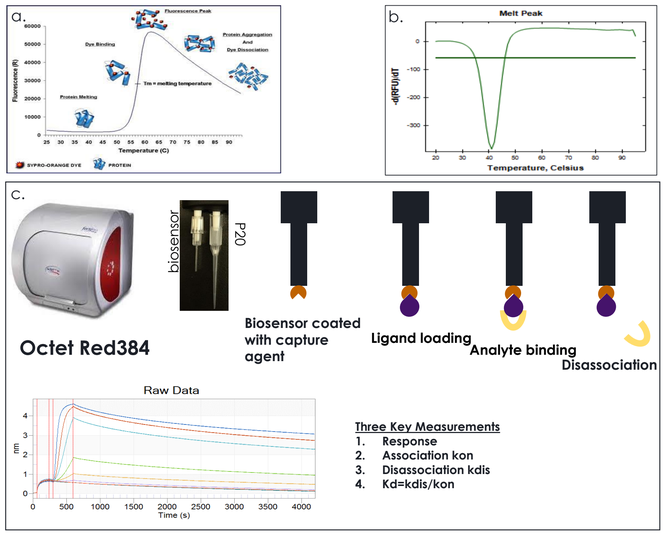
Towards developing a quantitative assay for gRNA folding we describe using bio-layer interferometry to measure the response rate (signal intensity) resulting from the interaction of folded and unfolded gRNA with the Cas9 protein.

Finally the in vitro biochemical activity and cellular potency of these RNP complexes were measured to correlate the biophysical measurements with activity of the complexes. Improperly folded gRNA lower the stability of RNP complexes and show lower biochemical and cellular potency.

Cas9



1. Differential scanning fluorimetry and Bio-layer interferometry



(a) Sypro-Orange dye binds to hydrophobic regions of proteins and emits florescence as protein becomes unfolded due to thermal melting.

(b) Melting temperature (Tm) of un-complexed SpCas9 was measured to be 41° C in solution.

(c) The Octet instrument measures the changes in binding of proteins and ligand to the optical sensor tip by interferometry. The rate of change of signal response is used to measure the kinetics of binding and the dissociation constant (Kd) of a protein-ligand interaction.

2. gRNA truncations alter RNP stability and activity

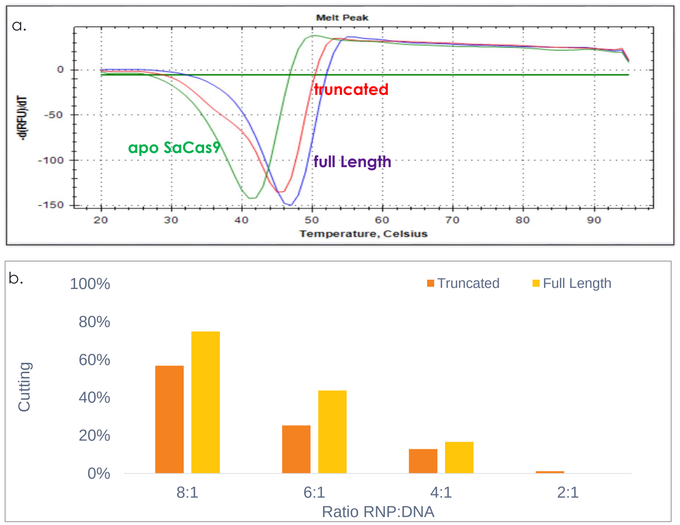
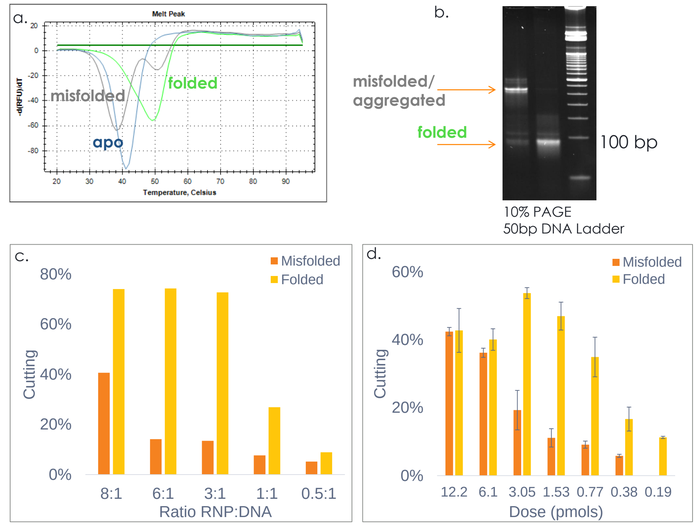


Figure 2: (a) Thermostability of RNPs formed with 3' truncated 75mer (red) and full-length 100mer (blue) *in vitro* transcribed gRNAs. We show that 3' truncation of the gRNA reduces SaCas9 RNP thermostability.

(b) Biochemical cutting activity of corresponding SaCas9 RNPs on synthetic substrate. Truncated gRNA shows decreased cutting activity.

3. Effect of gRNA folding on RNP stability and activity



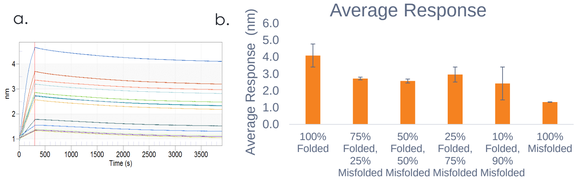
(a) Thermostability of RNP formed with misfolded gRNA indicates only a partial complexation of properly formed RNP as measured by DSF.

(b) Native-PAGE gel electrophoresis shows poor migration of misfolded gRNA indicating possible aggregation.

(c) In vitro cutting activity at various nuclease:substrate ratios shows less activity with misfolded gRNA.

(d) Cellular potency of RNP formed with folded and misfolded gRNA. Misfolded gRNA results in lower cellular efficacy.

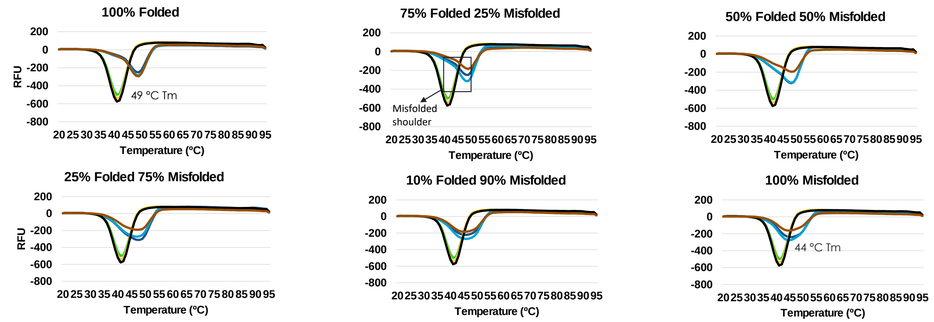
4a. BLI can be used to discern folded and mis-folded gRNA



a) BLI profiles measuring SpCas9 binding to varying concentrations of misfolded gRNA

b) Misfolded gRNA change the magnitude of the response but with limited dynamic range

4b. DSF can distinguish folded and mis-folded gRNA-RNPs



Conclusions

We demonstrate the use of DSF and BLI as two key methods of quality control to our RNP-based gene editing therapeutics. Furthermore, RNPs composed of folded or misfolded gRNA show differing stability and cellular editing efficacies

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