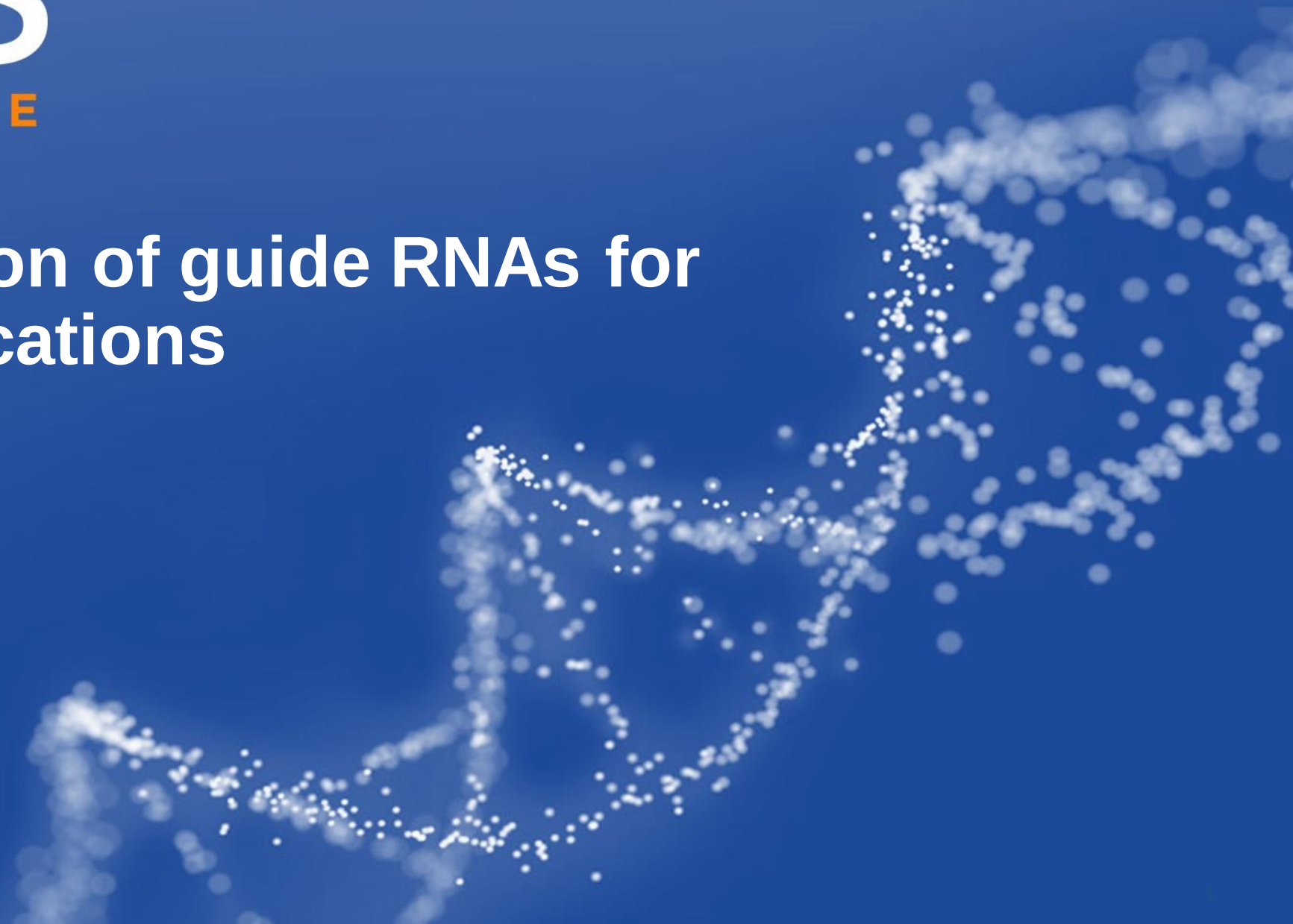
Characterization of guide RNAs for CRISPR Applications

Jean-Noël Lemercier



Disclosure

- I am an employee and shareholder of Editas Medicine

What is CRISPR?

CRISPR is a gene editing technology derived from the bacterial adaptive immune system that can revise, remove, and replace genes in a highly targeted manner.

In a therapeutic context, CRISPR uses a combination of 2 types of molecules to edit disease-related genes:

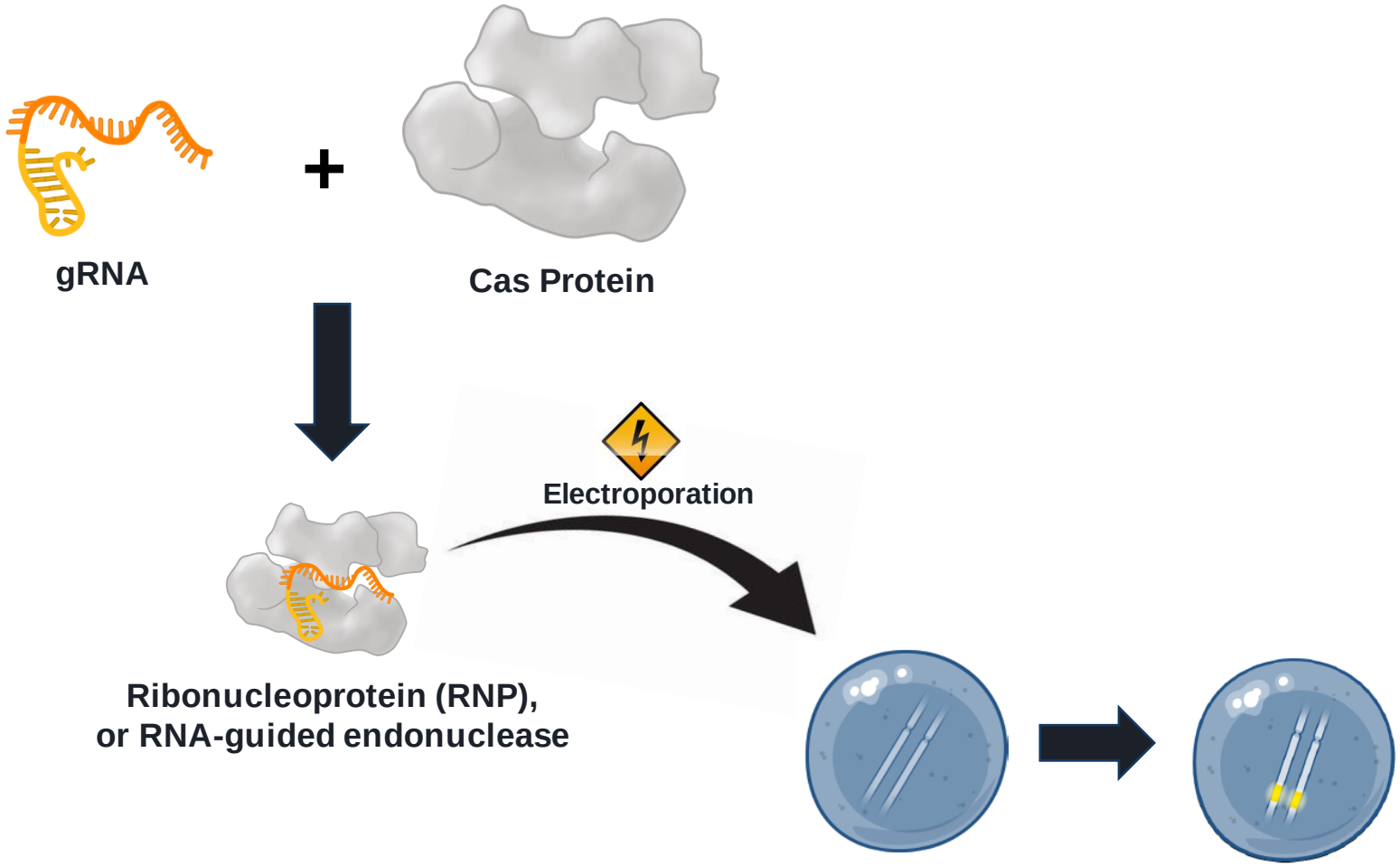
Cas Nuclease
the gene editor

guide RNA
helps the nuclease find the right place to edit

RNA can be designed to guide a nuclease to any DNA sequence

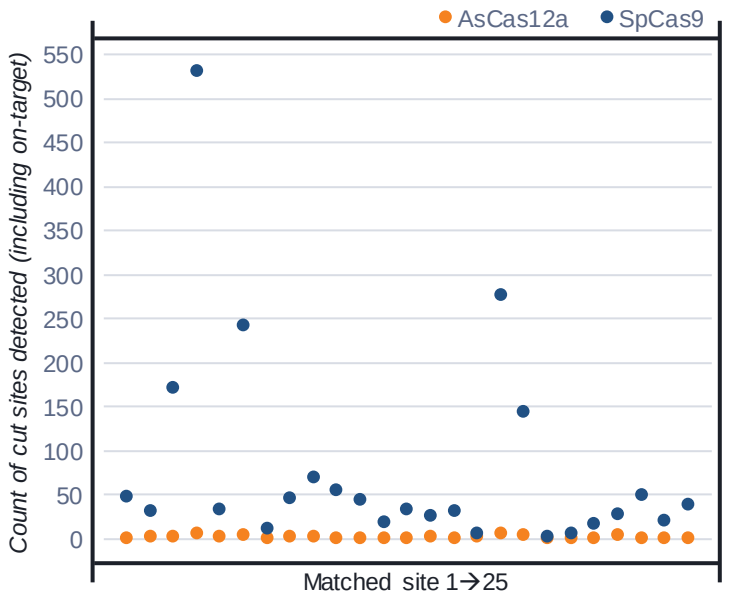
Specificity: CRISPR's ability to only edit intended DNA targets and avoid off-target editing. Achieving high levels of specificity requires the right combination of nuclease and guide RNA.

A CRISPR Application for ex-vivo Cell-Based Medicine



Editas Proprietary Engineered AsCas12a CRISPR Nuclease

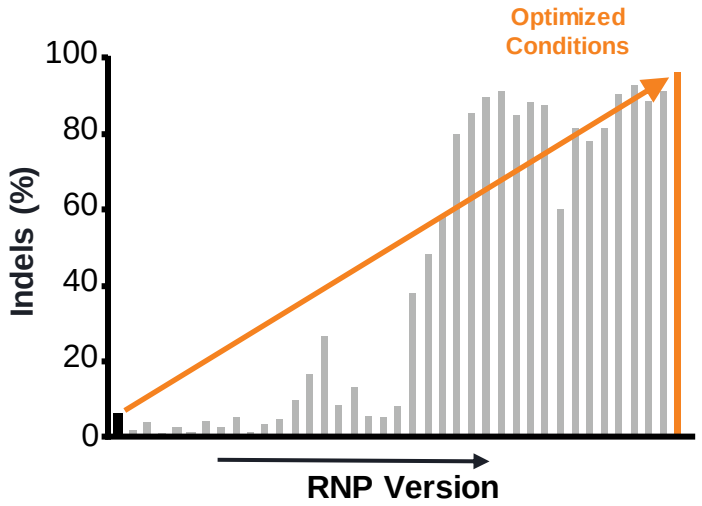
Editing Specificity[1]



Digenome-Seq with 25 matched sites*

- **AsCas12a is highly specific** primarily due to its DNA target engagement mechanism that is distinct from SpCas9

Editing Efficiency[2]



- Editas engineered AsCas12a RNP demonstrates **high editing efficiency** in CD34+ cells

Editas AsCas12a is highly efficient and more specific than spCas9

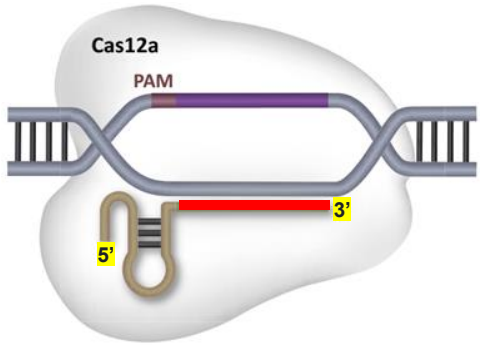
*Note: Matched target sites are 20mer protospacer sequences within the human genome with compatible PAM's for different nucleases (20-Ns)

[1] Editing specificity from Gotta et al. Cold Spring Harbor 2019; [2] Editing efficiency from De Dreuzy et al, ASH 2019;

Cas12a and Cas9 Require Different gRNAs

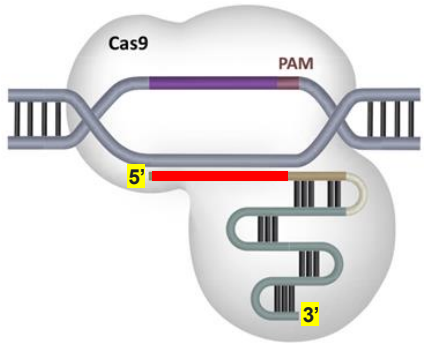
Detailed Characterization of the 2 types of gRNAs to follow

Cas12a[1]



- Shorter **41mer gRNA**
- Targeting sequence on the **3'-terminus of molecule**

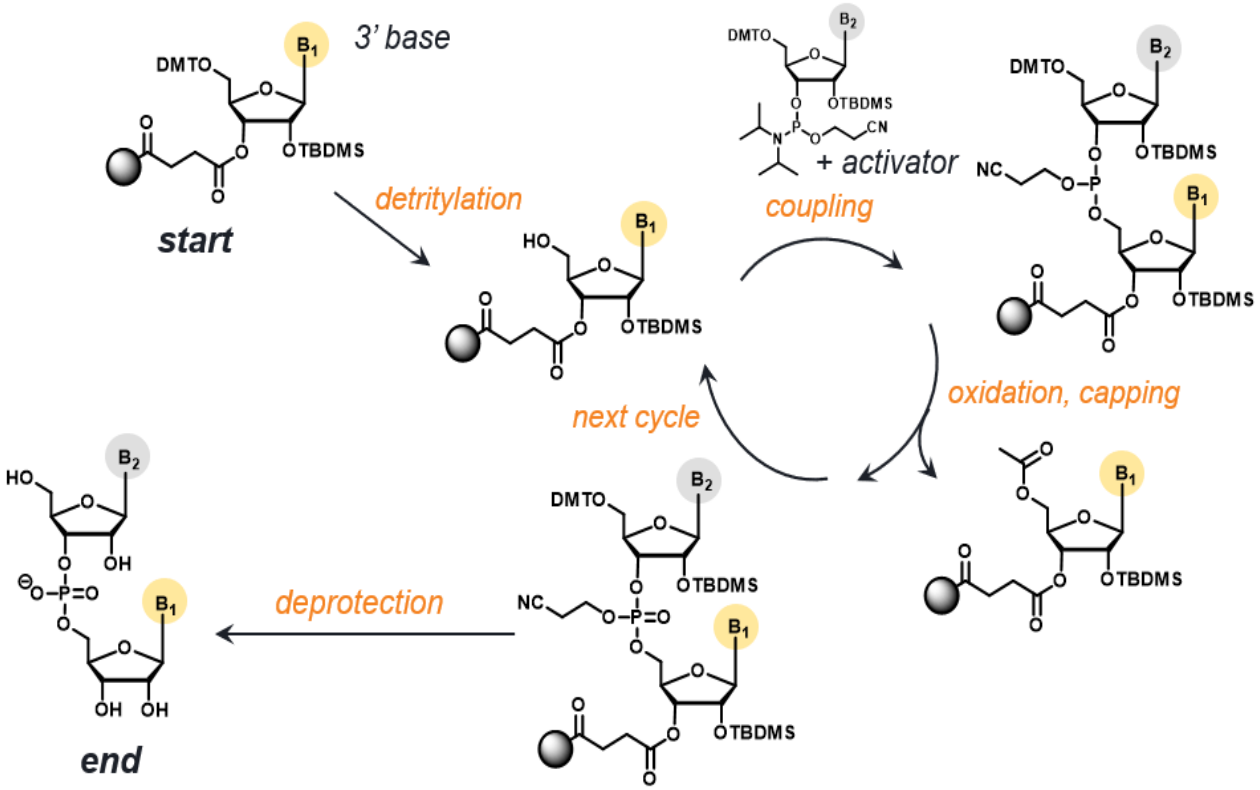
Cas9[2]



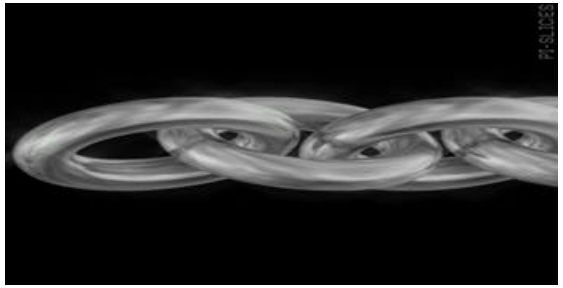
- Longer **100mer gRNA**
- Targeting sequence on the **5'-terminus of molecule**

[1] Cas 12a: Image adapted from Moon et al. (2019) Trends in Biotechnology 37(8): 870-881; [2] Cas 9: Image adapted from Moon et al. (2019) Trends in Biotechnology 37(8): 870-881

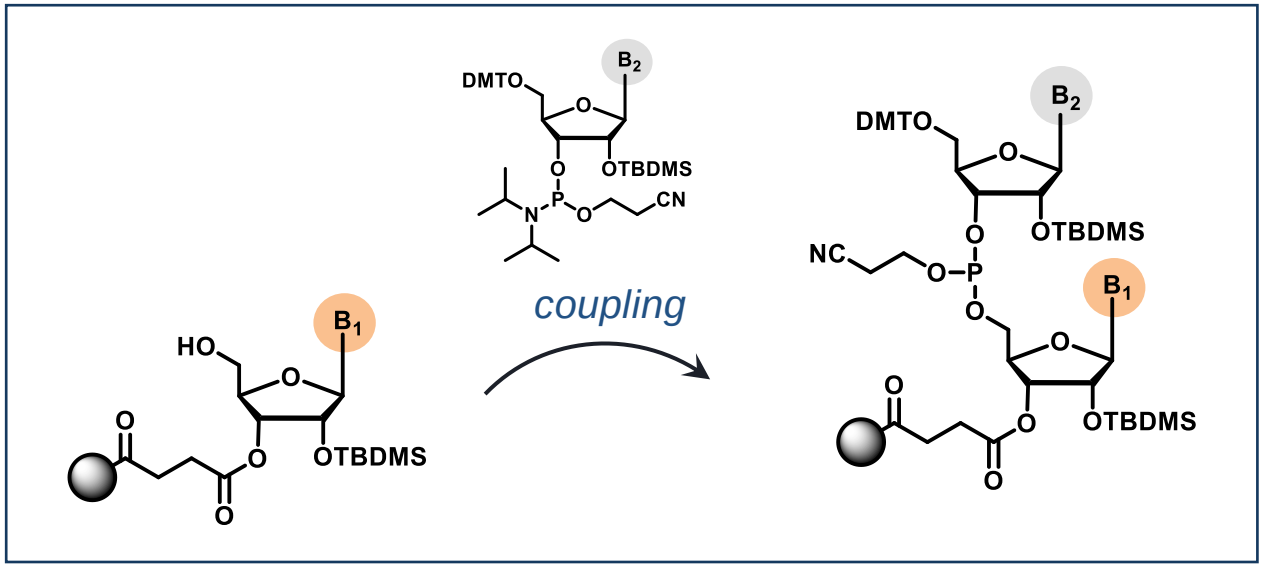
Oligonucleotide Synthesis Process

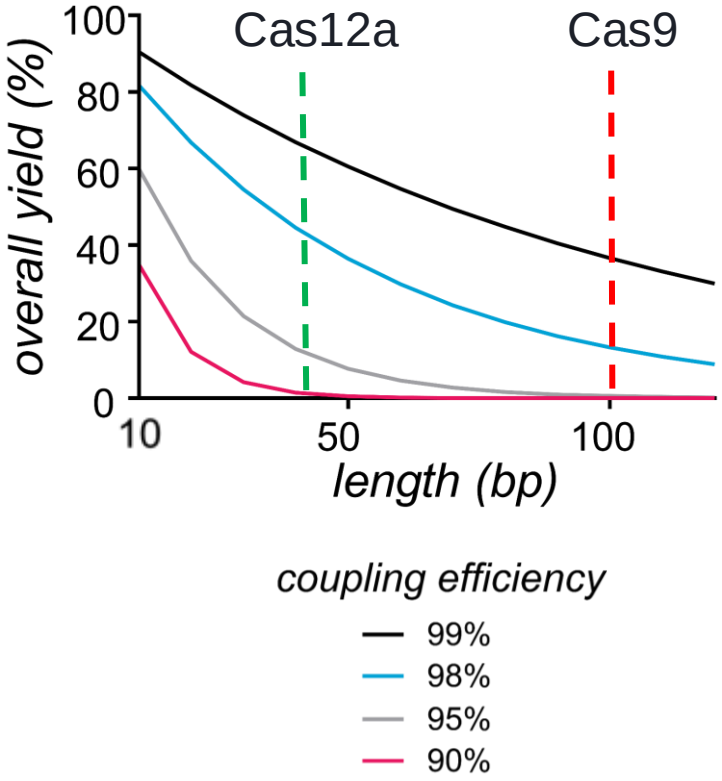


- Stepwise addition of nucleotides at the 5' terminus
- Each addition is a cycle made of 3 main steps + final cleavage and deprotection

5'-  -3'

Effect of Coupling Efficiency on Yield





Yield ~ (% coupling efficiency)^n

where n = # of nucleotide additions

$(0.99)^{40}$ = **67% theoretical crude yield**

$(0.99)^{99}$ = **37% theoretical crude yield**

Longer RNA = Lower Yield

Oligonucleotide Synthesis Impurities

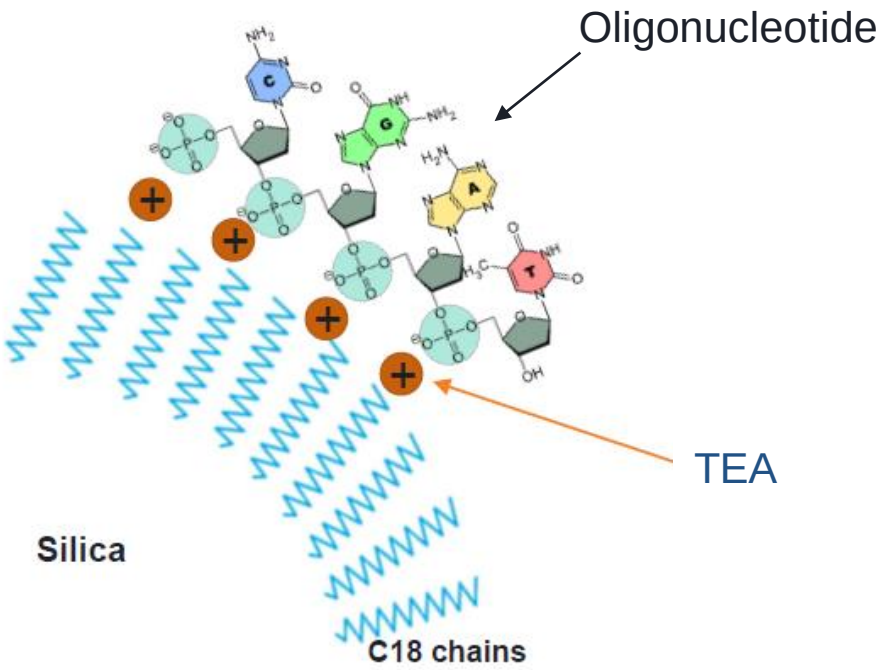
Impurity	Side Product
Truncations	n-x (n-1, n-2, etc.) depurination
Protecting groups	Failure to remove protecting groups
Synthesis errors	Double coupling Missed base Premature capping
Amidite impurities	Side reactions

1. Each synthesis step has the potential to introduce impurities
2. The number and complexity of impurities increase with gRNA length
3. Solid-phase RNA synthesis is carried out in a 3’ to 5’ direction

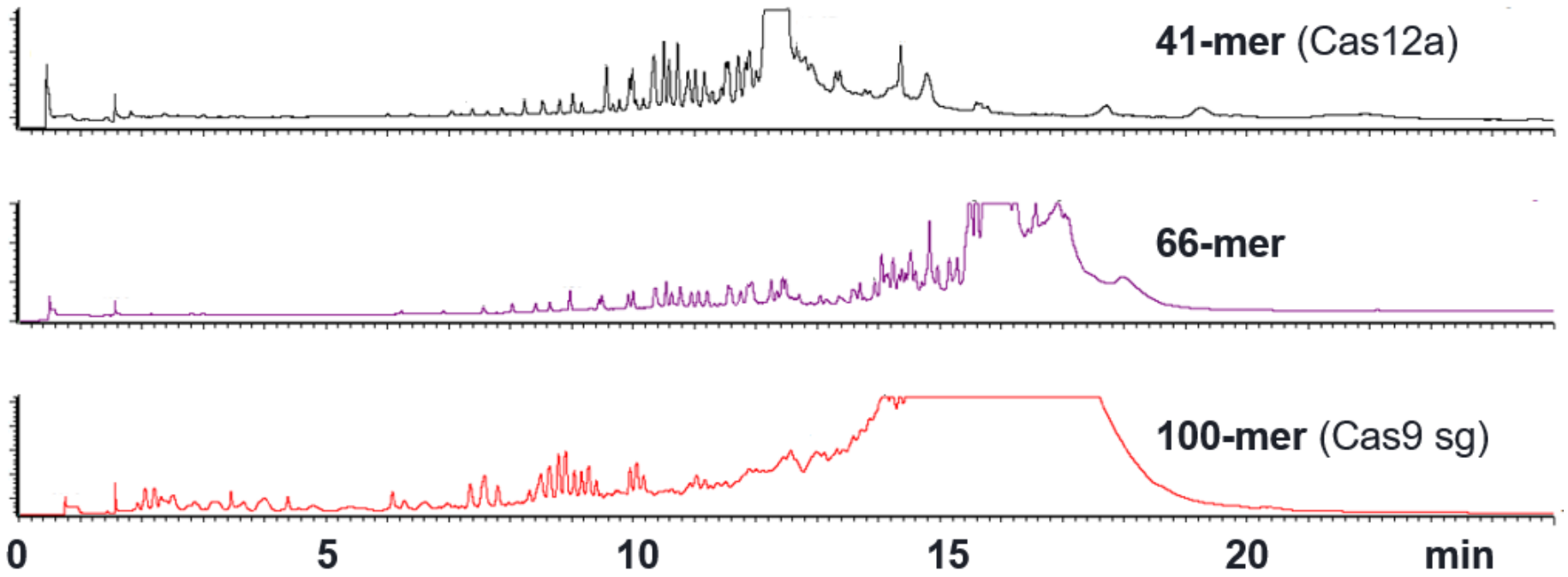
Sequence fidelity decays toward 5’ terminus

Purity Characterization – Liquid Chromatography

- Reverse-phase ion-pairing chromatography (RP-IP-UPLC) (industry standard)
- HFIP in mobile phase (chromatographic resolution and mass spec compatibility)
- UV Detection at 260 nm

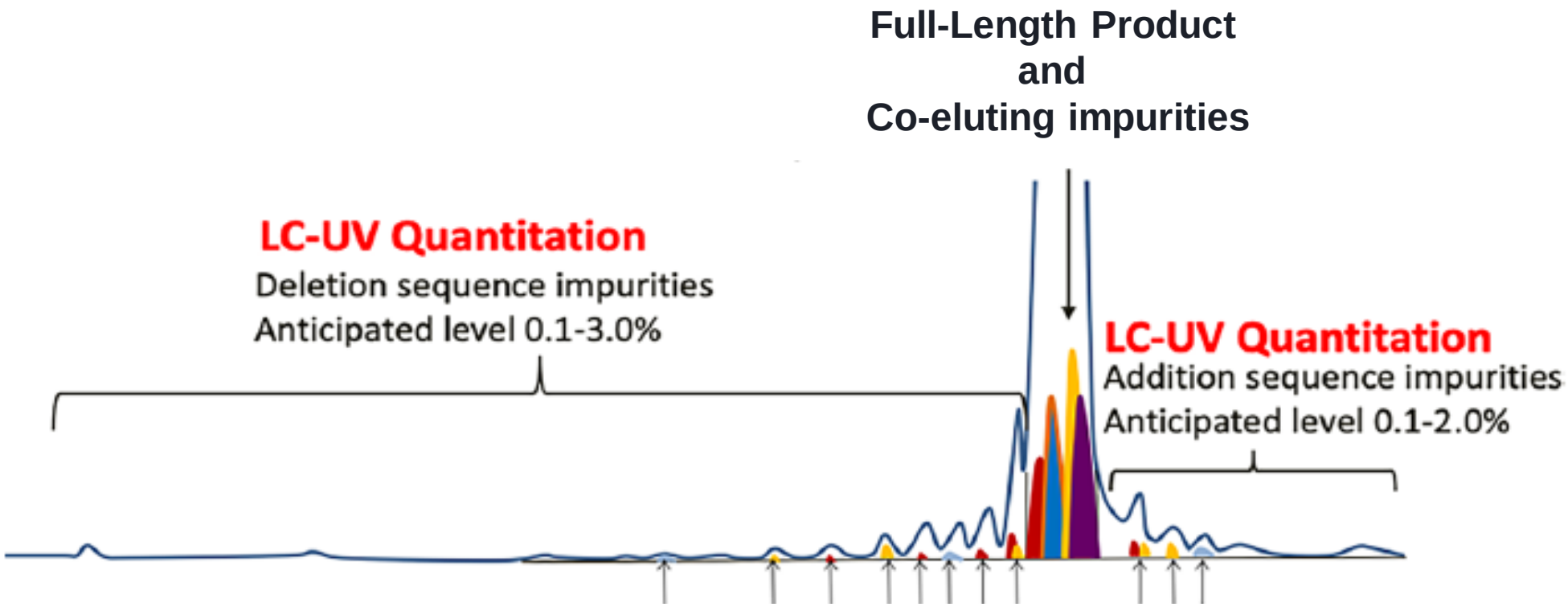


Analytics: Guide RNA Crudes vs. Length



Waters® Acquity Oligonucleotide BEH C18, 130Å, 1.7µm, 2.1 mm x 100 mm
Mobile phase A: 95 mM HFIP, 14 mM TEA, 1%ACN
Mobile phase B: ACN
Flow rate: 0.2 ml/min
Column temperature: 50 °C

The Reality of the Data: Purity Determination by LC-UV is Imperfect



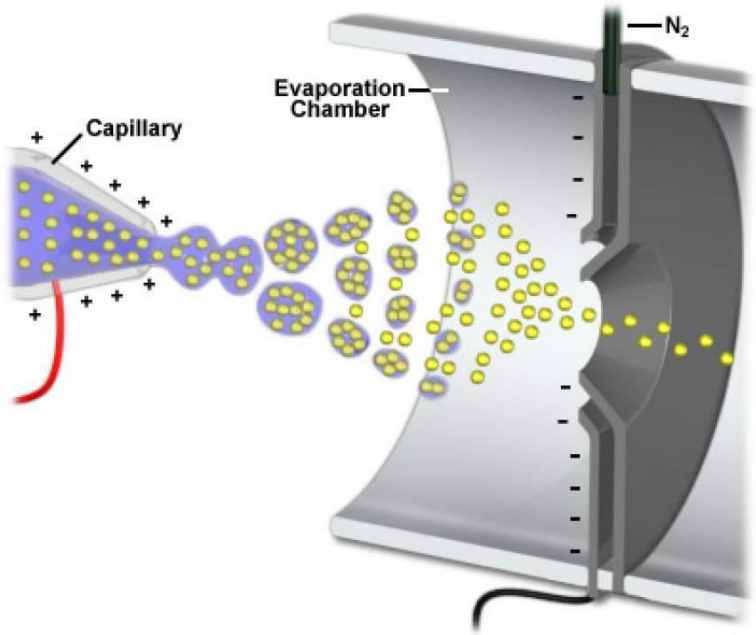
LC-UV-MS is required to obtain a full overview of all gRNA related substances

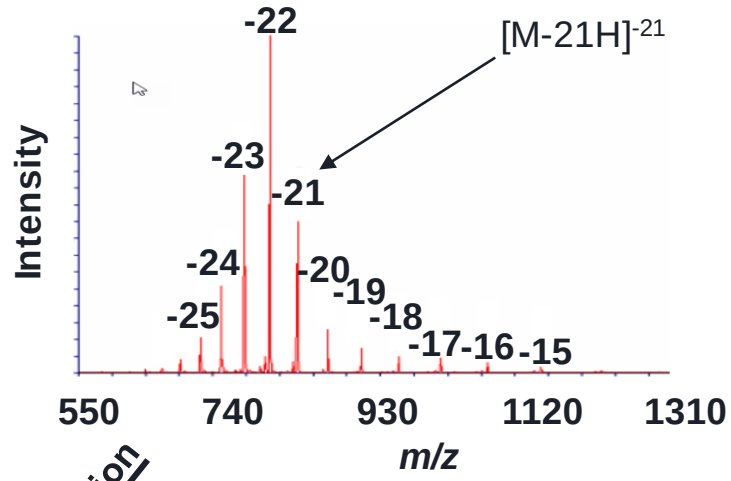
LC-UV-MS purity < LC-UV Purity

J. Am. Soc. Mass Spectrom. 2020, 31, 1775−1782

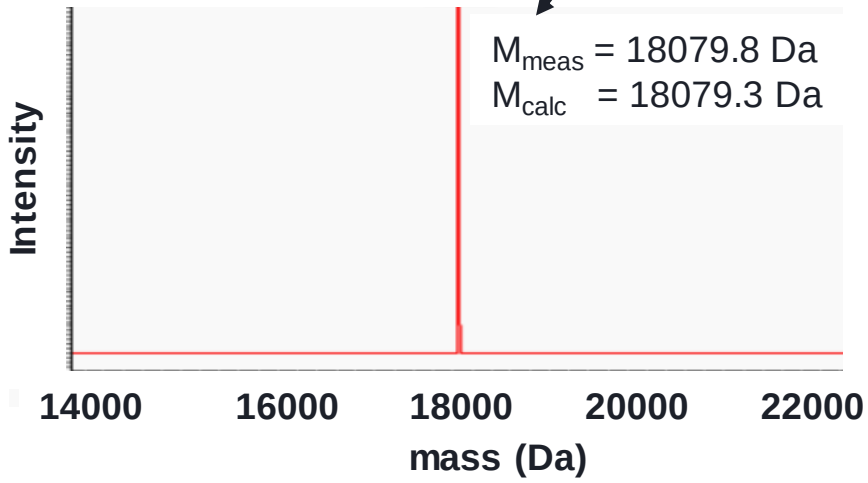
Identity: Determination by Mass Spectrometry

Biomolecule Characterization via ESI-MS detection





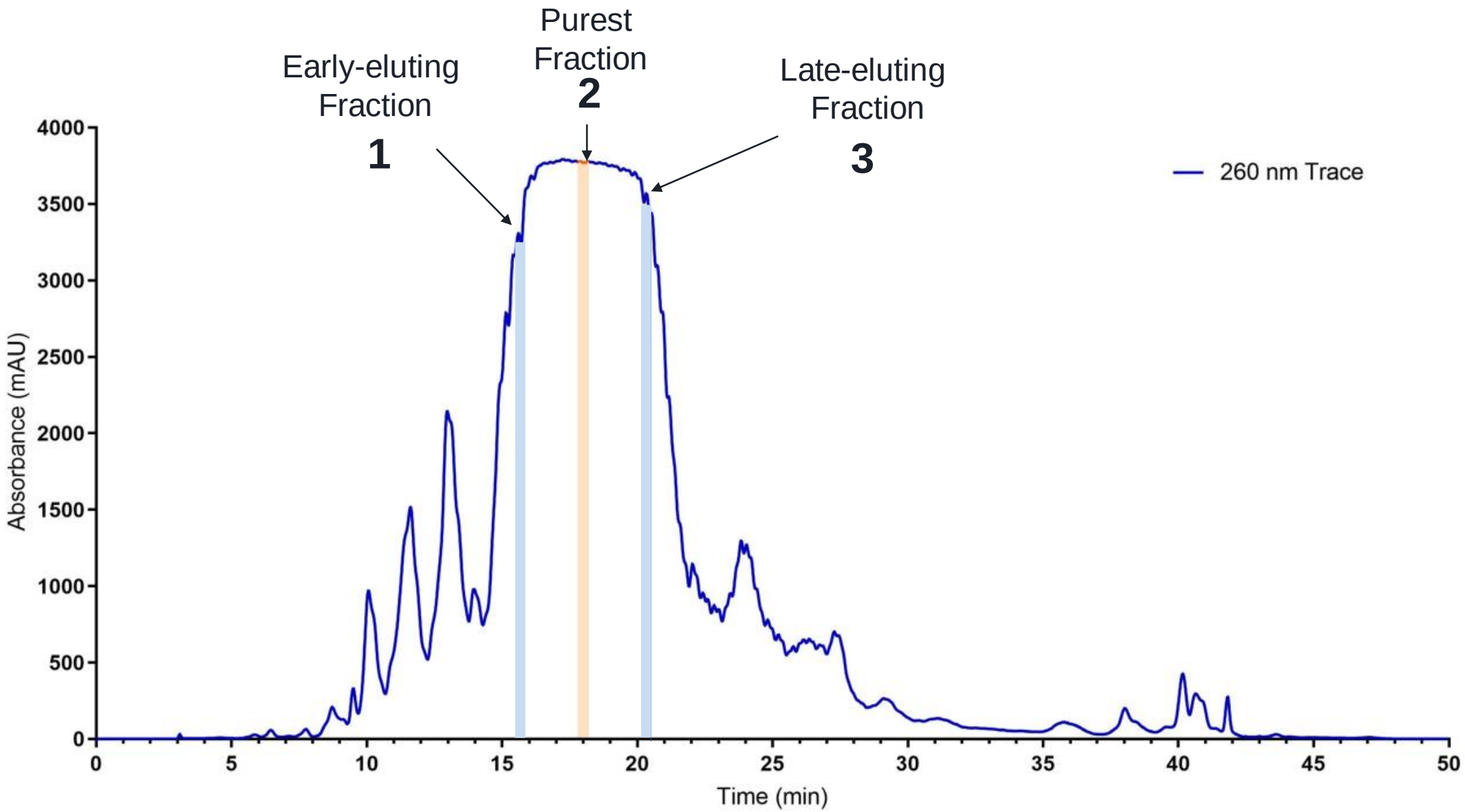
Deconvolution



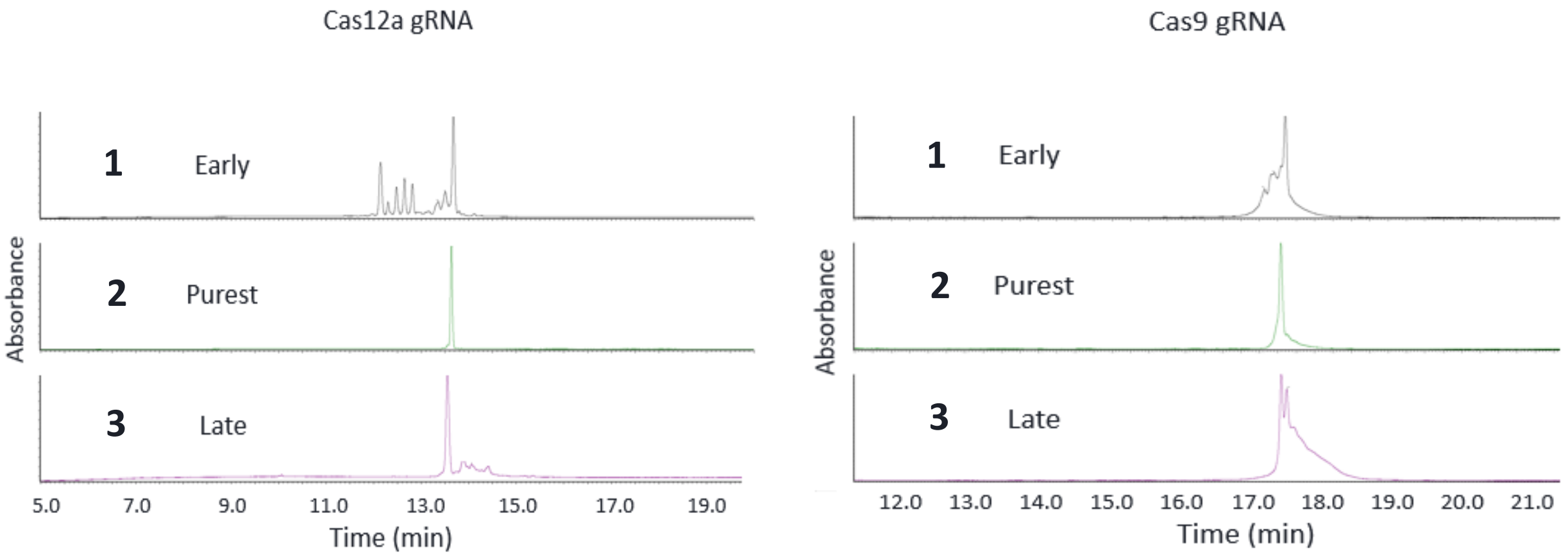
Electrospray ionization

- Revolutionary for biomolecule MS
- Nobel prize in Chemistry 2002
  - John Fenn and Koichi Tanaka

Preparative-Scale Purification of Crude by Reverse Phase Chromatography

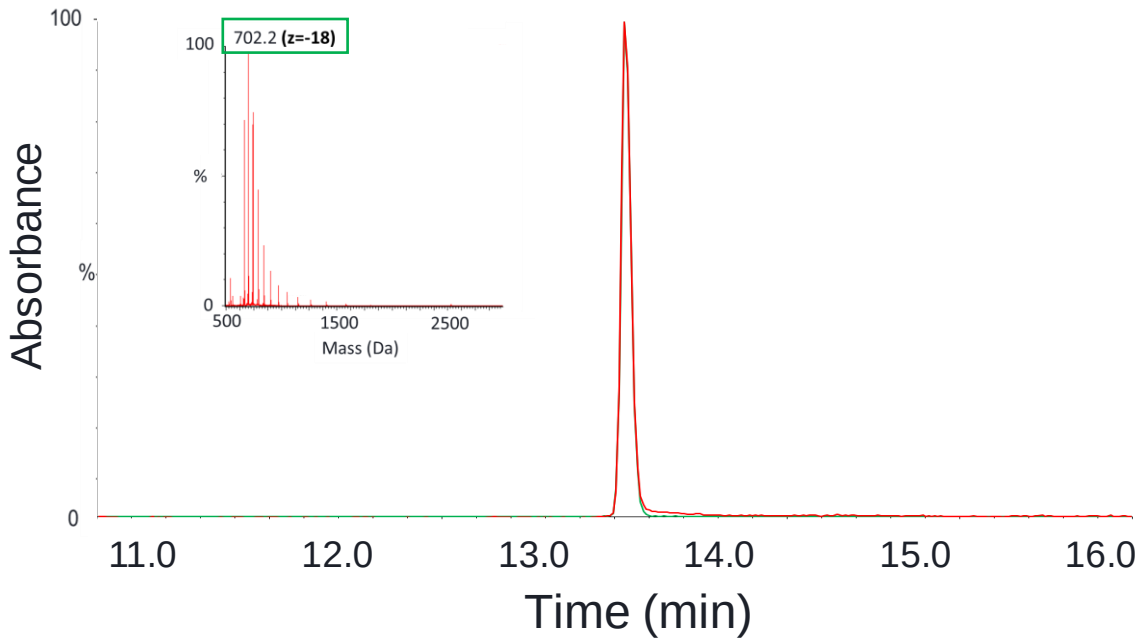


Cas12a and Cas9 Guide RNA Fractions

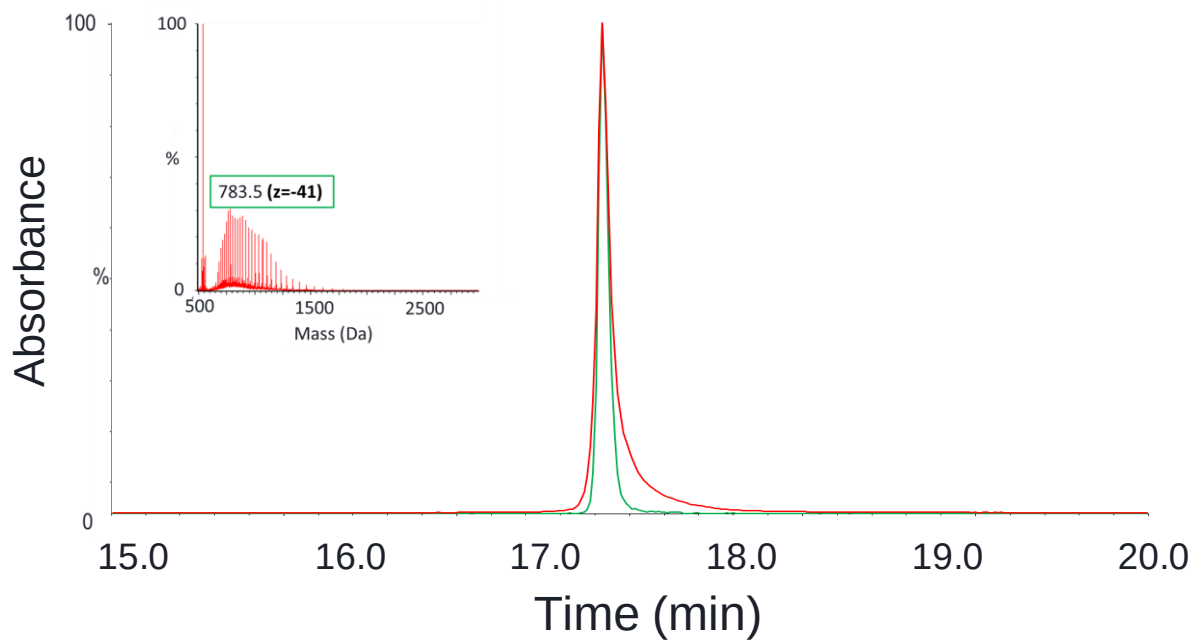


LC-UV Chromatograms at 260 nm

Guide RNA Purest Fractions: TIC vs EIC (Full-Length Product)



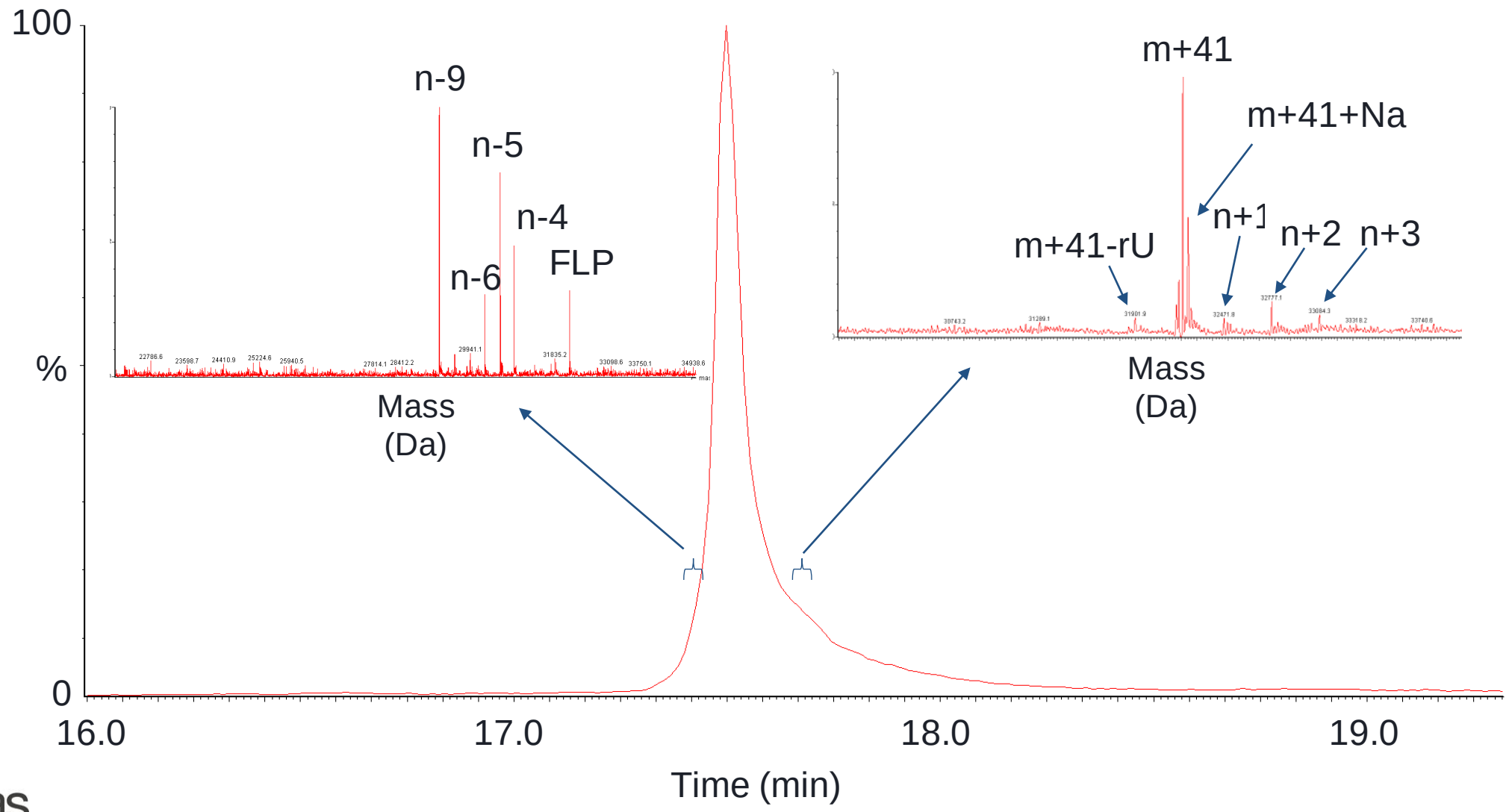
Cas12a



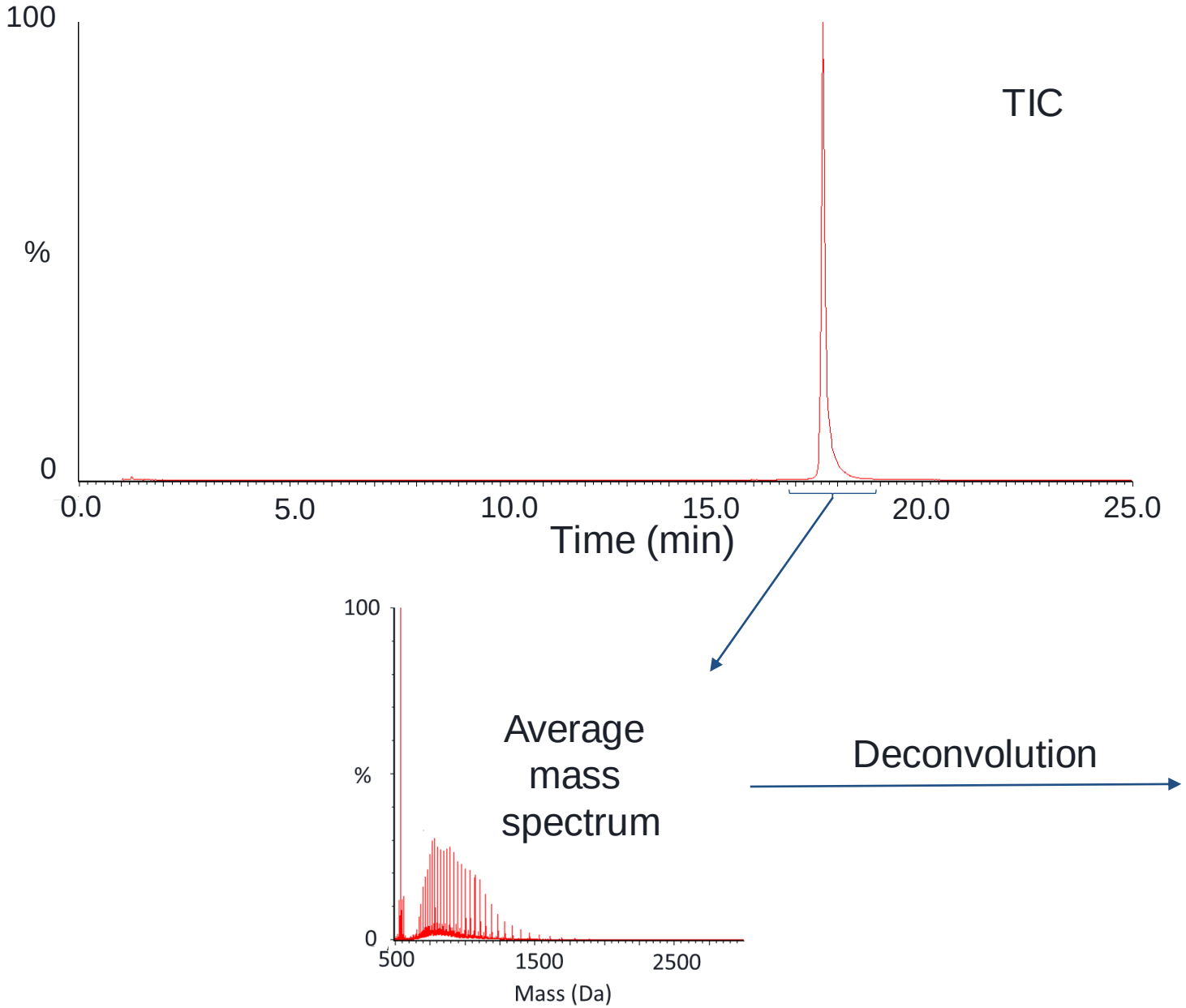
Cas9

- Total Ion Chromatogram
- Extracted ion chromatogram for full-length product (FLP)

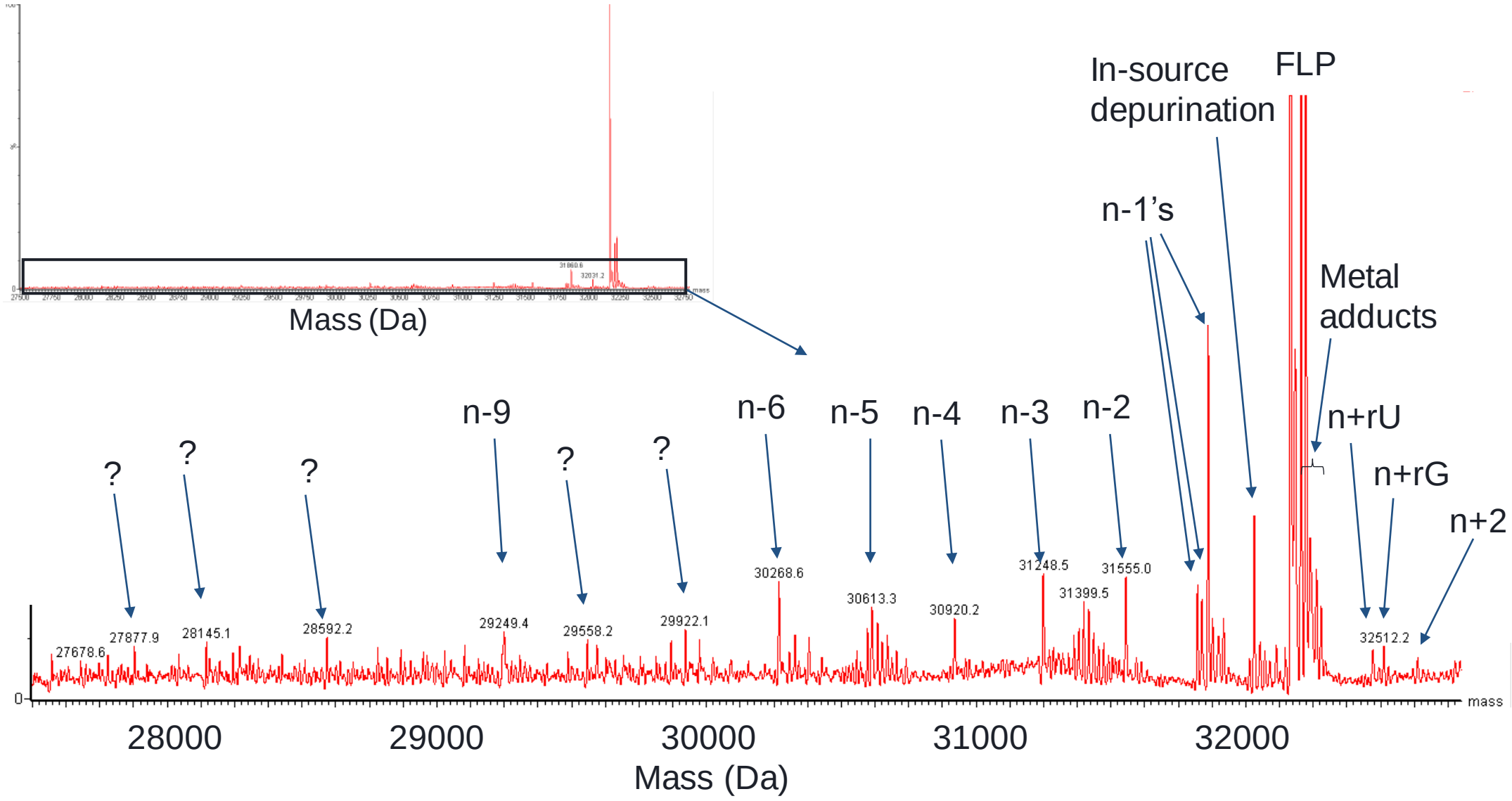
Guide RNA Purest Fraction: Cas9 Impurity Mining



Cas9 Guide RNA Purest Fraction LC/MS Analysis

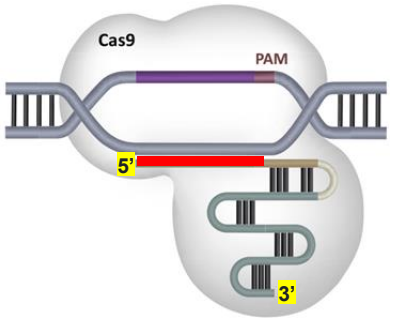


Cas9 Guide RNA Purest Fraction Deconvoluted Mass Spectrum

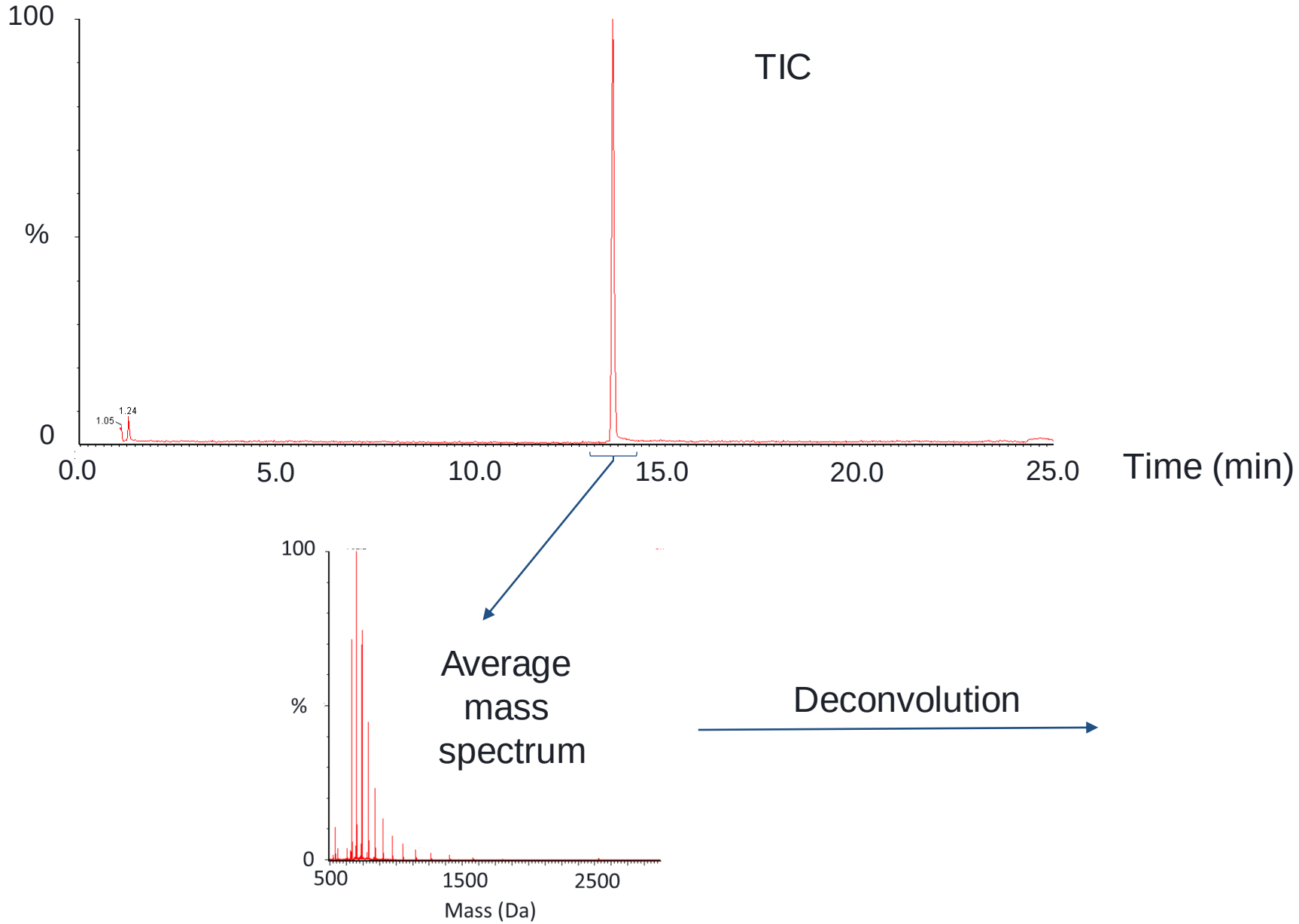


Cas9 gRNA Impurity Analysis Results

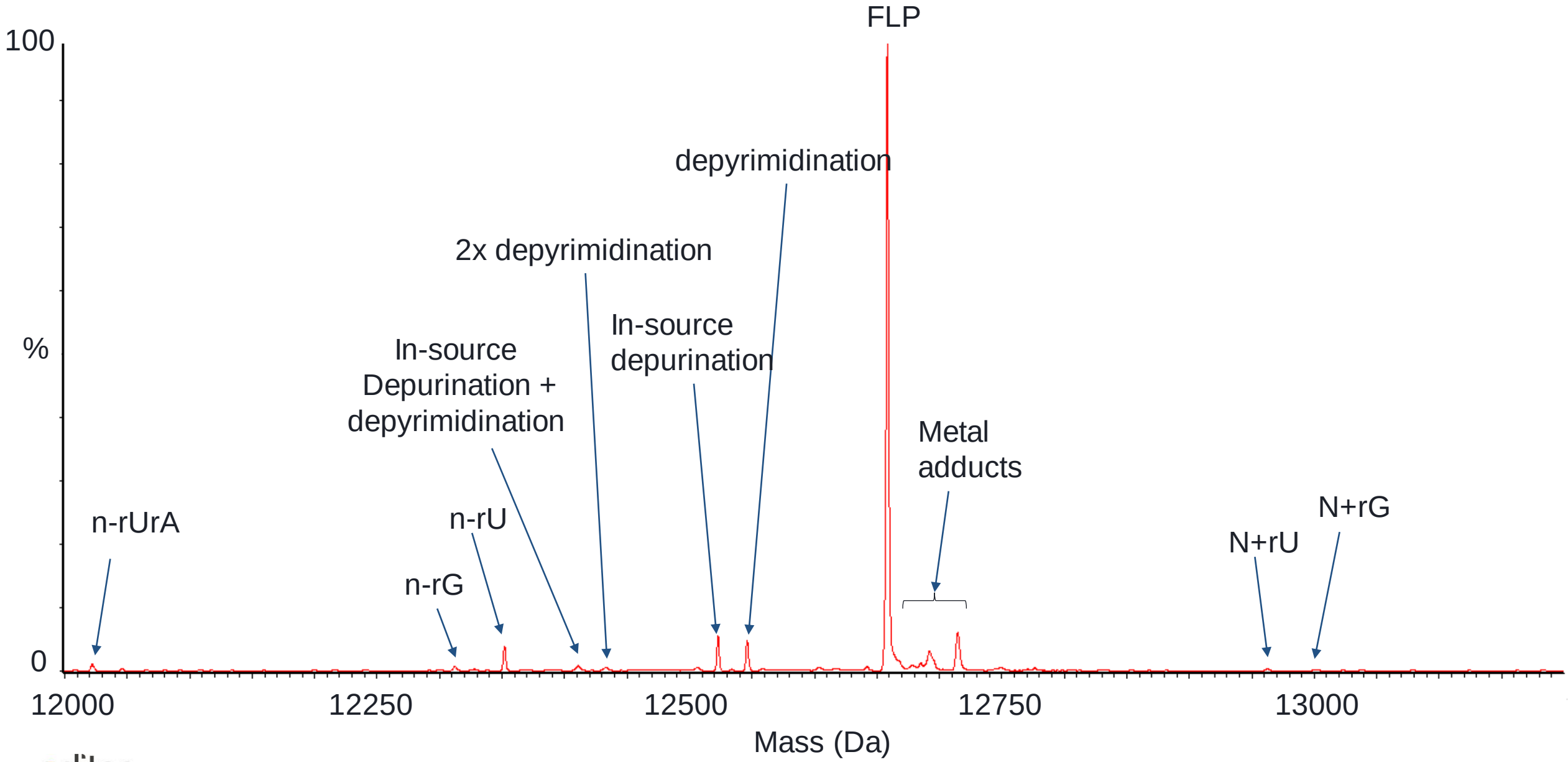
Mass Delta from FLP (Da)	Identity	Peak Height	%Peak Height	Sequence
-4288.8	?	180000	0.7	?
-4021.6	?	202000	0.8	?
-3574.5	n-12	224000	0.9	5'-rArU……-3'
-2917.3	n-9	232000	0.9	5'-rArGrUrArU……-3'
-2608.5	?	202000	0.8	?
-2244.6	?	238000	1.0	?
-1898.1	n-6	368000	1.5	5'-rArGrGrArGrUrArU……-3'
-1553.4	n-5	316000	1.3	5'-rGrArGrGrArGrUrArU……-3'
-1246.5	n-4	268000	1.1	5'-rUrGrArGrGrArGrUrArU……-3'
-918.2	n-3	407000	1.6	5'-rArUrGrArGrGrArGrUrArU……-3'
-611.7	n-2	382000	1.5	5'-rUrArUrGrArGrGrArGrUrArU……-3'
-345.9	n-rG (internal)	347000	1.4	?
-329.6	n-rA (internal)	302000	1.2	?
-306.9	n-1	1180000	4.8	5'-rCrUrArUrGrArGrGrArGrUrArU……-3'
0	**FLP**	**18300000**	**74.0**	5'-rCrCrUrArUrGrArGrGrArGrUrArU……-3'
17.9	+ H_2O	1120000	4.5	
304.7	n+rC	160000	0.6	?
345.5	n+rG	170000	0.7	?
609.6	n+2	127000	0.5	?



Cas12a Guide RNA Purest Fraction LC/MS Analysis

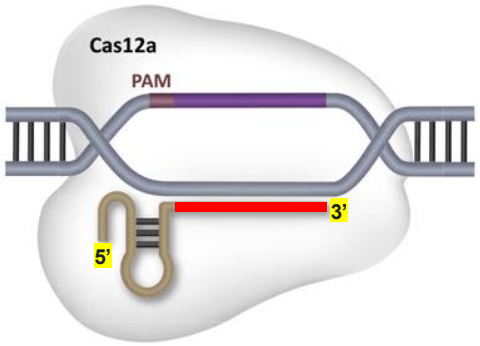


Cas12a Guide RNA Purest Fraction Deconvoluted Mass Spectrum



Guide RNA Purest Fraction: Cas12a

Mass Delta from FLP (Da)	Identity	Peak Height	%Peak Height	Sequence
-635.1	n-2 (-rUrA)	9.85E+05	0.5	5’-rA……-3’
-345.6	n-rG (internal)	8.78E+05	0.5	?
-306.3	n-1 (-rU)	4.04E+06	2.2	5’-rArA……-3’
-224.1	2x depyrimidination	9.94E+05	0.5	?
-111.9	depyrimidination	6.41E+06	3.6	?
0	**FLP**	**1.66E+08**	**92.3**	5’-UrArA……-3’
+305.7	n+rU	3.99E+05	0.2	?
+346.2	N+rG	2.88E+05	0.2	?

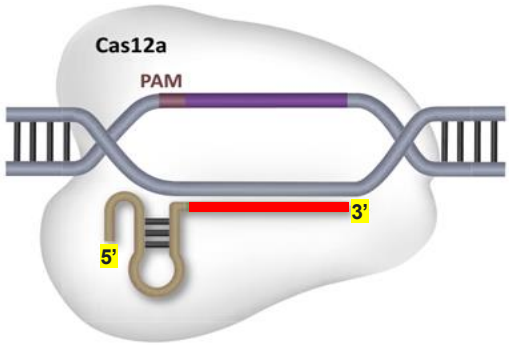


Higher purity
Impurities on the 5’ end -> impact on activity, not specificity

Cas12a Shorter gRNA: Higher Purity and Superior Fidelity

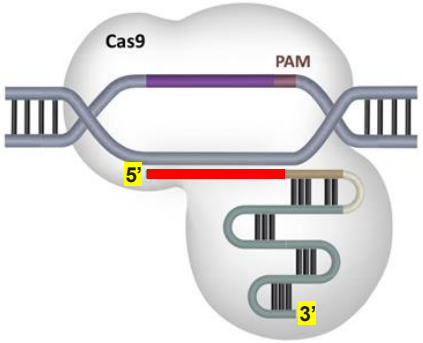
Improved Safety Profile due to Reduced Potential Off-Target Editing in Human Genome Caused by Errant Guide

Cas12a[1]



- Shorter **41mer gRNA** (higher purity & yield)
  - Significantly fewer low-level impurities
    - **Final Purity >80% achievable**
- Targeting sequence on the **3'-terminus of molecule**
  - 3'-terminus: beginning of synthesis → highest fidelity
  - Impurities likely to impact binding (activity) rather than result in off-target cuts

Cas9[2]



- Longer **100mer gRNA** (lower purity & yield)
  - More impurities, harder to separate chromatographically
    - **Final Purity >80% unlikely**
- Targeting sequence on the **5'-terminus of molecule**
  - 5' terminus: end of synthesis → lowest fidelity
  - **Possible problematic impurities → higher risk of catastrophic off-targets**
  - Possible solutions:
    - ligation of shorter RNAs (chemical or enzymatic)
    - In vitro transcription

[1] Cas 12a: Image from Moon et al. (2019) Trends in Biotechnology 37(8): 870-881.; [2] Cas 9: Image from Moon et al. (2019) Trends in Biotechnology 37(8): 870-881

Acknowledgments

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Thank you!

Questions?