

# In-Depth Impurity Assessment of Synthetic Oligonucleotides Enabled by HRMS

***SBIA 2022: Advancing Generic Drug Development:  
Translating Science to Approval***

***Day 1, Session 1B: Oligonucleotide Active Pharmaceutical Ingredient (API) Sameness and  
Impurity Assessment Considerations***

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CDER | US FDA

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# Learning Objectives

- Identify unique scientific and regulatory challenges in synthetic oligonucleotides
- Understand in-depth assessment of product-related oligonucleotide impurities
- Resolve complex impurity mixtures by high resolution mass spectrometry (HRMS)

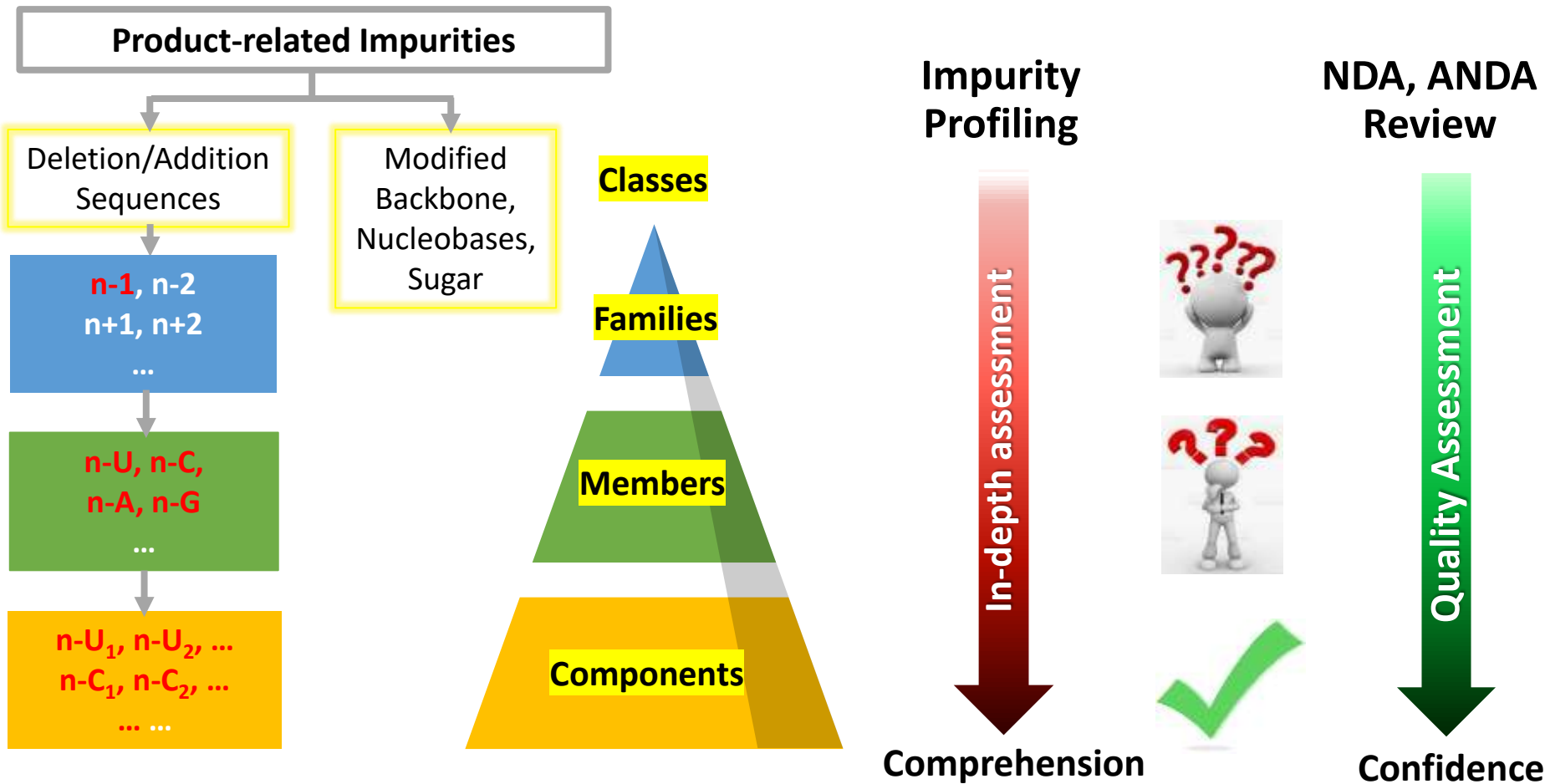
**Disclaimer: This presentation reflects the views of the authors and should not be construed to represent FDA's views or policies.**

# Challenges in Synthetic Oligonucleotides

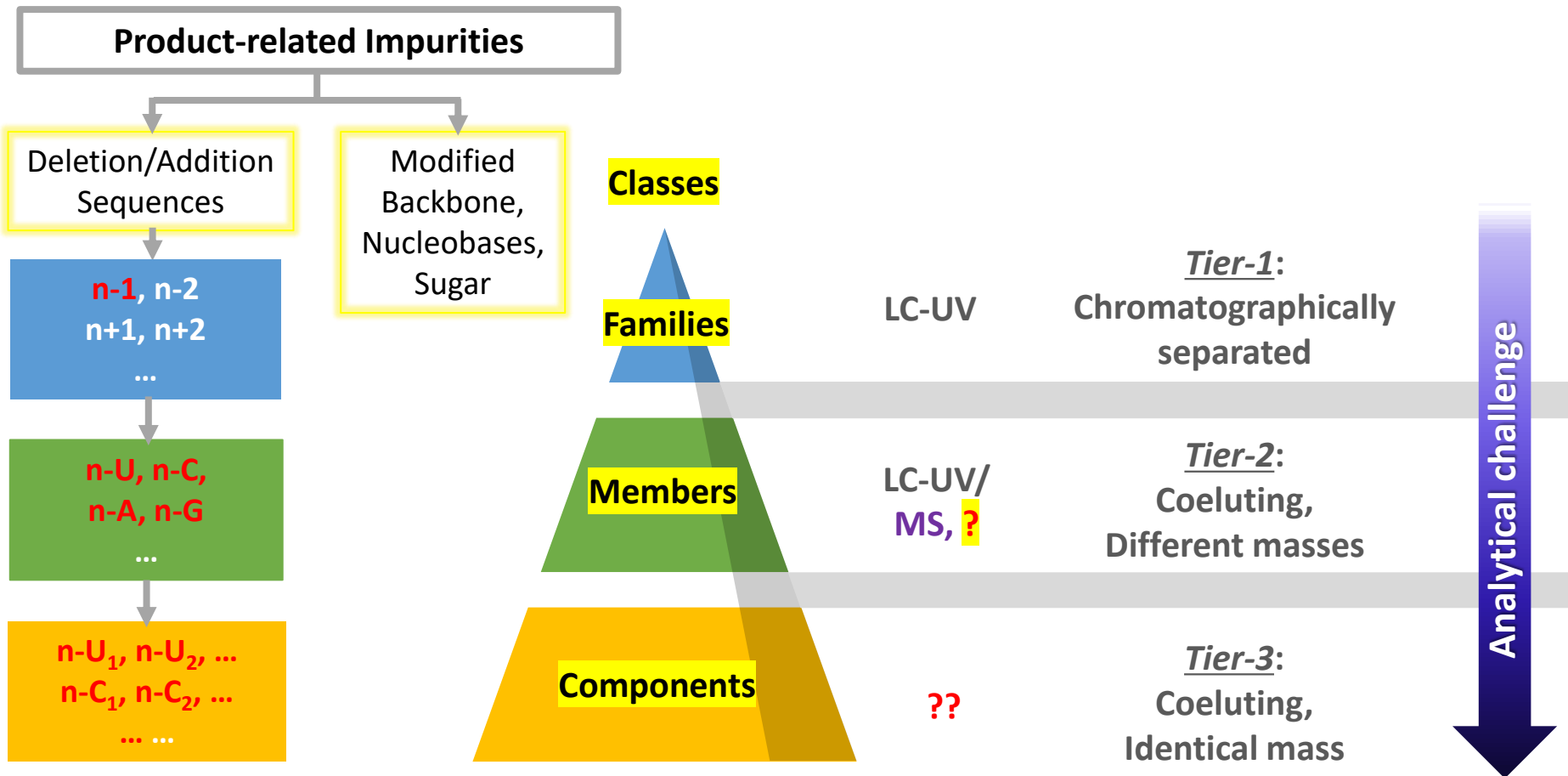


- *Target a broad range of mRNAs* that encode critical cellular proteins (“undruggable”; great variety)
- *Pose unique scientific and regulatory challenges* (“Big small molecules”; currently no ICH regulatory guidelines or FDA general CMC guidances)
  - ✓ 1<sup>st</sup> PSG – Nusinersen, draft guidance in Feb 2022

# Challenges in Impurity Assessment



# Challenges in Impurity Assessment



# Outline

- In-depth impurity assessment case study
  - HRMS-enabled identity and quantitation
  - Isobaric impurity case: deletion sequence variants
- High vs low resolution MS

# HRMS-enabled identity



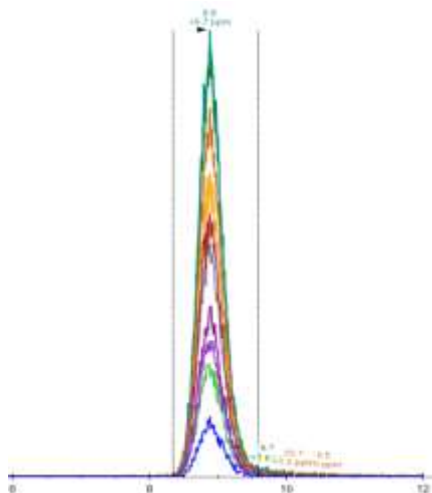
**Full-length product (FLP):** 2'-O-MOE, PS modified RNA 18-mer (nusinersen)

U-C-A-C-U-U-U-C-A-U-A-A-U-G-C-U-G-G (C and U are methylated)

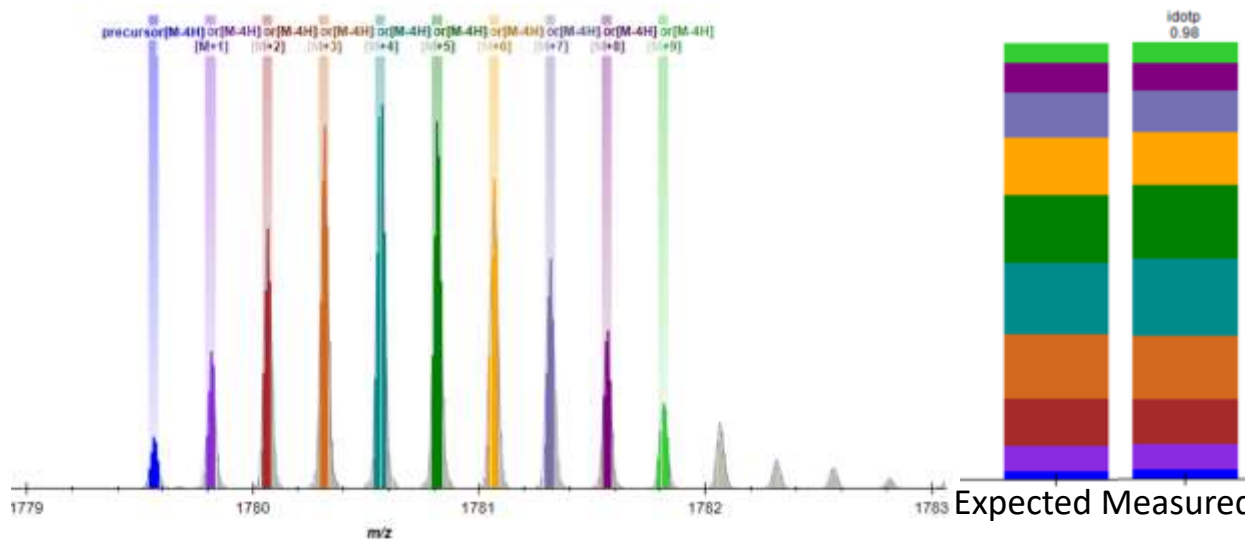
Exact mass: 7122.2763;  $m/z$  (-4 charge state): 1779.5618

precursor - 1779.5618[M-4H]	precursor [M+1] - 1779.8125[M-4H]	precursor [M+2] - 1780.0628[M-4H]
precursor [M+3] - 1780.3131[M-4H]	precursor [M+4] - 1780.5633[M-4H]	precursor [M+5] - 1780.8135[M-4H]
precursor [M+6] - 1781.0636[M-4H]	precursor [M+7] - 1781.3138[M-4H]	precursor [M+8] - 1781.5639[M-4H]
precursor [M+9] - 1781.8140[M-4H]		

## Accurate mass



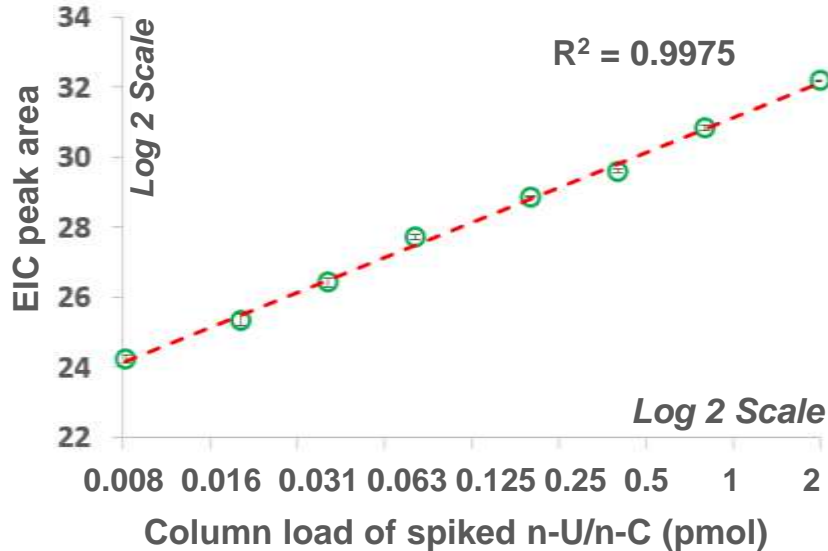
## Isotopic envelope / distribution



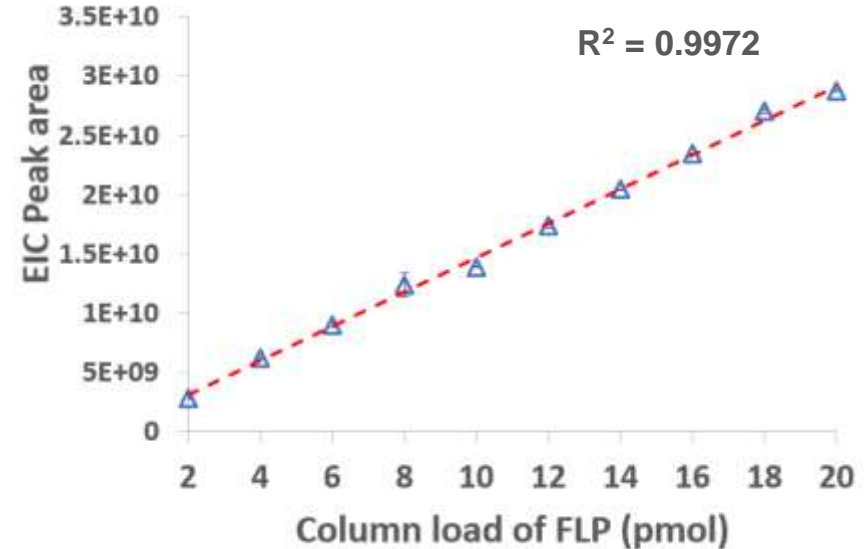
# HRMS-enabled quantitation



HILIC-HRMS: Extracted ion chromatogram (EIC) of isotopic peaks



RSD (%) of EIC peak area < 15%



RSD (%) of EIC peak area < 10%



A challenging scenario ...

## **Case study: resolve isobaric sequence deletion impurities**

## Isobaric: equal nominal mass

### Full-length product (FLP):

*2'-O-MOE, PS modified RNA 18-mer* (nusinersen)

U-C-A-C-U-U-U-C-A-U-A-A-U-G-C-U-G-G (C and U are methylated)

Exact mass: 7122.2763;  $m/z$  (-4 charge state): 1779.5618

### Isobaric sequence deletion impurities

Impurities	Exact mass (Da)	$m/z$ (-4 charge state)
<i>n-U</i>	6728.2163	<b>1681.0468</b>
<i>n-C</i>	6729.2003	<b>1681.2928</b>

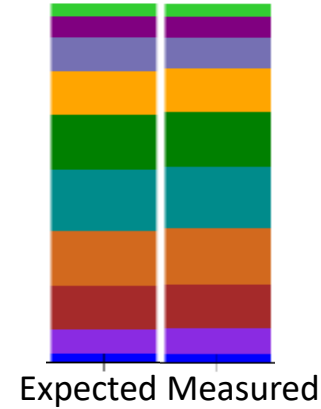
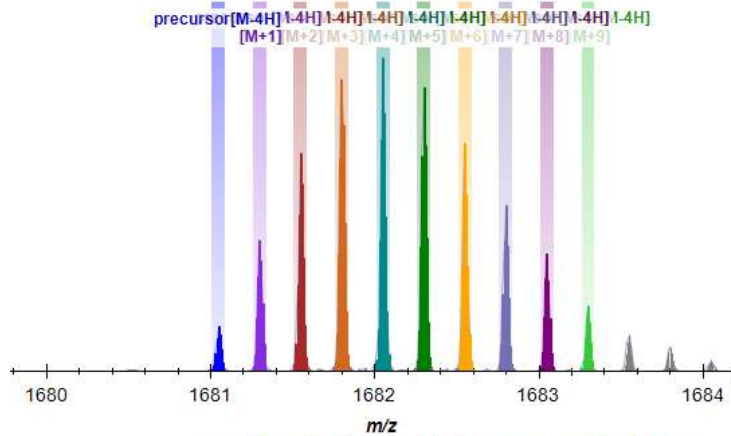
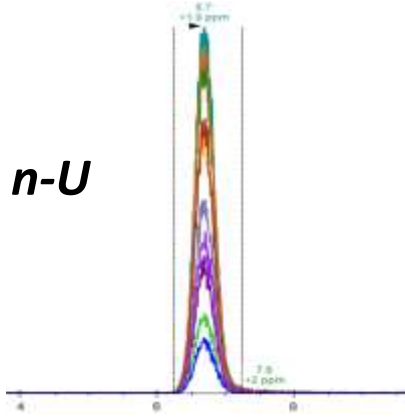
# Individual n-U and n-C



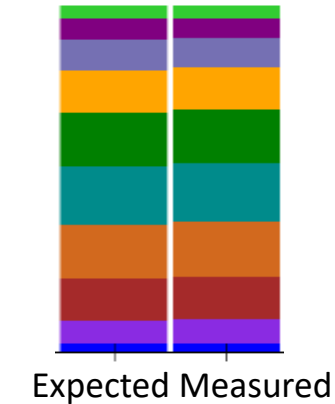
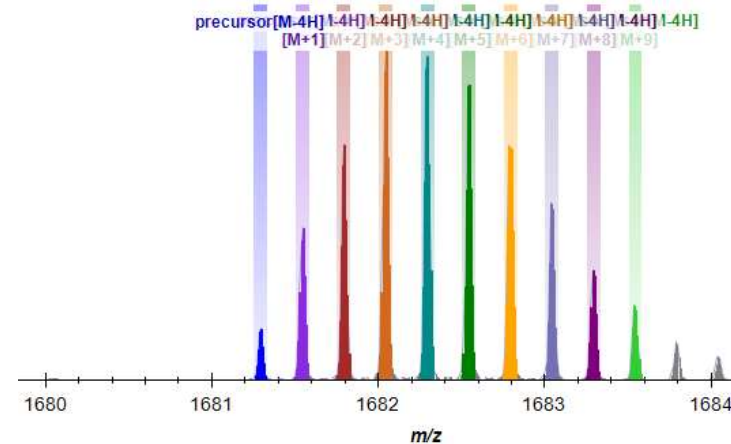
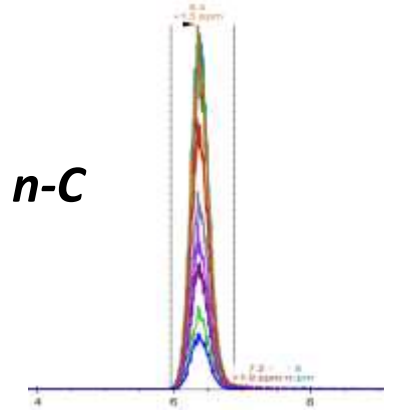
Accurate mass

Isotopic envelope / distribution

*n-U*



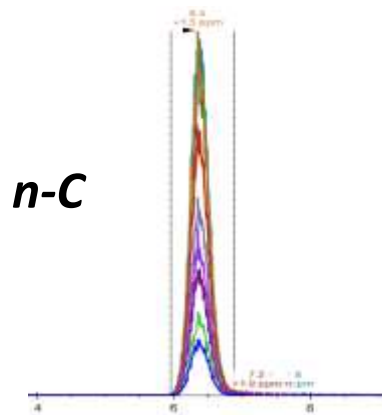
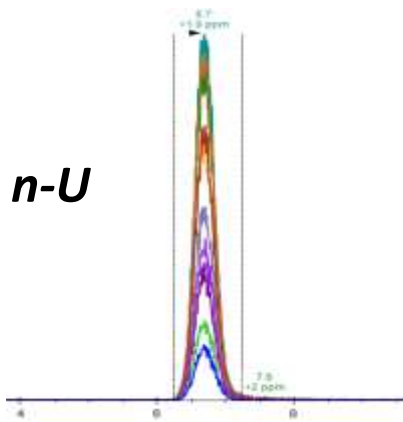
*n-C*



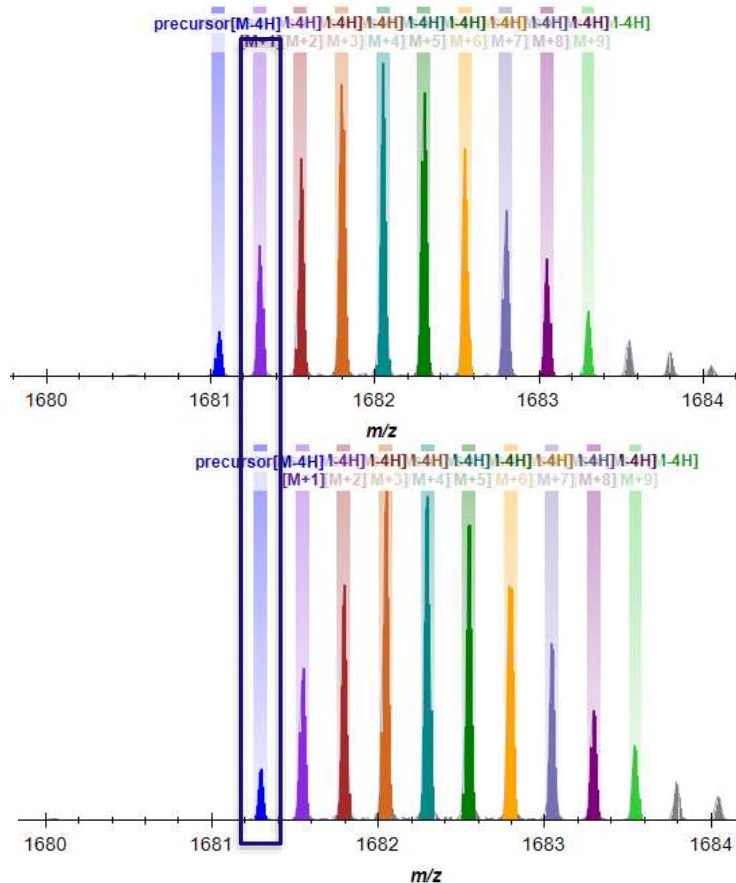
# Individual n-U and n-C



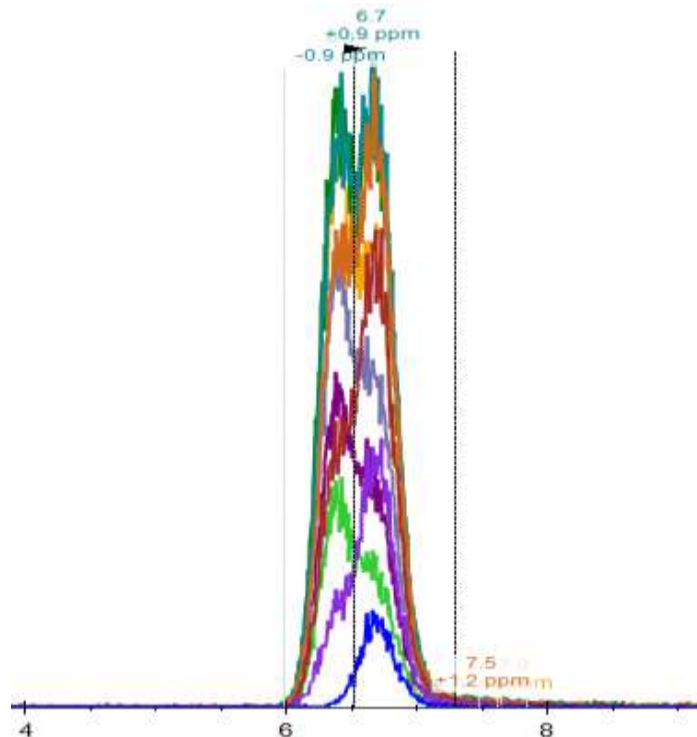
## Accurate mass



## Isotopic envelope / distribution



	<i>n-U</i>		<i>n-C</i>		$\Delta$
M	<b>1681.0468</b>				
M+1	1681.2975	M	1681.2928	0.0047	
M+2	1681.5478	M+1	1681.5435	0.0043	
M+3	1681.7981	M+2	1681.7938	0.0043	
M+4	1682.0483	M+3	1682.0441	0.0042	
M+5	1682.2984	M+4	1682.2943	0.0041	
M+6	1682.5486	M+5	1682.5445	0.0041	
M+7	1682.7987	M+6	1682.7946	0.0041	
M+8	1683.0488	M+7	1683.0447	0.0041	
M+9	1683.2989	M+8	1683.2948	0.0041	



## Coexisting *n-U* / *n-C* in a mixture:

- Not chromatographically separated
- Not mass differentiated
- Not individually reported in QC testing or specification

**n-p(MOE<sup>Me</sup>C) / n-p(MOE<sup>Me</sup>U)**

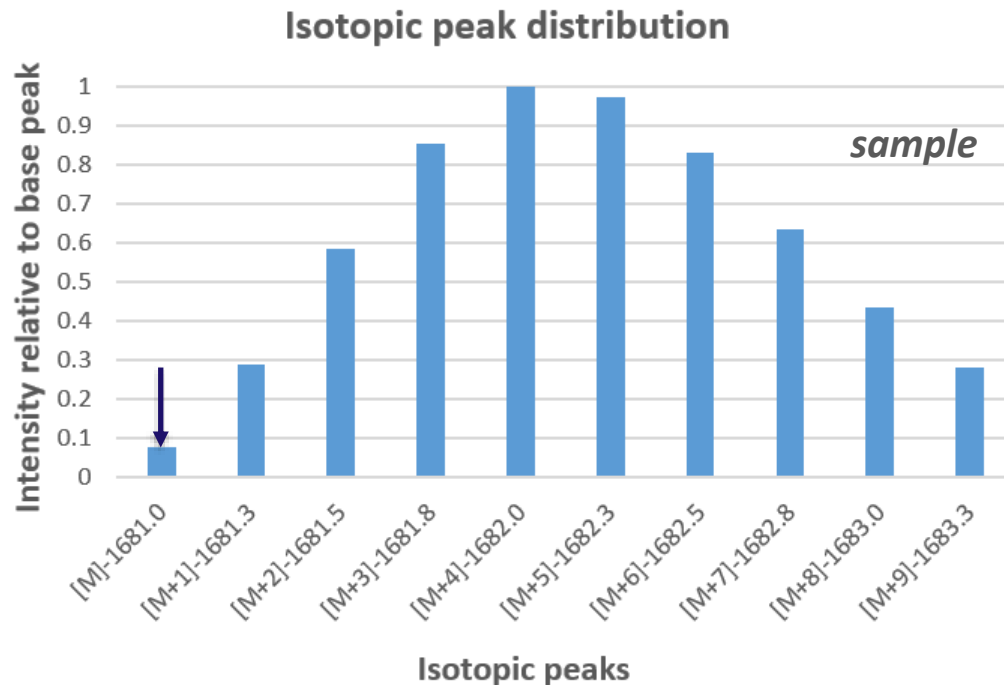
**@ m/z 1682.2**

# n-U / n-C mixtures



<i>n-U</i>		<i>n-C</i>	
M	1681.0468		
M+1	1681.2975	M	1681.2928
M+2	1681.5478	M+1	1681.5435
M+3	1681.7981	M+2	1681.7938

- Monoisotopic peak at 1681.0 indicates **the presence of n-U**

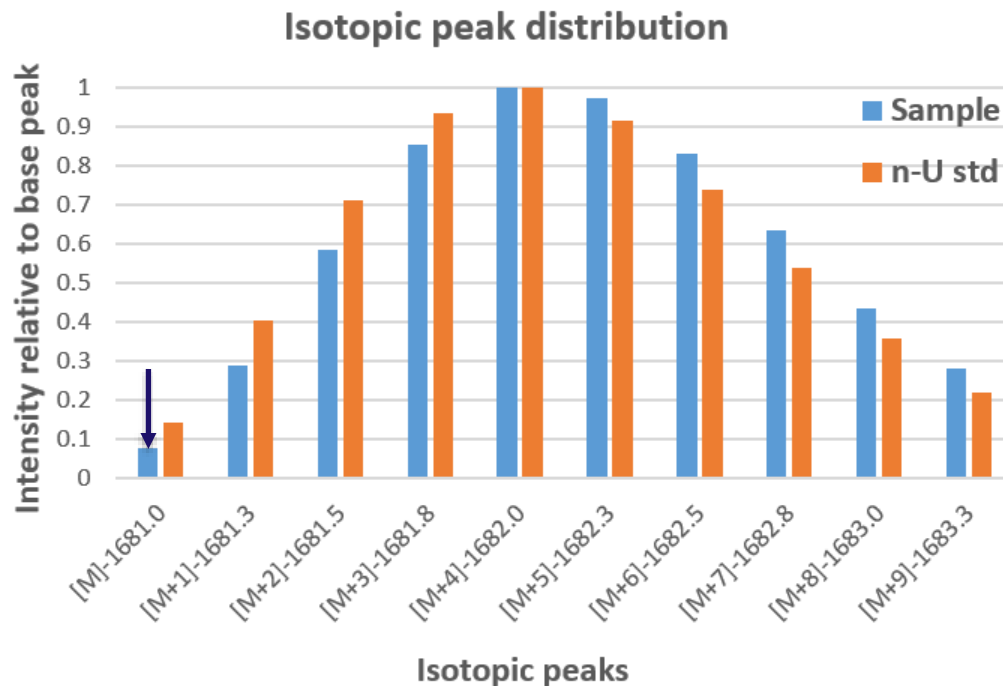


# n-U / n-C mixtures



<i>n-U</i>		<i>n-C</i>	
M	1681.0468		
M+1	1681.2975	M	1681.2928
M+2	1681.5478	M+1	1681.5435
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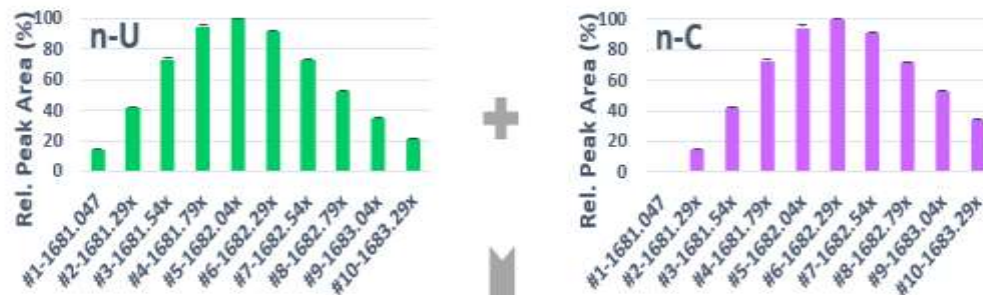
- Monoisotopic peak at 1681.0 indicates **the presence of n-U**
- Mismatching isotopic pattern indicates **the coexistence** of n-U with n-C in the mixture



# Calculated (predicted) vs Measured isotopic distributions

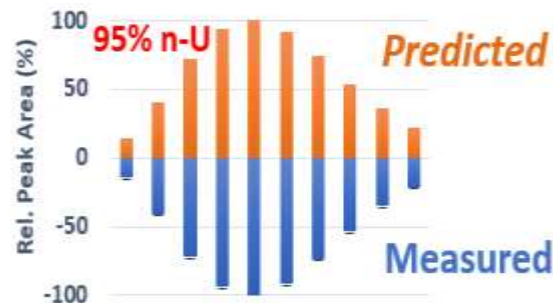
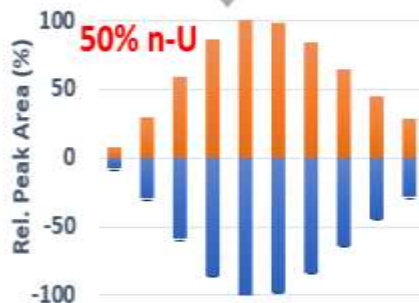
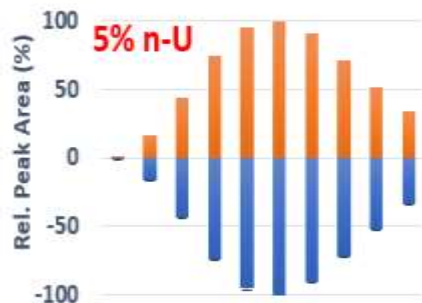


Individual  
n-U and n-C



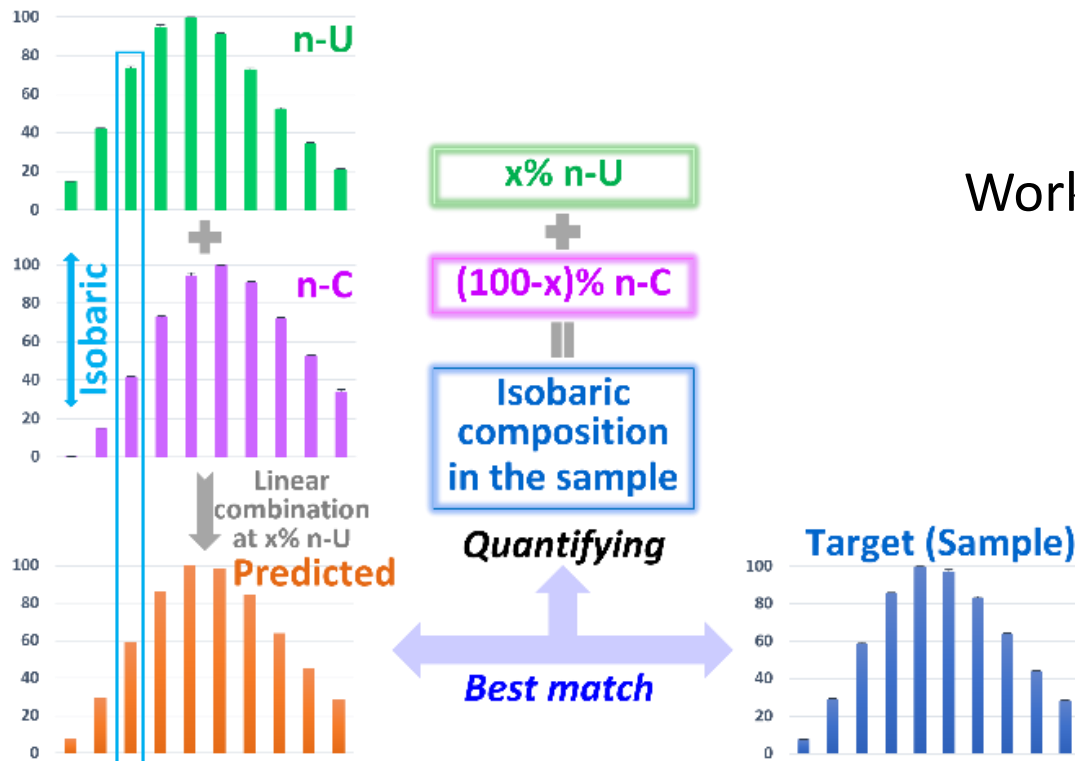
Linear combination

n-U / n-C  
mixtures





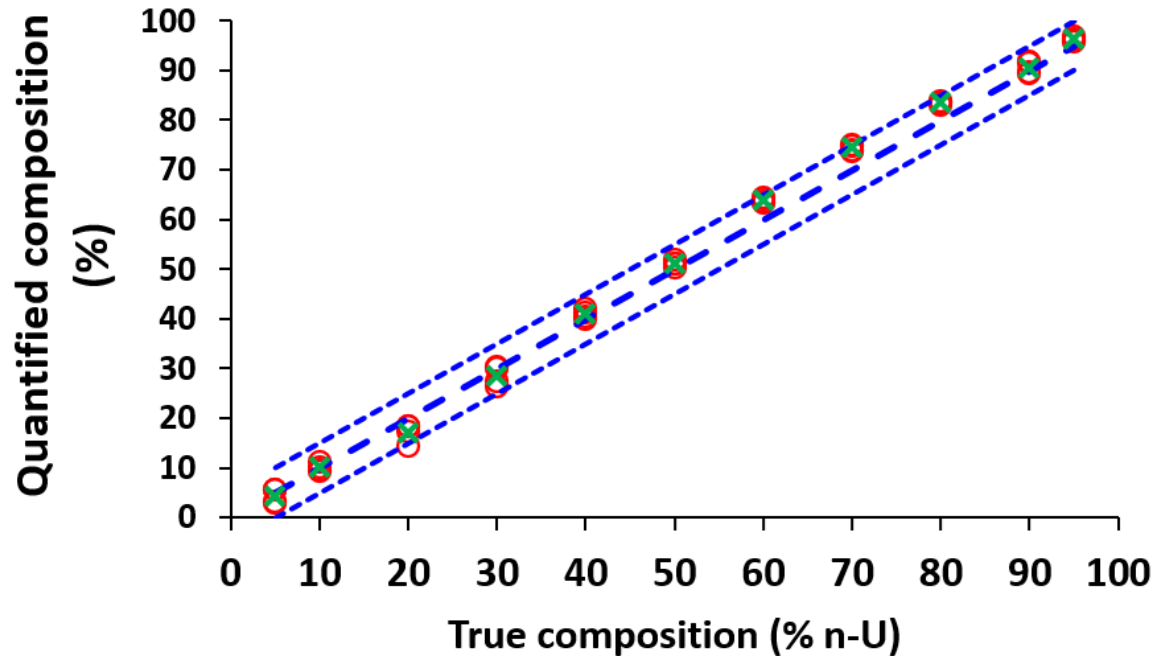
# Quantification of isobaric composition of n-U/n-C mixtures



**Best match:**

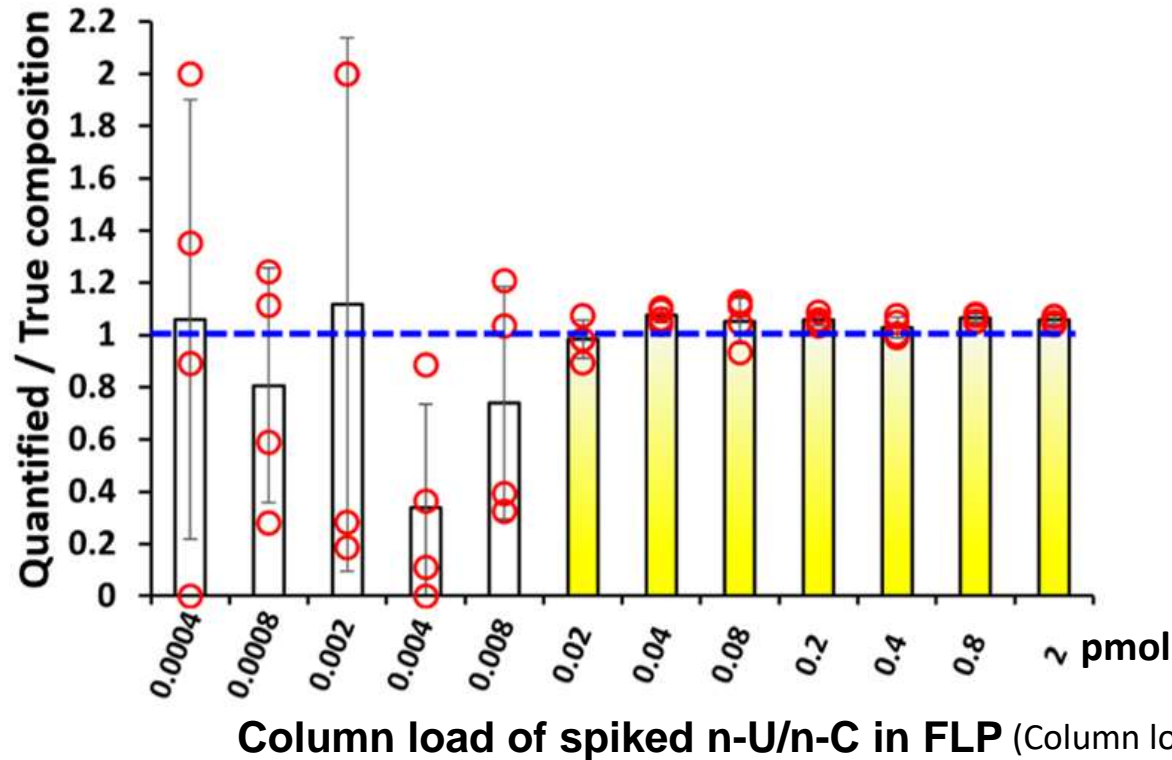
**Defined by:** the difference between the predicted and the targeted is minimum.

# Quantification of isobaric composition of n-U/n-C mixtures (covering 5%/95% n-U/n-C to 95%/5% n-U/n-C)



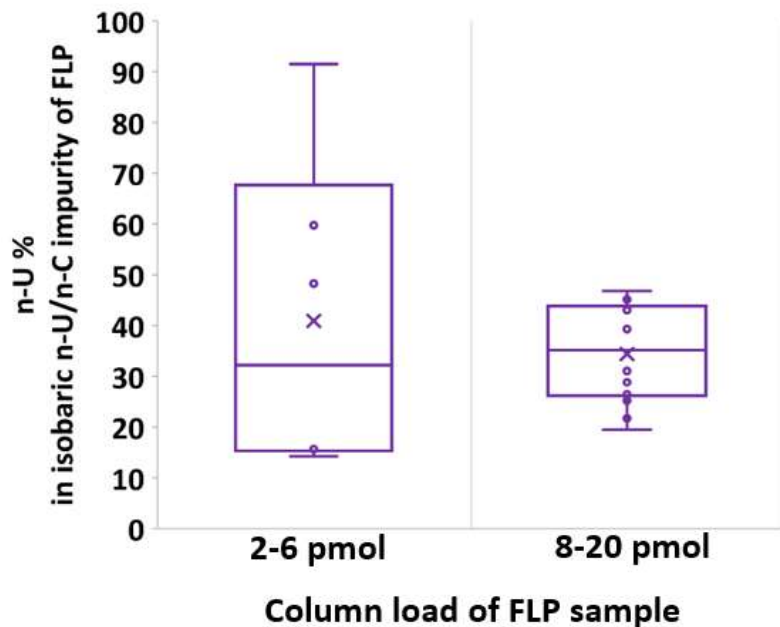
Unpublished data: A.M. Abdullah, C. Sommers, J. Rodriguez, D. Zhang, D. Kozak, J. Hawes, M. Sapru, and K. Yang. Manuscript under revision.

# Quantification of isobaric composition of the n-U/n-C standard *spiked* in FLP



Unpublished data: A.M. Abdullah, C. Sommers, J. Rodriguez, D. Zhang, D. Kozak, J. Hawes, M. Sapru, and K. Yang. Manuscript under revision.

# Quantification of isobaric composition of the n-U/n-C impurity ***present*** in FLP



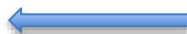
On-column n-U/n-C  
< 0.02 pmol      > 0.02 pmol

## FLP:

Custom synthesized by vendor:

- Synthesis – Solid phase phosphoramidite synthesis
- Purification method – RP HPLC

Total n-U/n-C in FLP quantified at  
 $0.27 \pm 0.02\%$  of FLP



# High vs low resolution MS

	HRMS	Unit- or low-resolution MS
<b>Isotopic peak-resolved envelope</b>	Yes	No
<b>Identification of coexisting n-U and n-C (isobaric)</b>	Yes	No (n-U/n-C)
<b>Quantitation of coexisting n-U/n-C ratio</b>	Yes	No (sum of n-U/n-C)

# Summary



- Synthetic oligonucleotides, as an evolving class of therapeutics, pose unique scientific and regulatory challenges.
- In-depth impurity assessment offers comprehensive impurity evaluation and is crucial for comparative impurity analysis in generic drug development.
- High resolution MS demonstrates advantages in resolving complex oligonucleotide impurities, such as isobaric sequence variants.

# Acknowledgement



- **Office of Testing and Research (OTR)**
  - ✓ Oligo research team  
A.M. Abdullah
  - ✓ Division of Complex Drug Analysis (DCDA)
  - ✓ OTR Management  
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Jason Rodriguez  
David Keire
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  - Deyi Zhang
  - Darby Kozak
- **FDA Critical-Path Research Grants (FY20, FY22)**
  - ✓ PI: Kui Yang
  - ✓ Collaboration offices:  
OPQ, OGD, OND, NCTR
- **OTR-led Oligo SME team / working group**
  - ✓ Participating offices:  
OPQ, OGD, OND, NCTR, OTS

# Challenge Question #1

**Which of the following statements is NOT correct for synthetic oligonucleotide therapeutics (ONTs)?**

- A. ONTs target a broad range of mRNAs.
- B. Like mRNAs, ONTs are regulated as biologics by FDA.
- C. Currently no ICH regulatory guidelines specifically address quality aspects for ONTs.
- D. FDA published the first PSG draft guidance for ONTs in 2022.



# Challenge Question #2

**True or False?**

**In-depth impurity assessment by HRMS is recommended in PSG draft guidance for Nusinersen published in Feb 2022.**

- A. True
- B. False

# Questions?

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