Characterization of gRNAs and Ribonucleoproteins for CRISPR Applications

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Editas Medicine

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Disclosures

Steven Wolk is an employee and shareholder of Editas Medicine, Inc.

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- Keith Jarvis and the Editas Boulder PD Team

Outline

Introduction to CRISPR and RP-IP-UPLC Purity Methods

- Brief Introduction to CRISPR
  - gRNAs and RNPs

Characterization of Secondary Structure and Multimer Formation

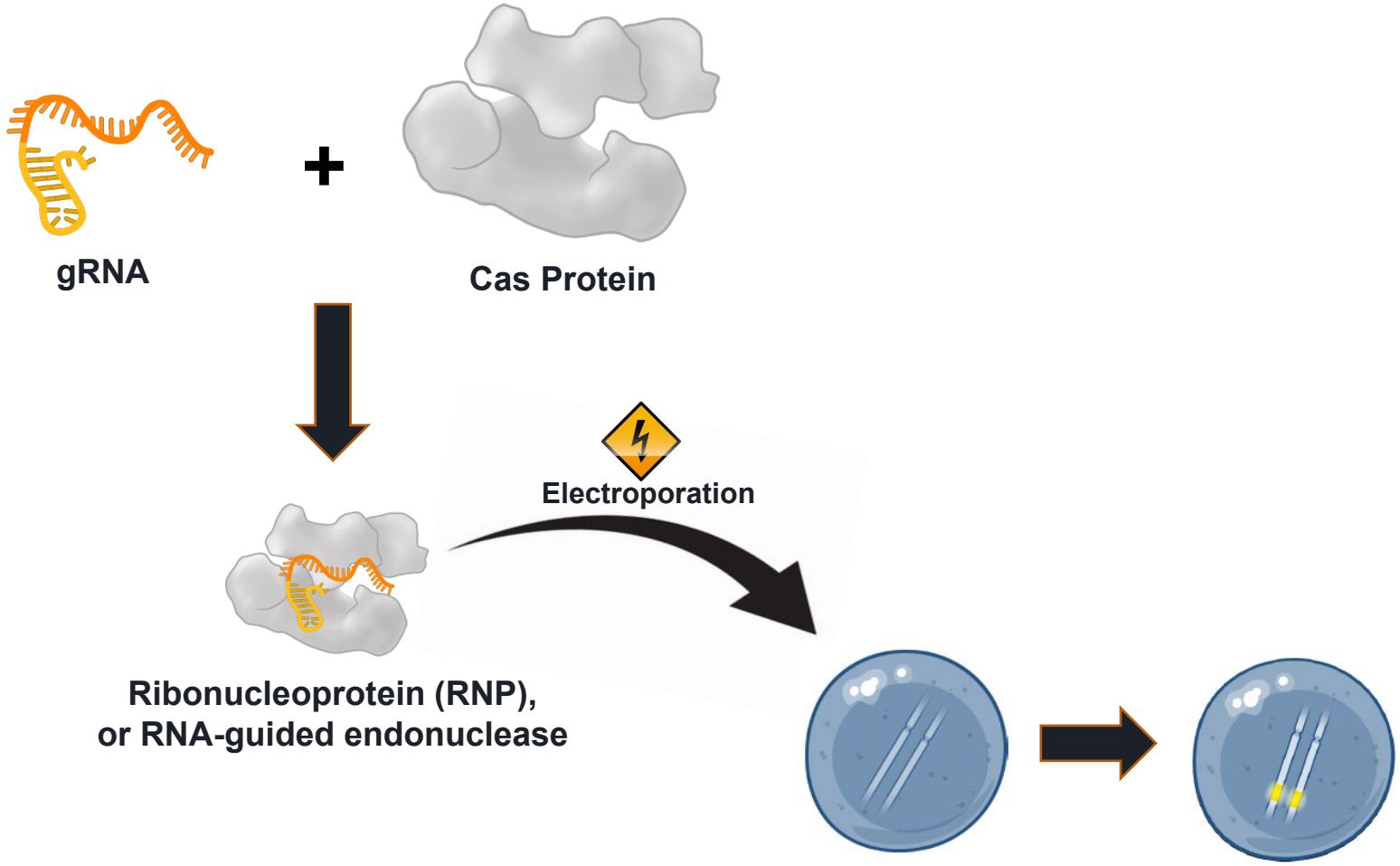
- gRNAs
  - methods for characterizing intramolecular folding and multimer formation
  - secondary structure predictions, PAGE, SEC, Ion Mobility

- CRISPR enzyme complex (RNP)
  - impact of these structures on complex formation
  - PAGE, SEC, Ion Mobility, IEX

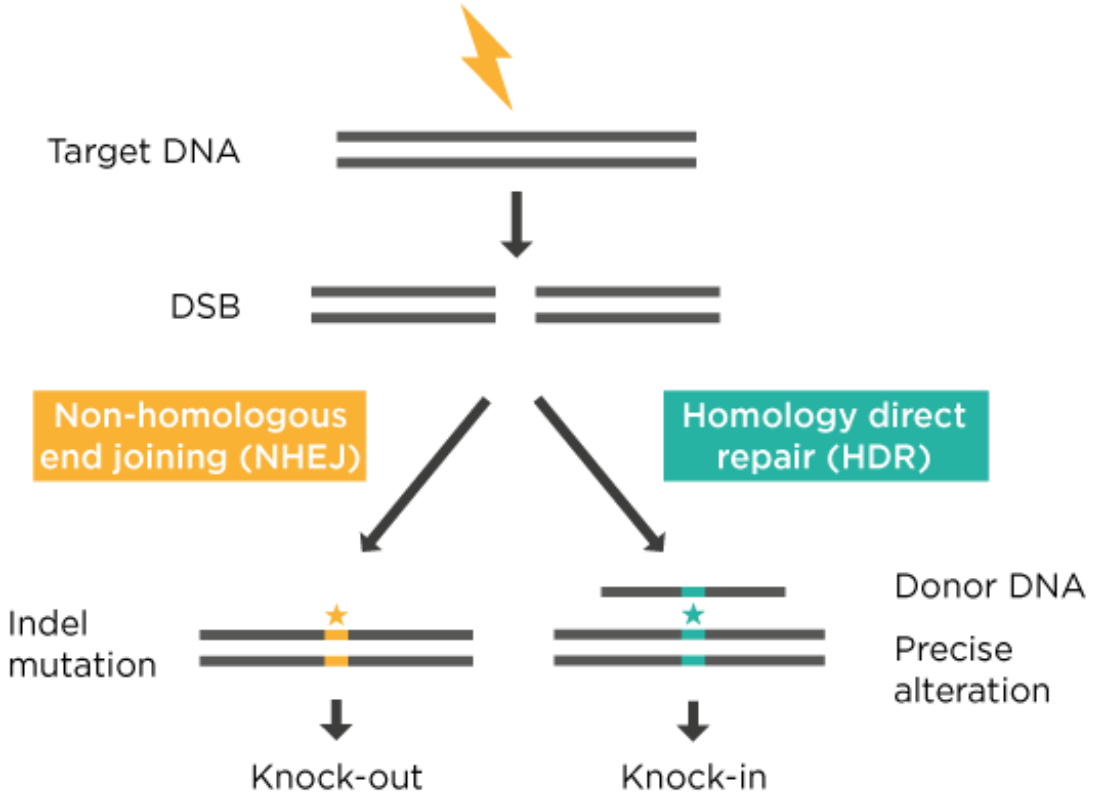
Concluding Remarks and Acknowledgements

CRISPR Basics

RNP: The Simple View of a Cell Based Medicine



Knock-outs and Knock-ins



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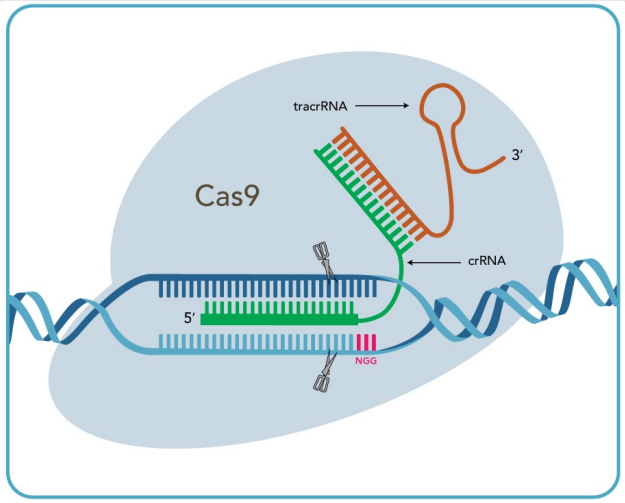
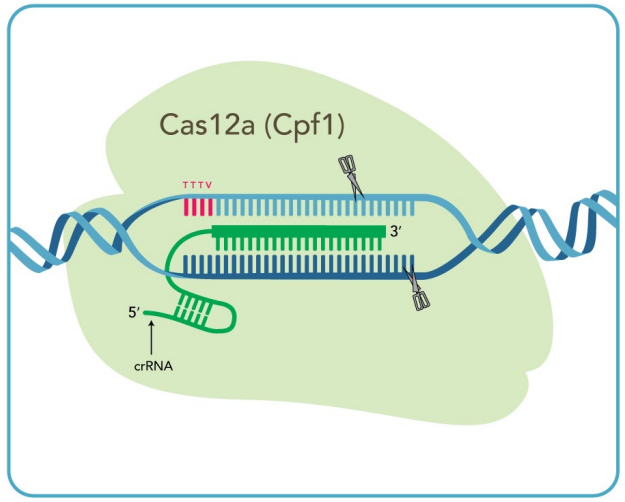
Cas9 vs. Cas12a



Cas9

- Separate crRNA and tracrRNA (can be synthesized as single guide RNA (sgRNA) (~100mer)
- G-rich PAM: NGG
- Blunt-ended cut
- Endonuclease domains:
  - RuvC-like (cleaves non-target strand)
  - HNH (cleaves target strand)
- Target sequence on 5’-terminus of the gRNA



Cas12a

- Single, shorter crRNA guide (40mer)
- T-rich PAM: TTTN
- staggered DNA cut with 4-5 nt overhangs
- Endonuclease domain: RuvC-like only
- Target sequence on 3’-terminus of gRNA
- **Shorter guides → higher purity, fewer competing structures**
- **3’-target region → better specificity**

Tang & Fu (2018) Cell Biosci 8:59.
https://www.idtdna.com/pages/products/crispr-genome-editing
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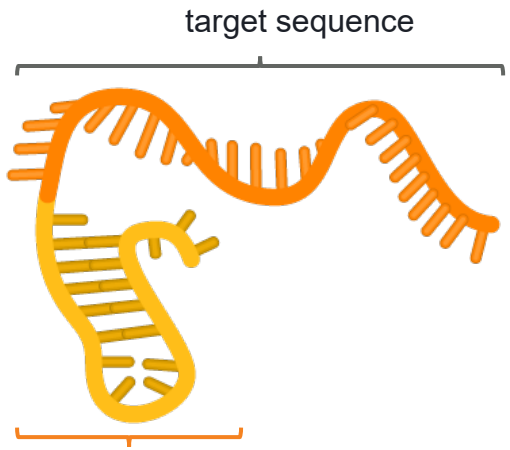
gRNA Characterization

It's Complicated……

RNA Secondary Structure

- This is how we like to think the gRNA looks:
- Which it does in the RNP crystal structures



RNA Secondary Structure

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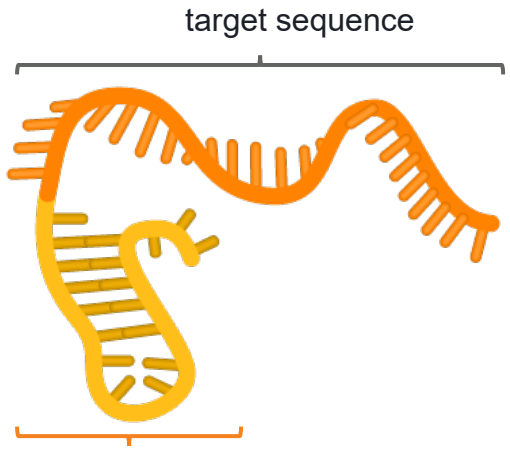
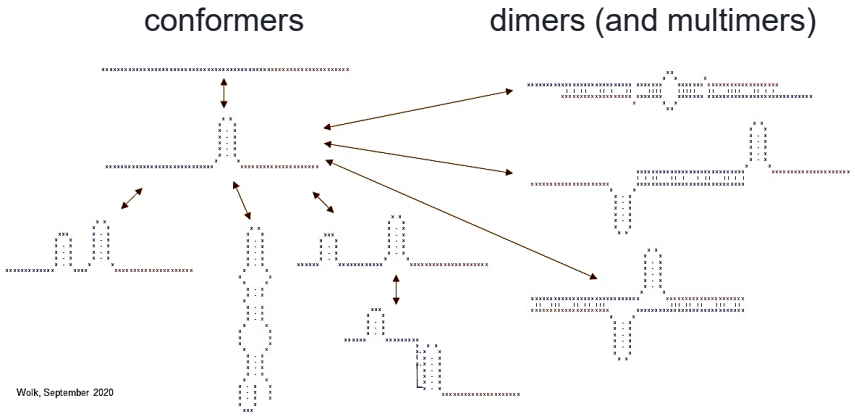
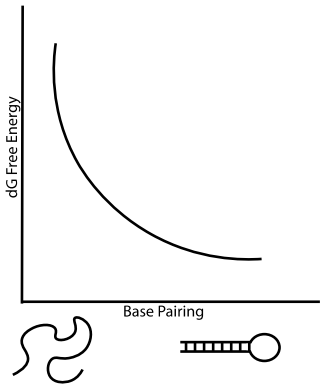


- The situation is likely much more complicated
- Gets more complicated with increasing length

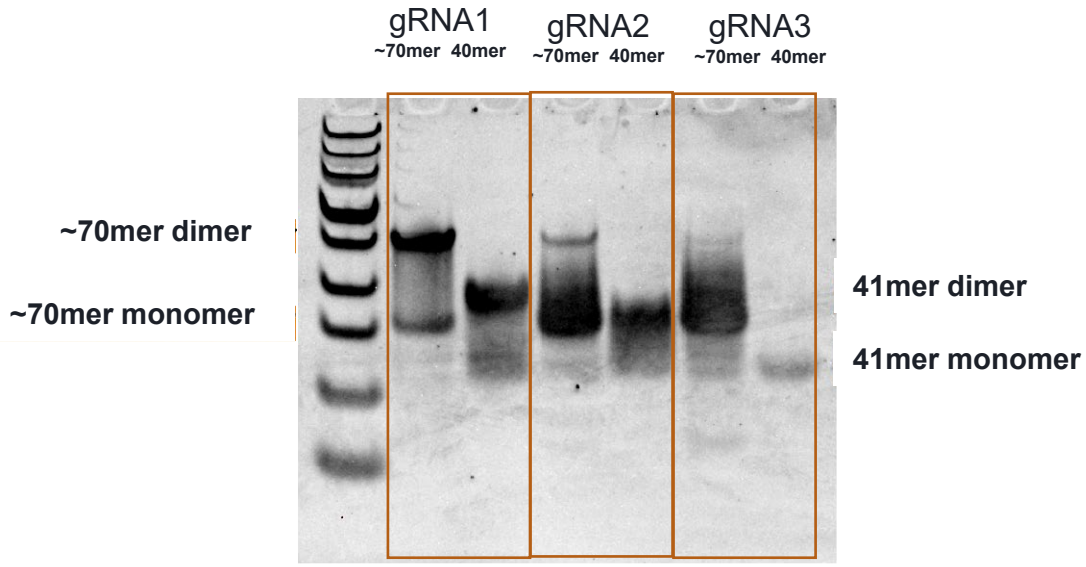




Characterization Platform

- **Guide Characterization**
  - (Purity/impurity Profile by LC/MS – not addressed today)
  - Conformational Analysis
    - secondary structure predictions
    - PAGE
    - SEC
    - ion mobility

Dimerization for gRNA is Sequence Dependent



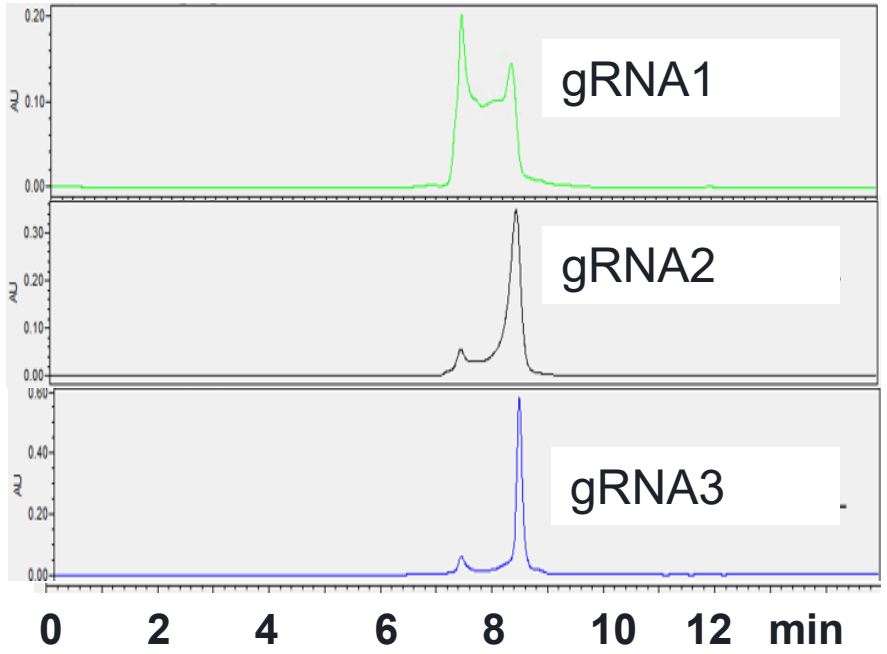
34 mM Tris, 66 mM HEPES, 75 mM NaCl, 2 mM $MgCl_2$, pH 7.5

guide	41mer	~70mer
gRNA1	dimer	dimer
gRNA2	dimer	monomer
gRNA3	monomer	monomer

SEC of gRNAs

Impact of gRNA Multimers

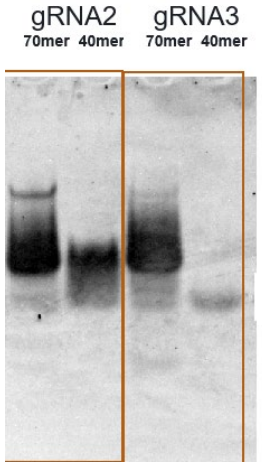
SEC of 3 gRNAs (~70mer versions): only change is the target sequence within the gRNA (gRNAS analyzed on a Waters UPLC-SEC column)



PAGE Results:

guide	41mer	~70mer
gRNA1	dimer	dimer
gRNA2	dimer	monomer
gRNA3	monomer	monomer

HOS for apo-gRNA is Sequence Dependent

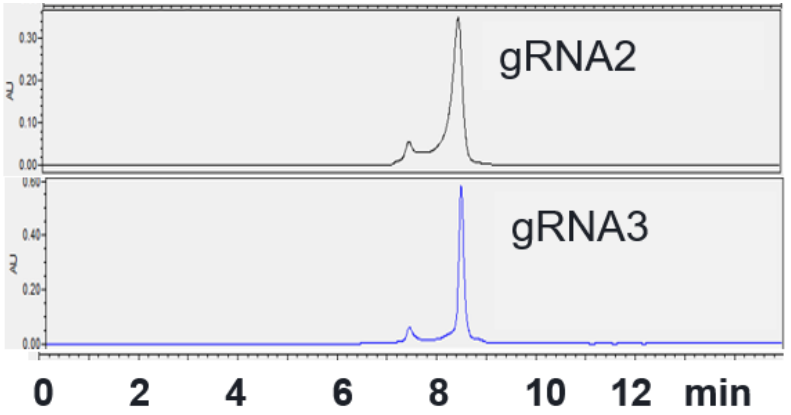


41mer dimer

41mer monomer

34 mM Tris, 66 mM HEPES, 75 mM NaCl, 2 mM $MgCl_2$, pH 7.5

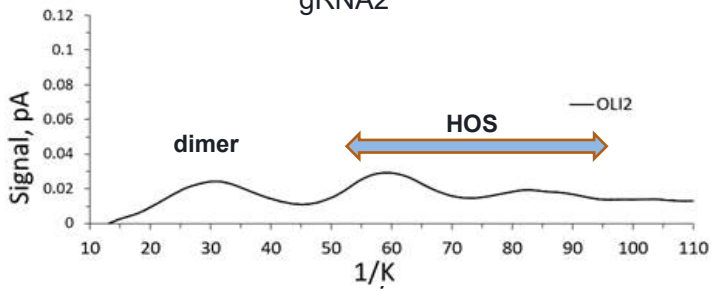
SEC (~70mers)



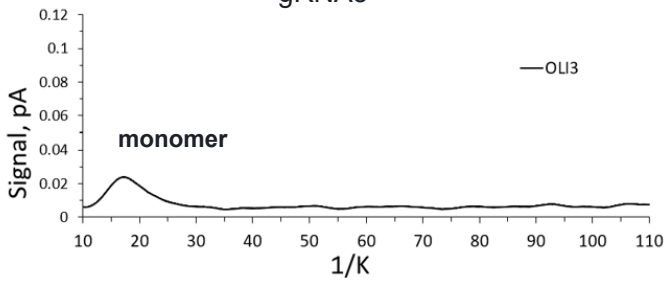
150 mM NaCl, 100 Na-Phos, pH 7.4

Ion Mobility Data (~70mers), 5 mM $(NH_4)OAc$, pH 7.4
IonDX IMgenius

gRNA2



gRNA3



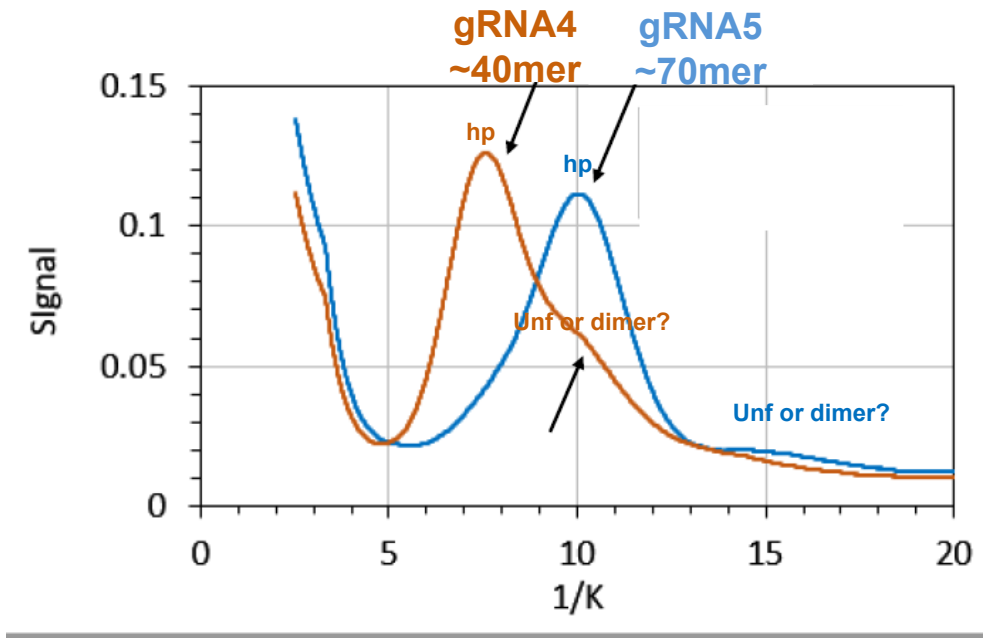
1/K ∝ collisional cross-section (size)

Detection of gRNA Folding via IM

Detection by IM

Two additional gRNAs (40mer and ~70mer)

- 25 mM $(NH_4)_2CO_3$, pH 8.5
- earlier IM hardware parameters (resulting in different 1/K values)



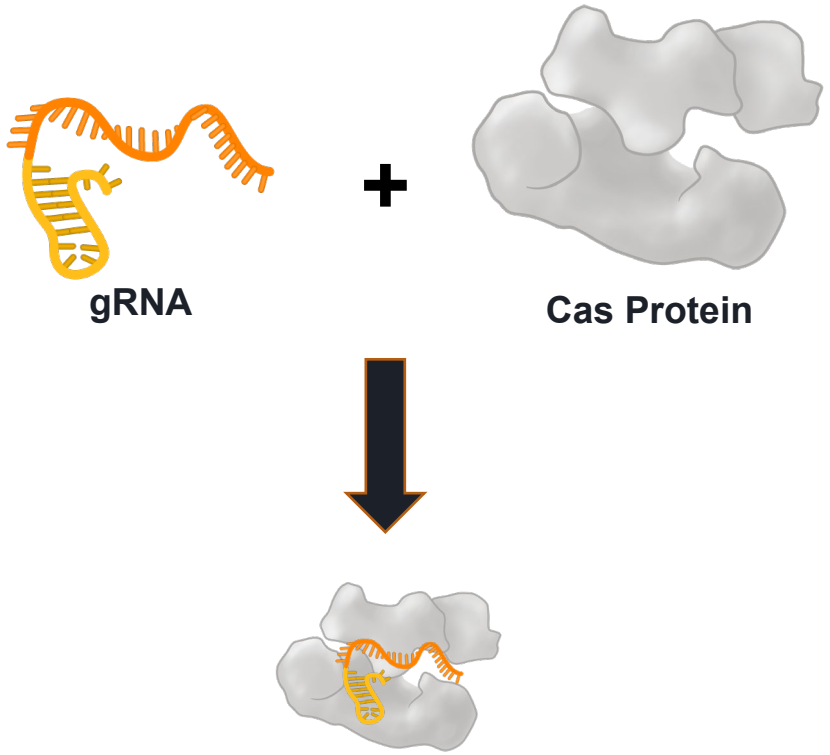
Summary of gRNA Data

guide	Secondary structure prediction	PAGE	SEC	IM
	Vienna RNA-fold	75 mM NaCl 34 mM Tris 66 mM HEPES 2 mM $MgCl_2$ pH 7.5	150 mM NaCl 100 Na-phos pH 7.4	5 mM $(NH_4)OAc$ pH 7.4
gRNA2	prone to multimer formation	mostly monomer	mostly monomer (exchange observed)	all HOS
gRNA3	not prone to multimer formation	monomer	mostly monomer	monomer

RNP Characterization

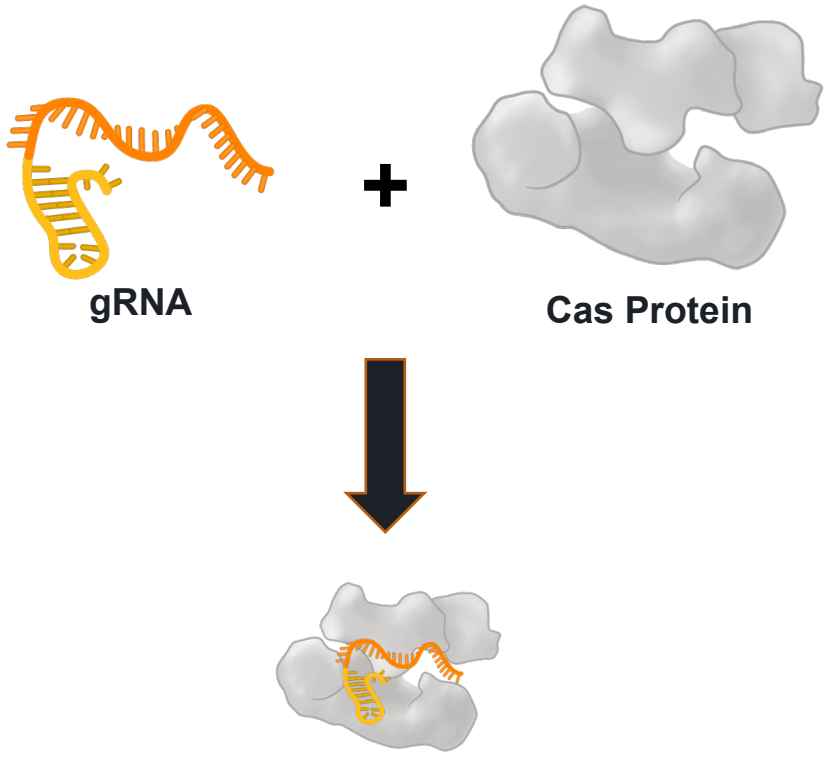
It's More Complicated…..

RNP: The Simple View



RNP: The Less Simple View

Impact of Conformers and multimers



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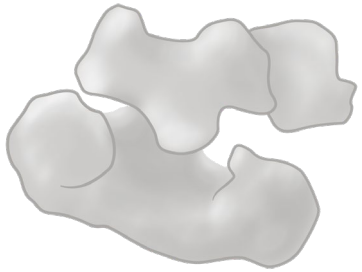
RNP: The Less, Less Simple View

Impact of Conformers and multimers

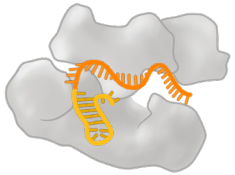
gRNA

+



Cas Protein

- truncates
- PTMs
- disulfide variants
- aggregates (soluble/insoluble)
- degradants (oxi, deamidation)



Ribonucleoprotein (RNP), or RNA-guided endonuclease

- multiple stoichiometries
- aggregates (soluble/insoluble)
- dynamics/exchange

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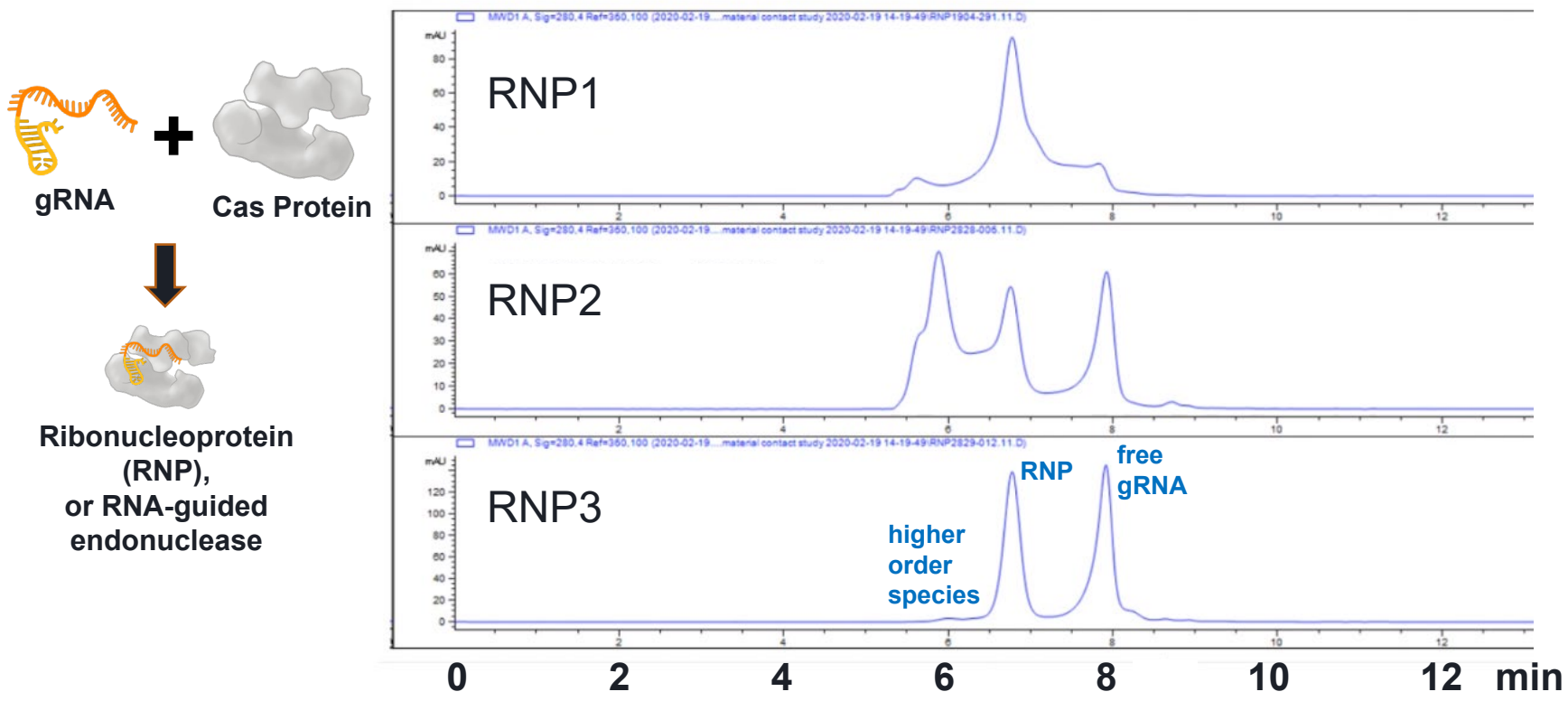
SEC of RNPs

Impact of gRNA Multimers

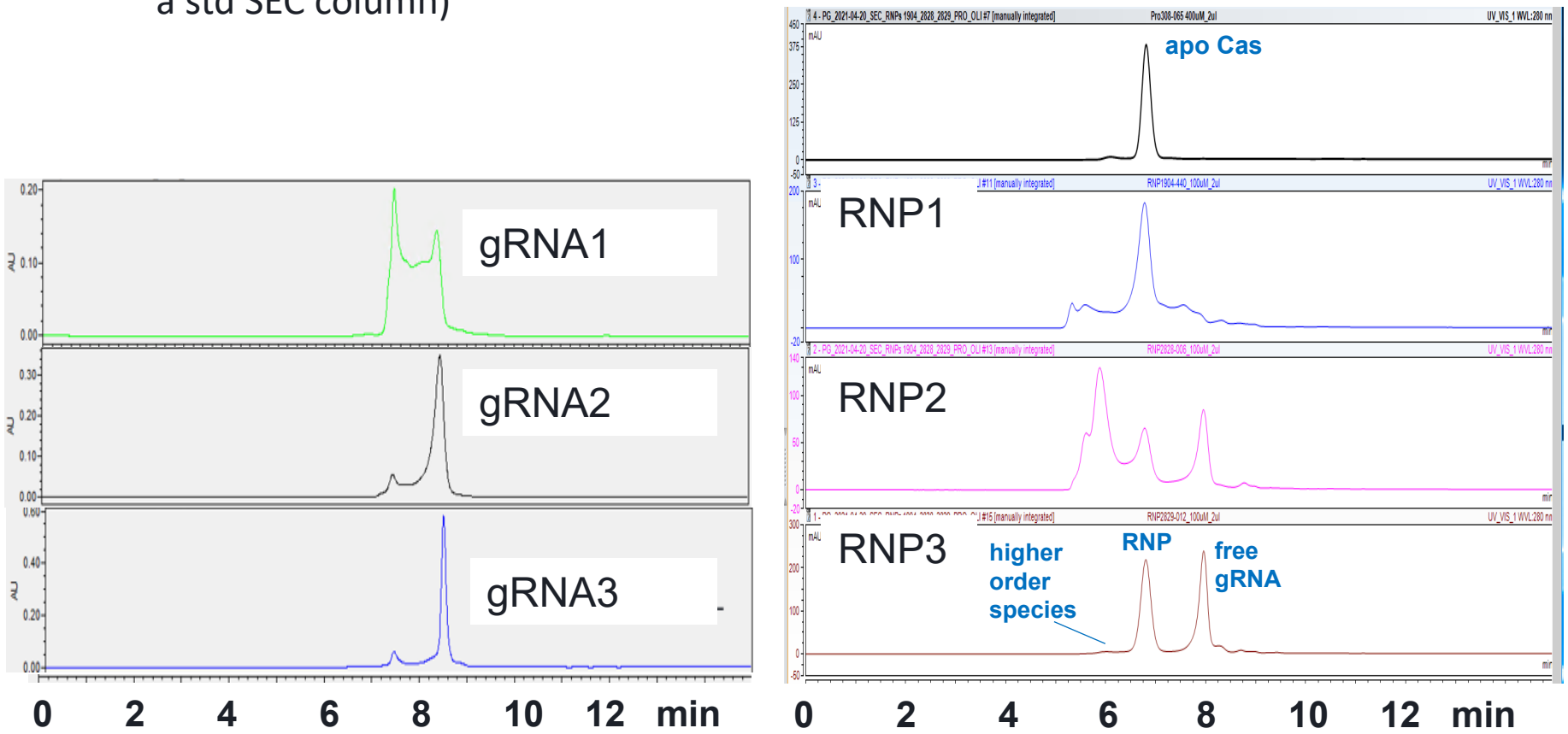
SEC of 3 RNPs: only change is the target sequence within the gRNA



SEC of RNPs

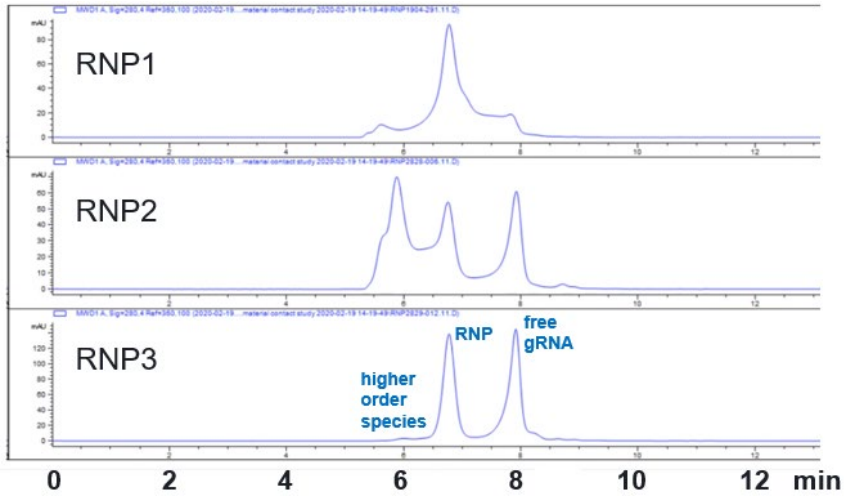
Impact of gRNA Multimers

SEC of 3 RNPs: only change is the target sequence within the gRNA (gRNAS analyzed on a Waters UPLC-SEC column; RNPs analyzed on a std SEC column)

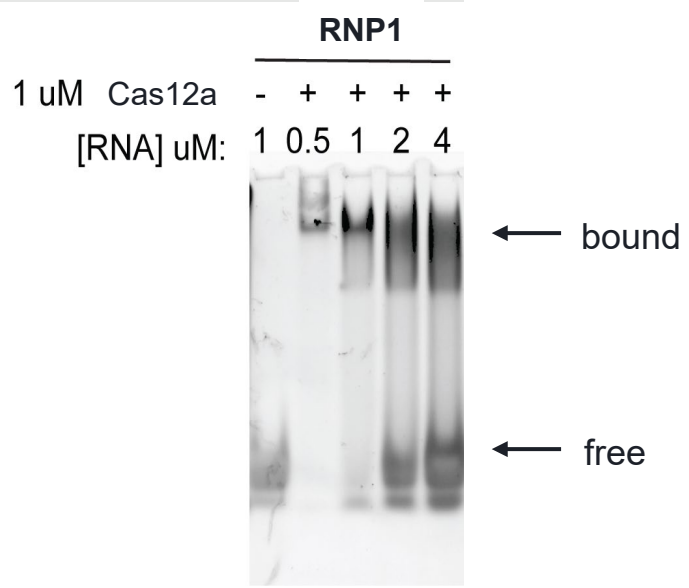


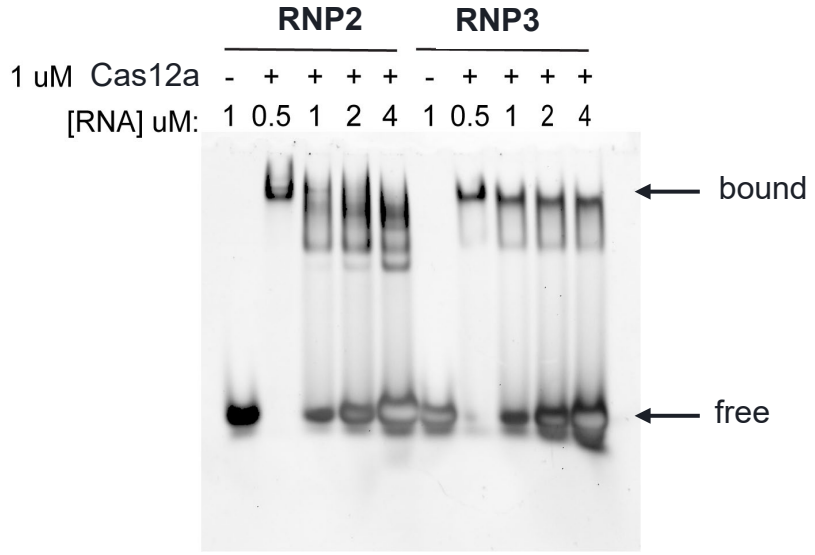
SEC and Gel Shift Data Correlate

SEC



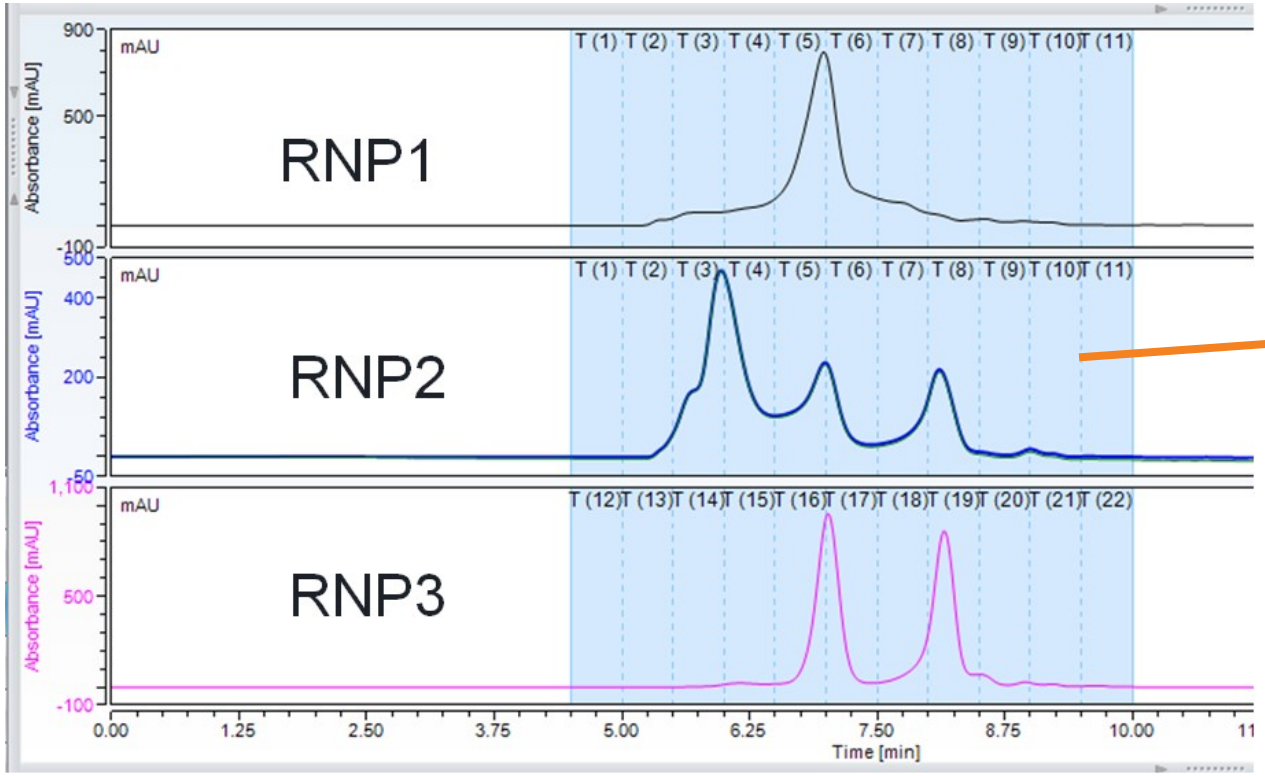
SEC analysis of 2:1 gRNA:PRO

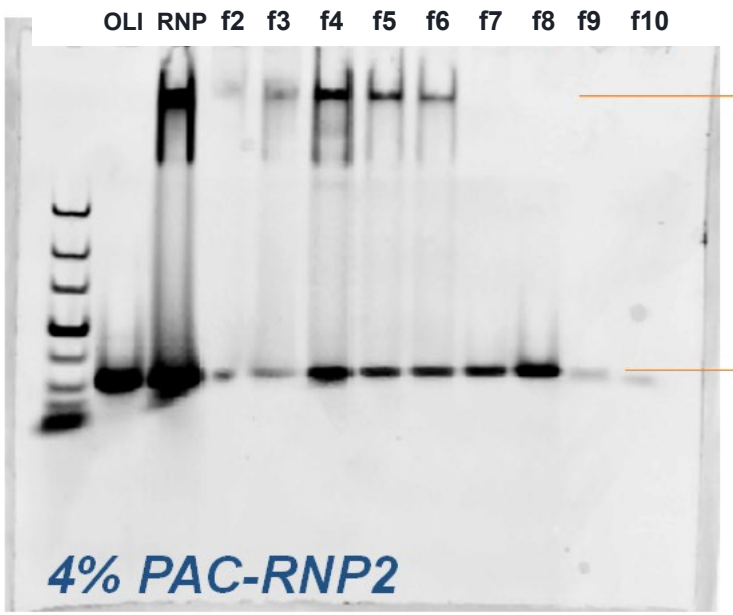




RNP2: SEC+PAGE

See Poster T.1 (Ghude et al.)



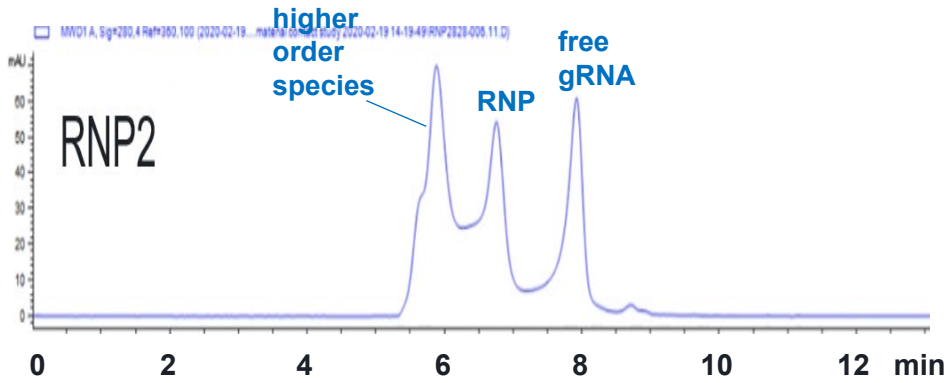


Complexed gRNA in RNP2

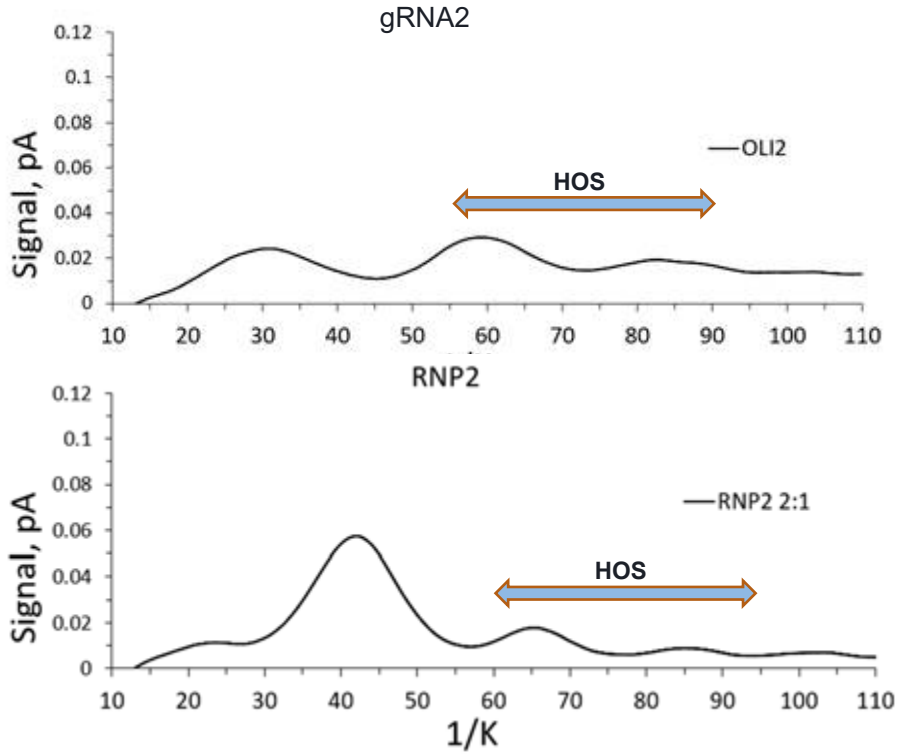
Free gRNA monomer in RNP2

- Lane 1: LMW DNA ladder
- Lane 2: gRNA2 (unfractionated)
- Lane 3: RNP2 (unfractionated)
- Lane 4-12:SEC-Fractions of RNP2 (F2-F10)

RNP2: SEC vs. IM



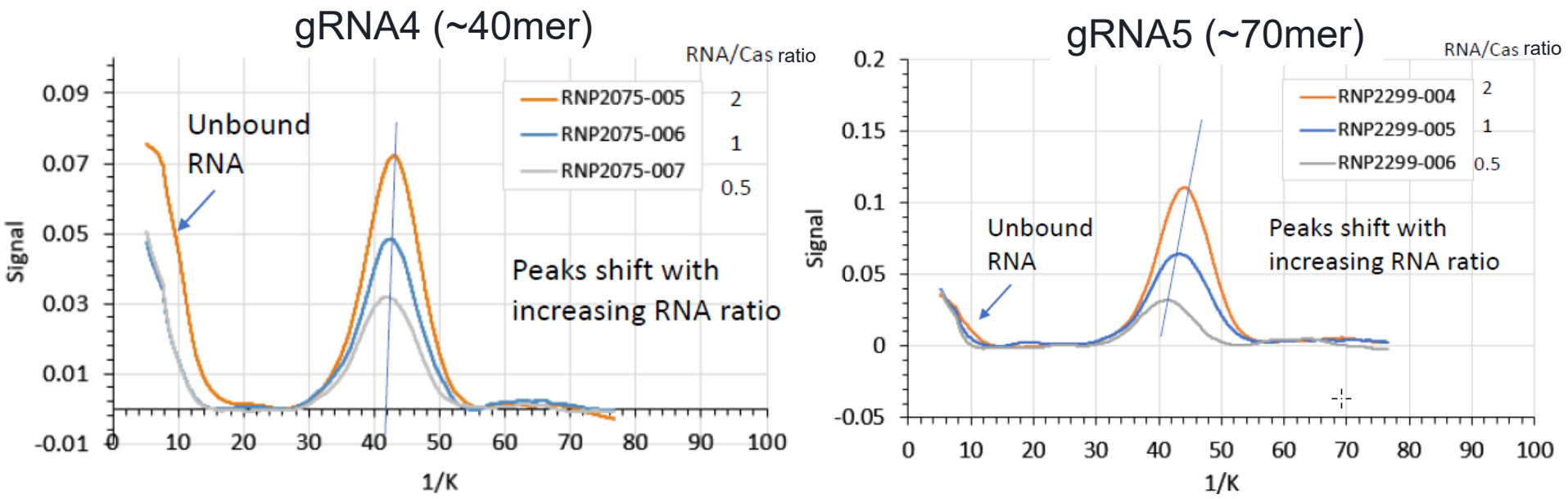
IM data in 5 mM NH_4OAc, pH 7.4



1/K	MW_{calc}
41.97	159 kDa
65.08	332 kDa
85.15	519 kDa

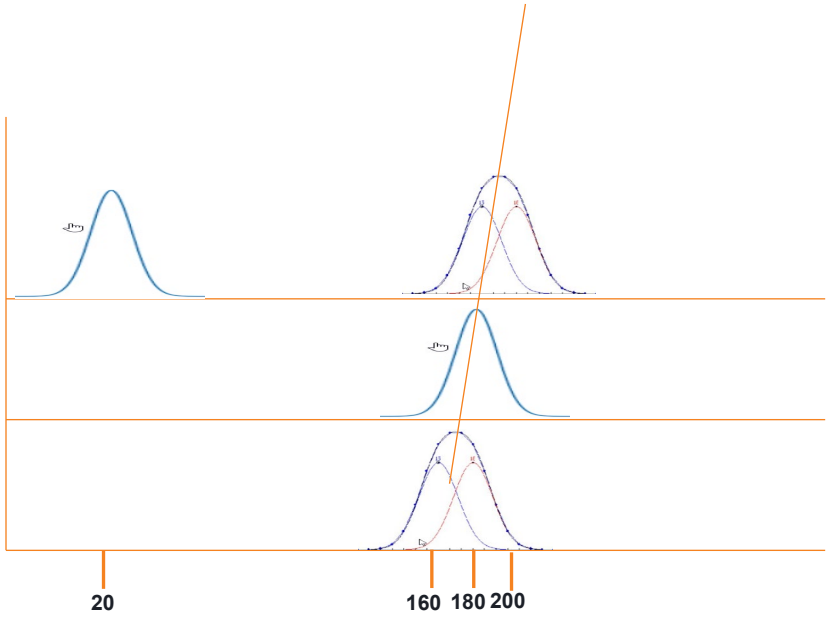
RNPs via IM: Varying the gRNA:Cas12a ratio

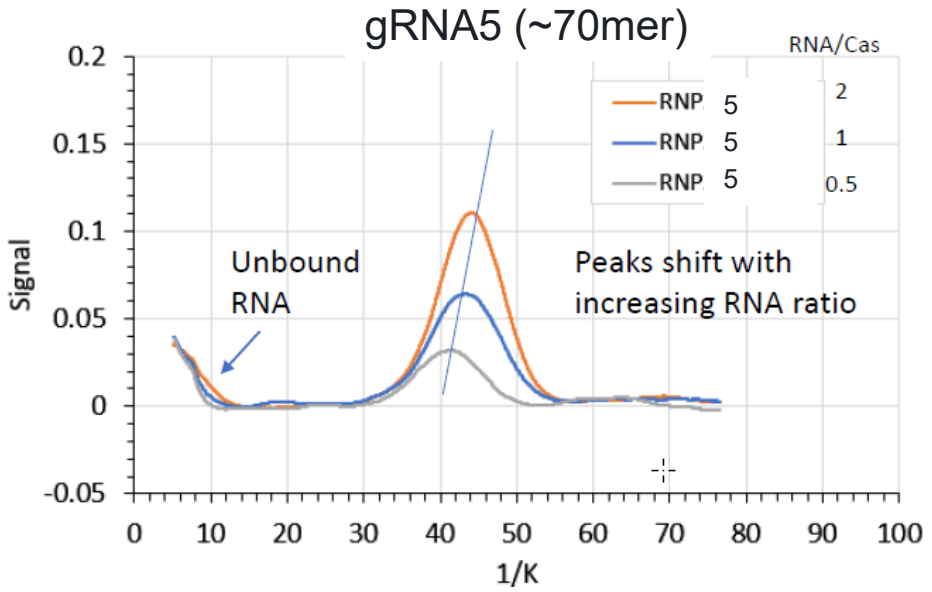
RNPs in 25 mM $(NH_4)_2CO_3$, pH 8.5



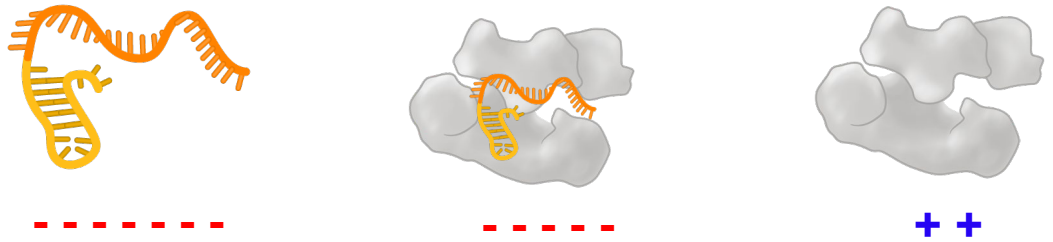
RNPs via IM: Varying the gRNA:Cas12a ratio

- Likely interpretation of the slope in the observed 1/K:
  - 0.5:1 = superposition of two slightly resolved peaks
    - apo protein (160 kDa) and 1:1 (180 kDa))
  - 1:1 = mostly 1:1
  - 2:1 = superposition of two slightly resolved peaks
    - 1:1 (180 kDa) and 2:1(200 kDa) + apo-guide
  - Δ(1/K) < peak width





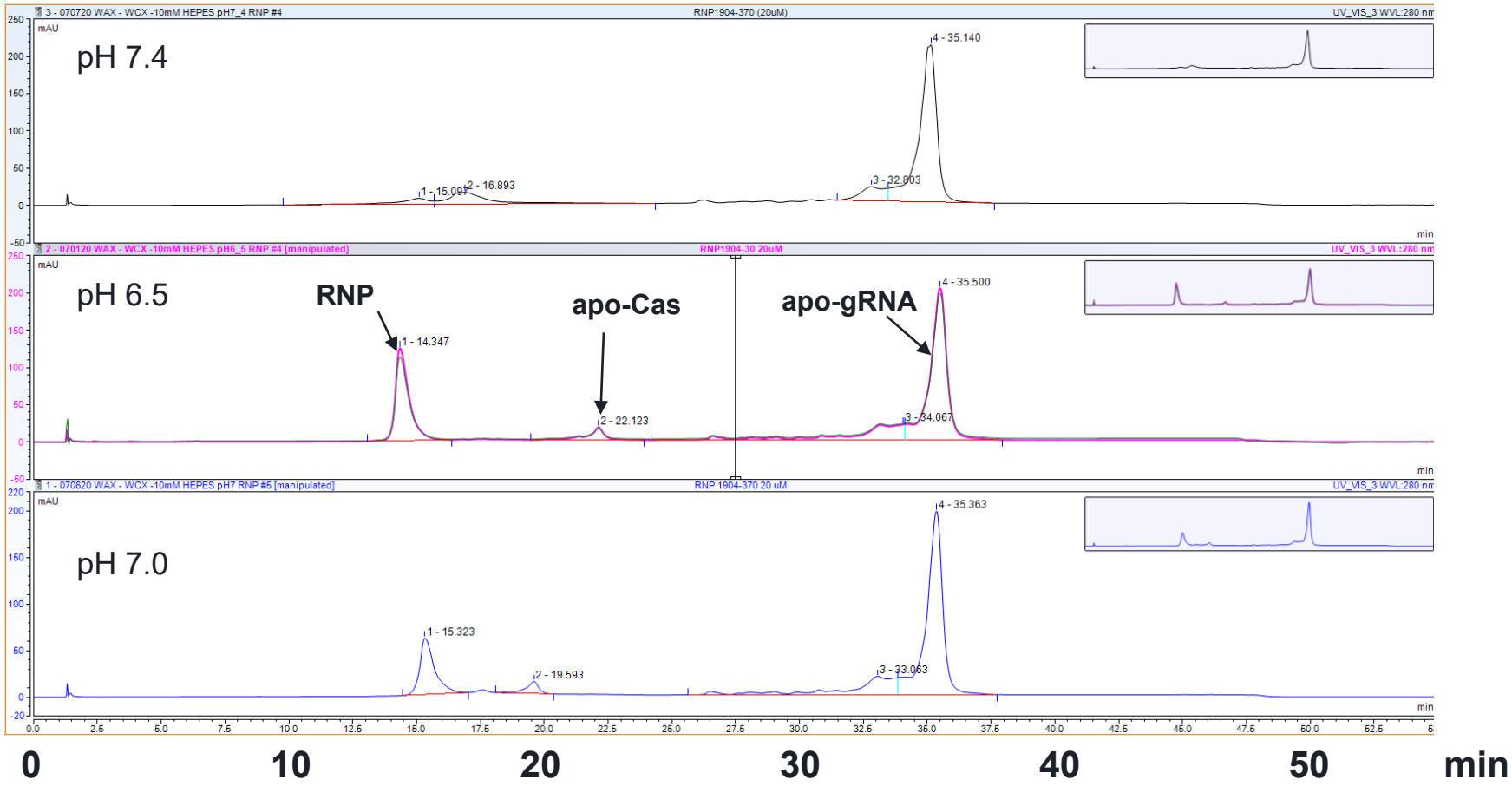
Ion Exchange Analysis of an RNP



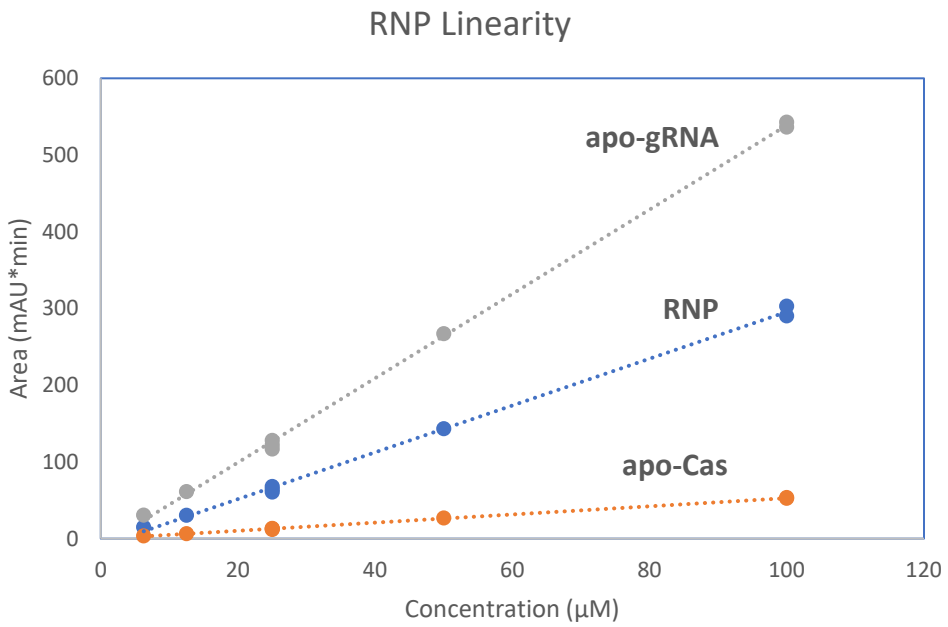
mode	gRNA	RNP	Cas
WCX	void	in between	retain
WAX	retain	in between	void
WCX-WAX	retain	retain	retain

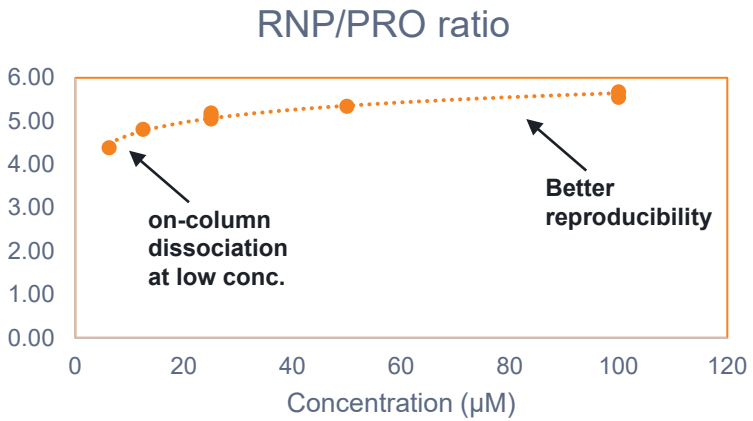
Analysis of RNPs via WCX-WAX

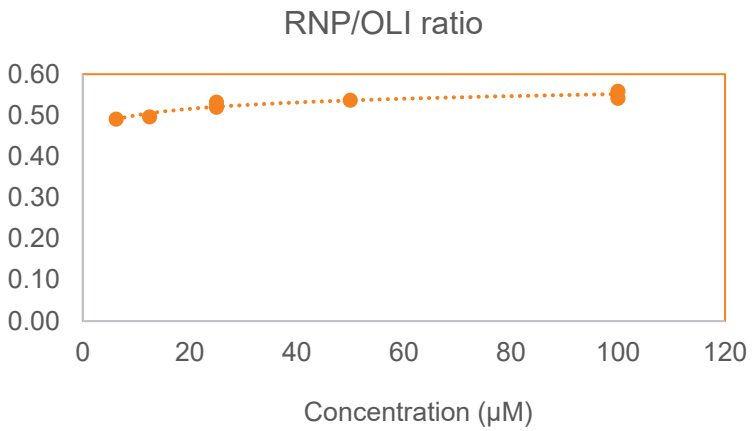
See Poster T.13 (Lemercier et al.)



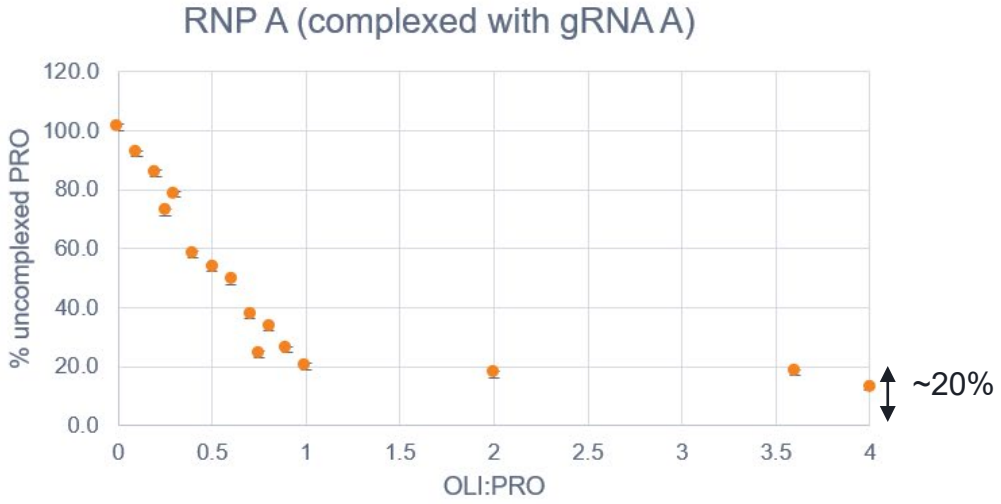
RNP Linearity via WCX-WAX

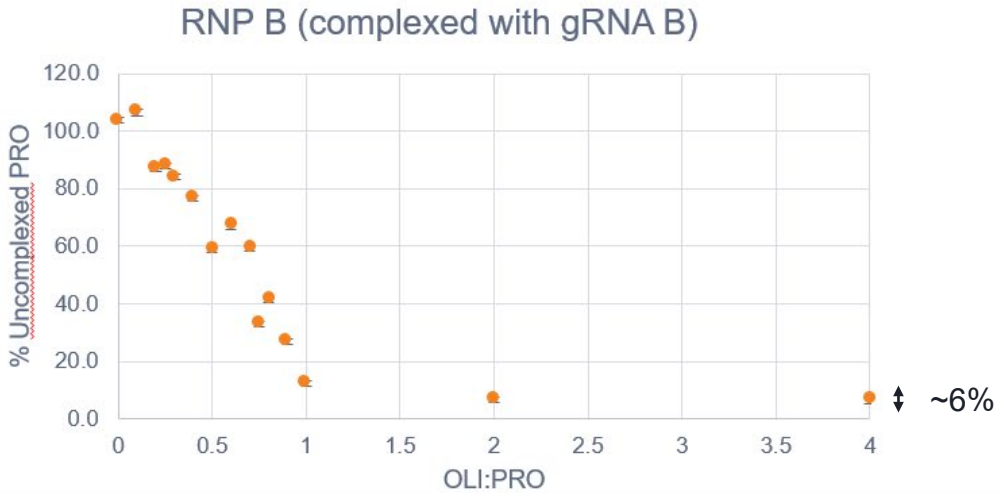


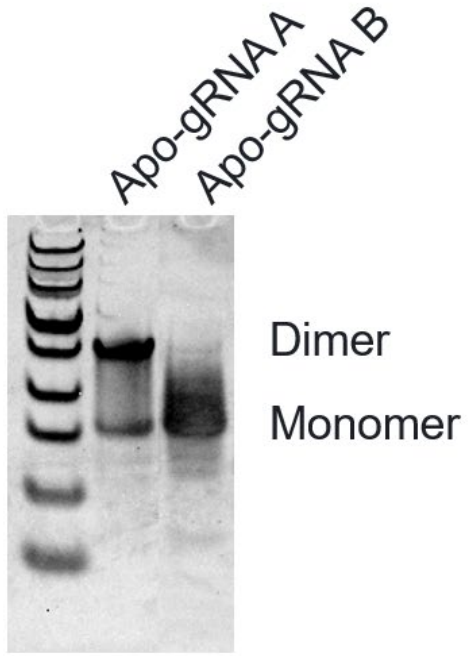




Accuracy via WCX-WAX (% Uncomplexed PRO vs OLI:PRO Ratio)





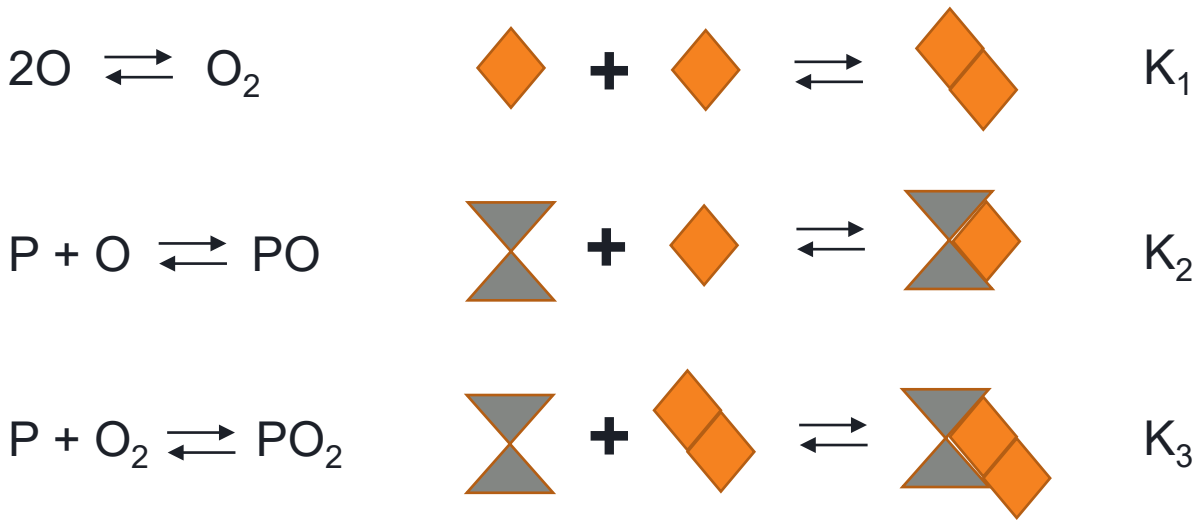


% Uncomplexed PRO vs OLI:PRO Ratio

Different Plateaus for gRNA Monomer vs. Dimer/Multimer?

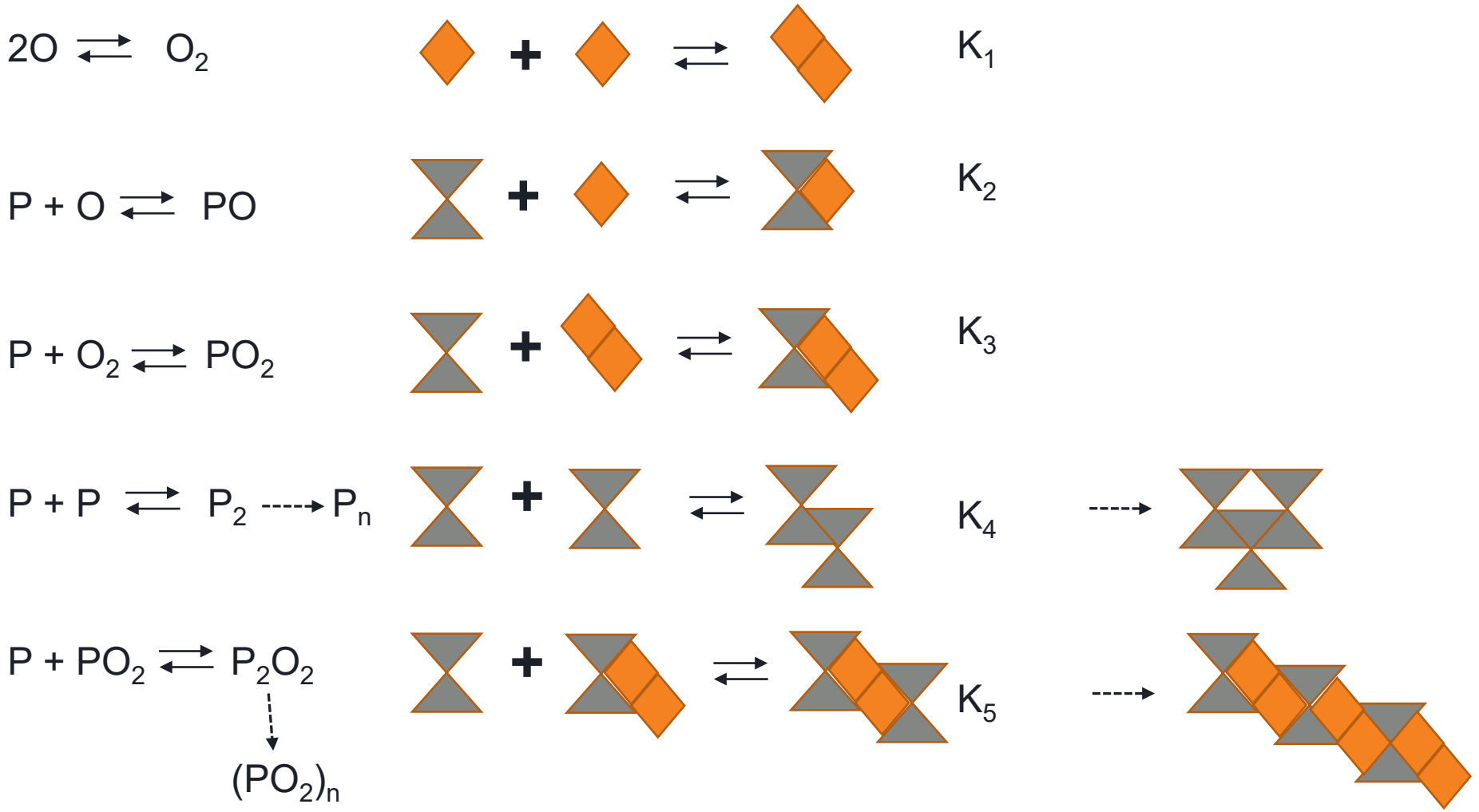
Possible Explanations:

- kinetically driven
  - *on-column dissociation* $k_{-3} > k_{-2}$
- thermodynamically driven
  - *e.g.*, K_1 , $K_2 >> K_3$
- *modeling not adequate to reproduce data*



% Uncomplexed PRO vs OLI:PRO Ratio

It’s probably a bit more complicated… (analysis in progress)



Summary & Concluding Remarks

Analytical Characterization of gRNAs and RNPs

- gRNA Conformers and Multimers
  - formation is sequence dependent, and more likely for longer sequences
  - manifestation is condition dependent (gRNA conformers, stoichiometries, dynamics, and analysis method)
- RNPs
  - another level of complexity due to noncovalent nature of complex (truncates etc., conformers, stoichiometries, dynamics, and the analysis method)
  - SEC, IEX, PAGE, IM data shows that the secondary structures formed by the guides impact the complexation
  - some method dependence of results, which is not surprising for these noncovalent complexes of large biomolecules
  - “Combo” WCX/WAX method is promising as a characterization and possible release method

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Thank You