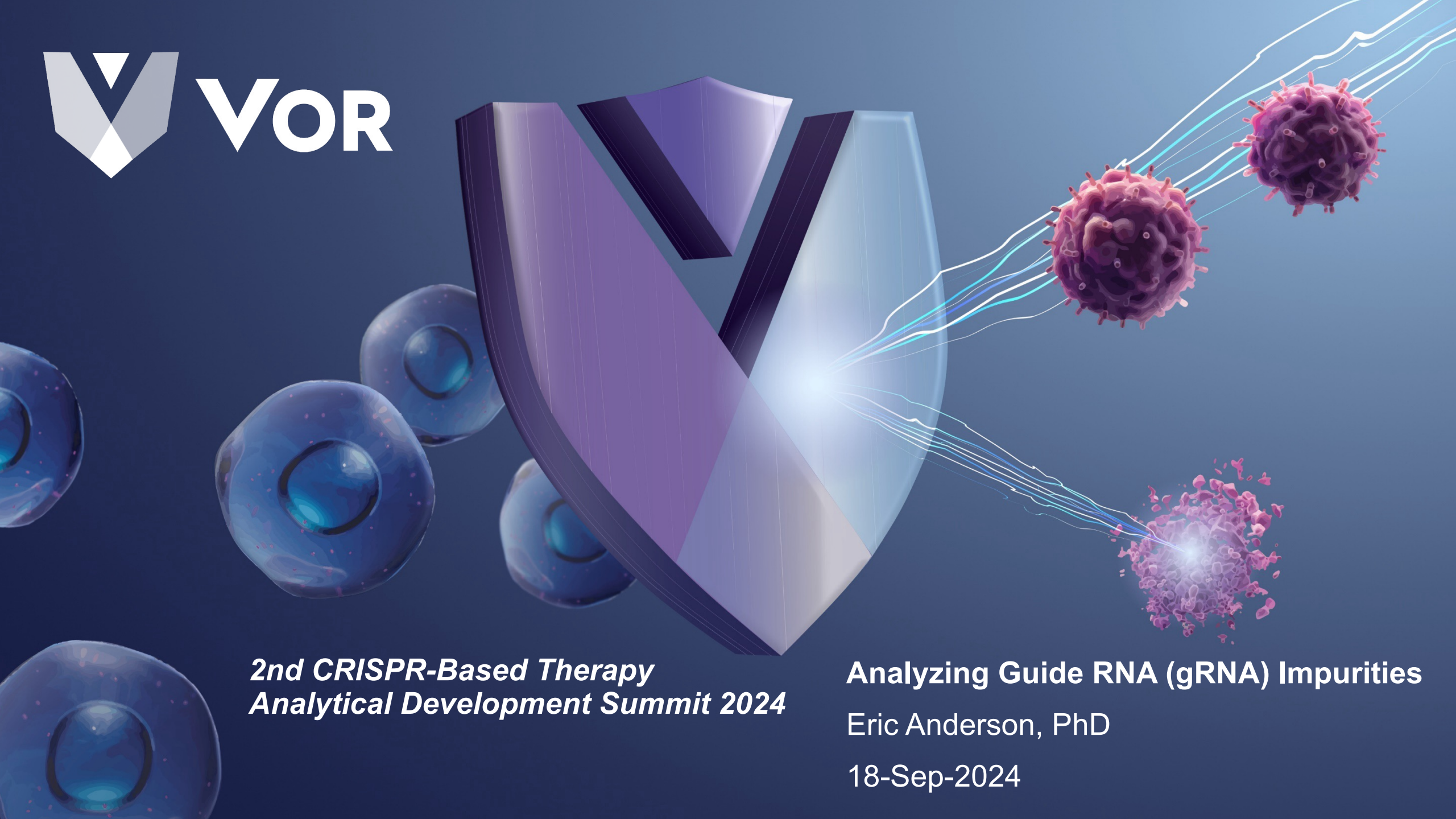


2nd CRISPR-Based Therapy Analytical Development Summit 2024

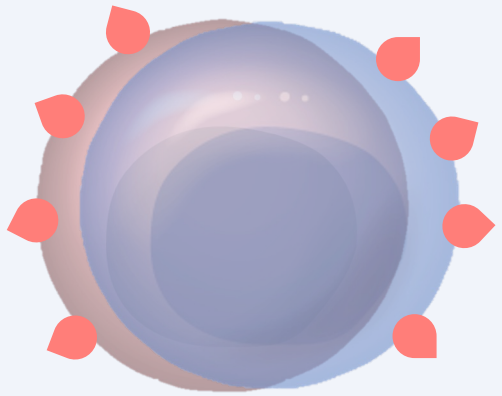
Analyzing Guide RNA (gRNA) Impurities

Eric Anderson, PhD

18-Sep-2024

We use genome engineering to make healthy cells invisible to drugs

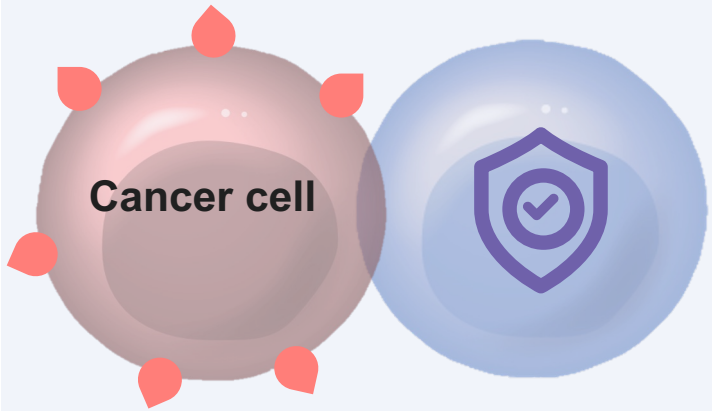
Problem



Few unique cancer antigens, so drugs kill both cancer and healthy cells through **on-target toxicity**

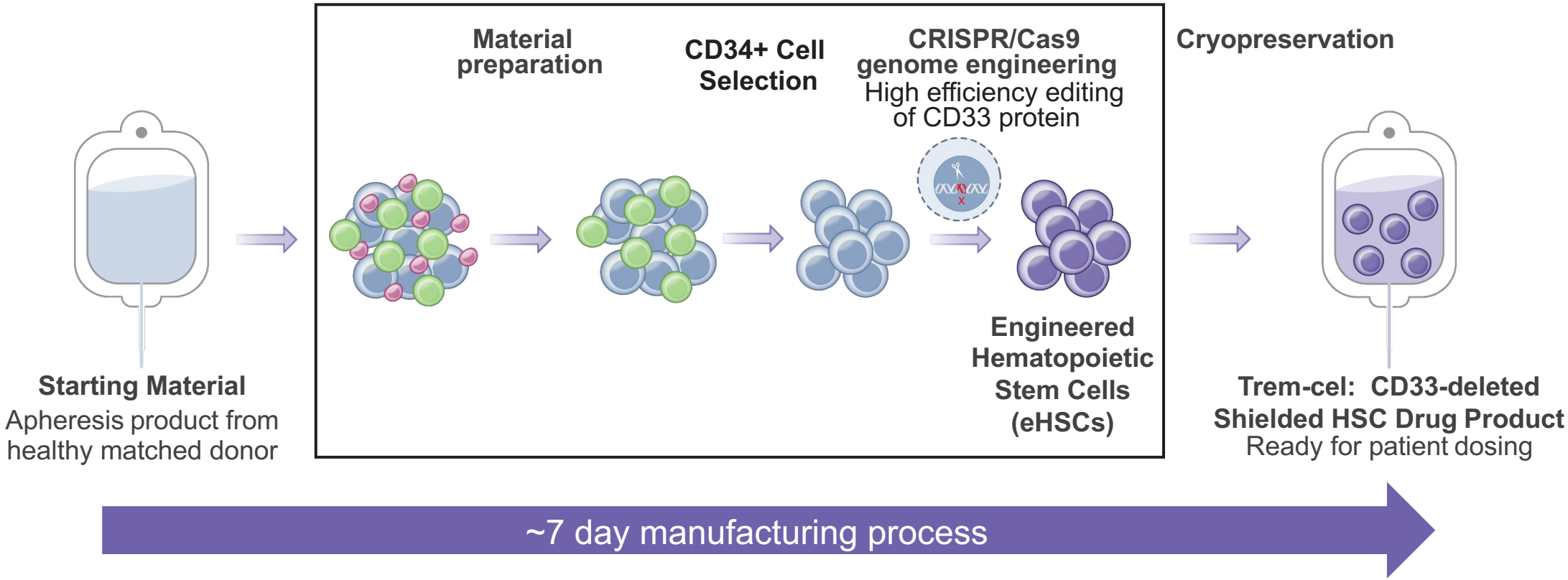
Genome engineering

Vor Paradigm: Engineered HSCs (eHSCs)

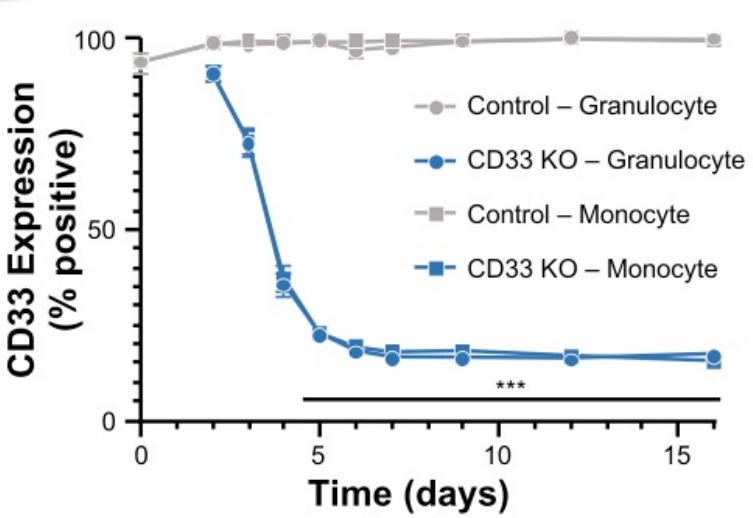


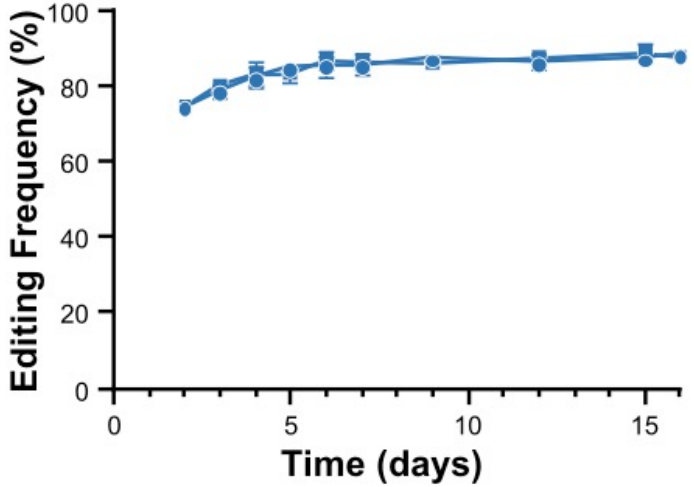
Remove target expression on healthy cells so that killing is **cancer-specific**

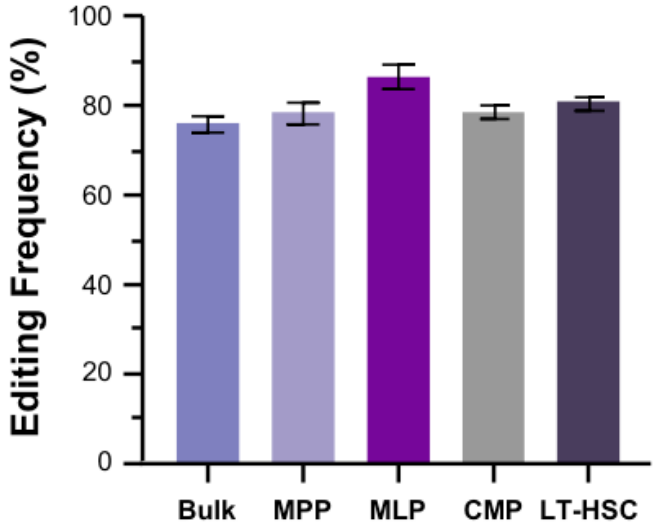
Trem-cel uses CRISPR/Cas9 editing to delete CD33 in hematopoietic cell transplants during treatment of Acute Myeloid Leukemia



CRISPR/Cas9 effectively and safely edits loss of CD33 from CD34+ cells







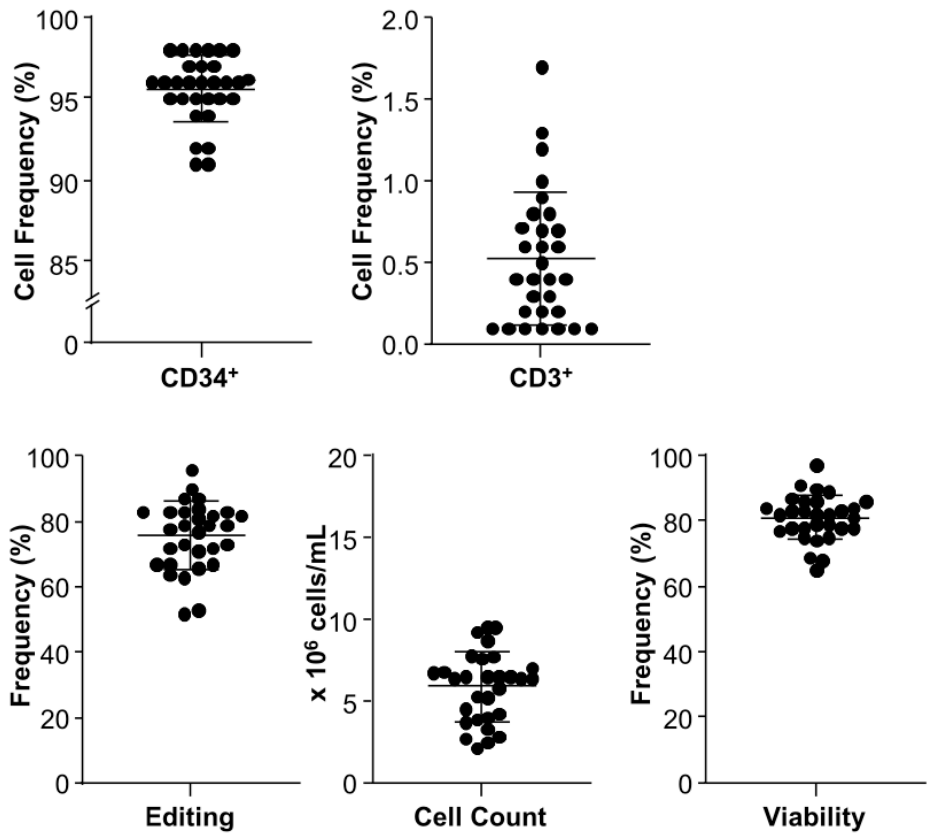
Trem-cel has no significant off-target effects

Nomination	Validation	Results
***in silico* off-target prediction** up to 5 mismatches 3076 Sites **Cell-based nomination assay** 19 sites	**Hybrid Capture** 3085 sites 7 trem-cel batches tested	**0 off-target sites** with editing above detection limit (≥0.2%) 71.27% average on-target editing

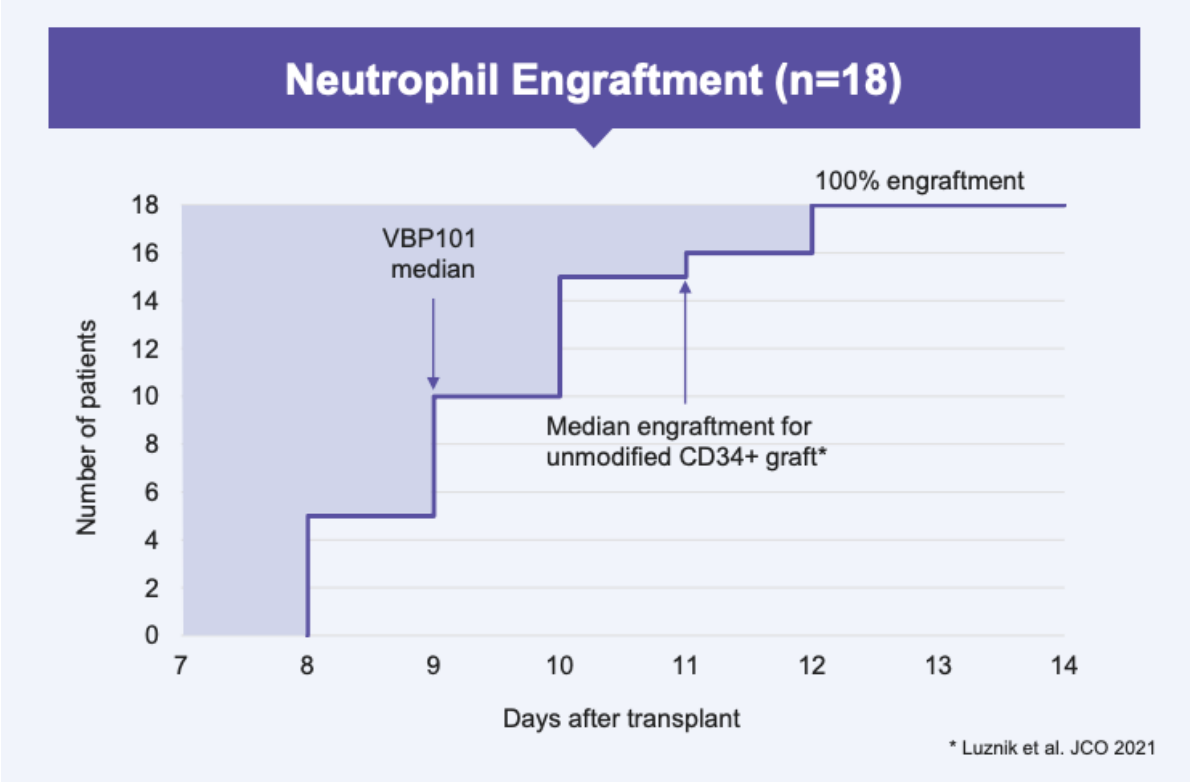
Figures from: J. R. Lydeard *et al.*, *Mol Ther Methods Clin Dev.* (2023)

Trem-cel is manufactured at clinical-scale with robust CD33 editing and demonstrates 100% engraftment in 18 patients

Trem-cel clinical-scale manufacturing (30 batches)



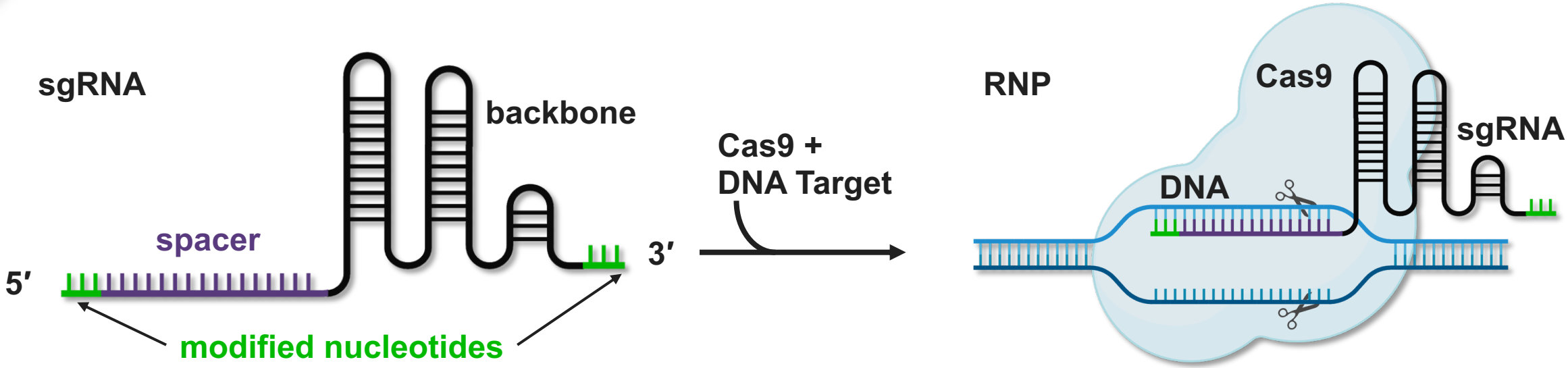
Trem-cel demonstrates 100% engraftment



High CD33 editing efficiency (median 89%, range 71-94%)

Figures from: J. R. Lydeard *et al.*, *Mol Ther Methods Clin Dev.* (2023)
Vor Bio Corporate Presentation, Sep 2024

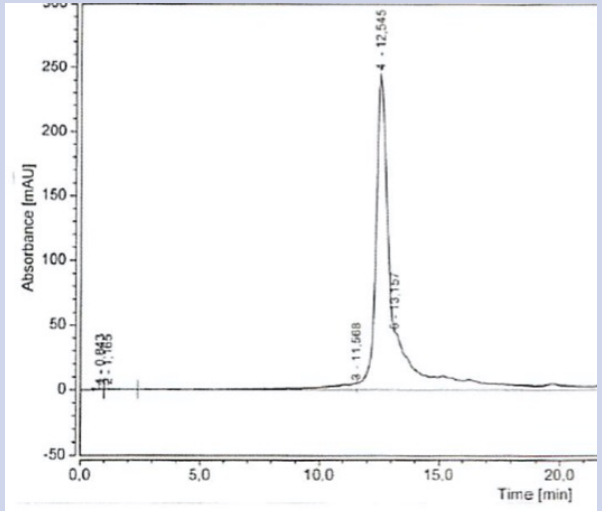
sgRNA is critical for RNP formation and on-target editing



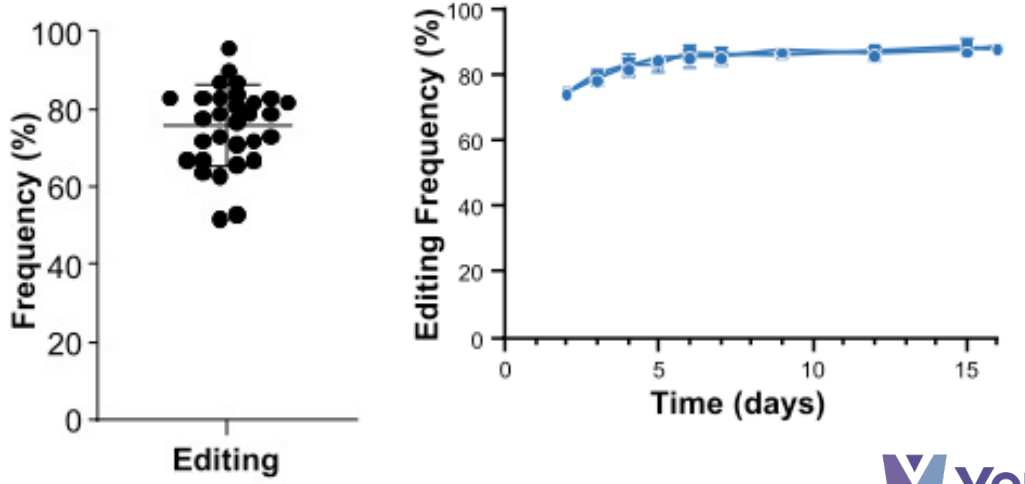
Identity

mGsmAsmGsmUCCGA
GCAGAAGAAGAAGUU
UUAGAGCUAGAAAUA
GCAAGUUAAAAUAAG
GCUAGUCCGUUAUCA
ACUUGAAAAAGUGGC
ACCGAGUCGGUGCmU
smUsmUsU

Purity

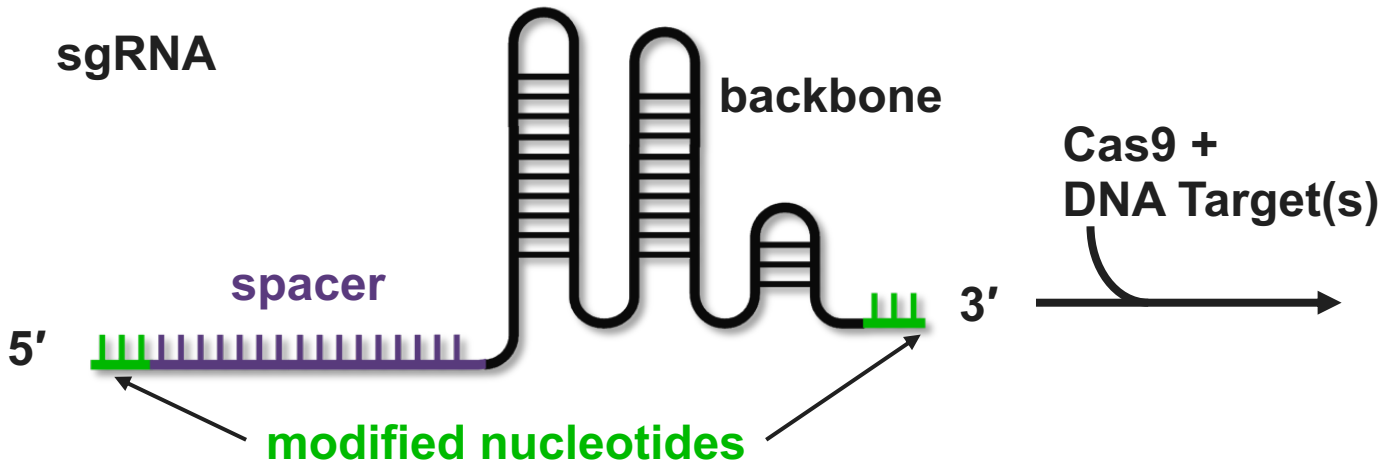


Potency



J. R. Lydeard *et al.*, *Mol Ther Methods Clin Dev.* (2023).

sgRNA is critical for on-target editing specificity

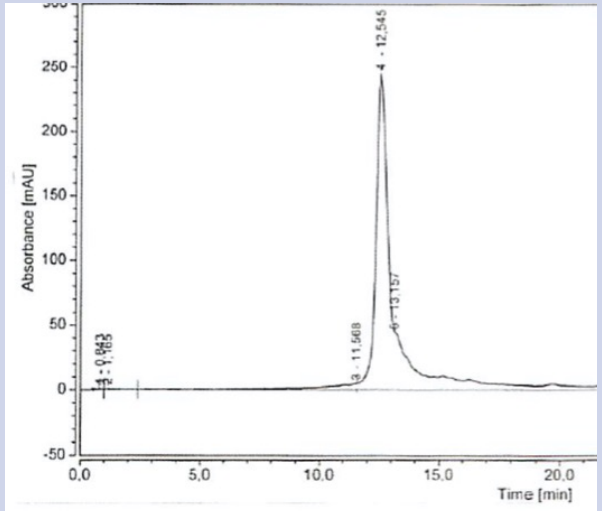


Cas9 +
DNA Target(s)

Identity

mGsmAsmGsmUCCGA
GCAGAAGAAGAAGUU
UUAGAGCUAGAAAUA
GCAAGUUAAAAUAAG
GCUAGUCCGUUAUCA
ACUUGAAAAAGUGGC
ACCGAGUCGGUGCmU
smUsmUsU

Purity



Safety

	spacer sequence	PAM
sgRNA	GAGUCCGAGCAGAAGAAGAA	NGG
DNA	CTGAGTCCGAGCAGAAGAAGAA	GGGCT

On-target with perfect matches

	GAGUCCGAGCAGAAGAAGAA	NGG
TTGA**A**TCCGAG**A**AGAAGAAGAA	GGGAC	

Off-target with **2** mismatches

sgRNA impurity
G>A substitution

	GA**A**UCCGAGCAGAAGAAGAA	NGG
TTGA**A**TCCGAG**A**AGAAGAAGAA	GGGAC	

Off-target with **1** mismatch

What is the FDA guidance for sgRNA as a critical component?

Question-and-Answer

What are FDA's recommendations for gRNA purity analysis?

In general, concern is with safety:

Optimize the GE components to reduce the potential for off-target genome modification, to the extent possible.

sgRNA Purity Recommendations

- ≥ 80% purity (HPLC)
- Identify impurities ≥ 1%

If < 80% purity

- Justify purity
- Conduct risk assessment for off-target

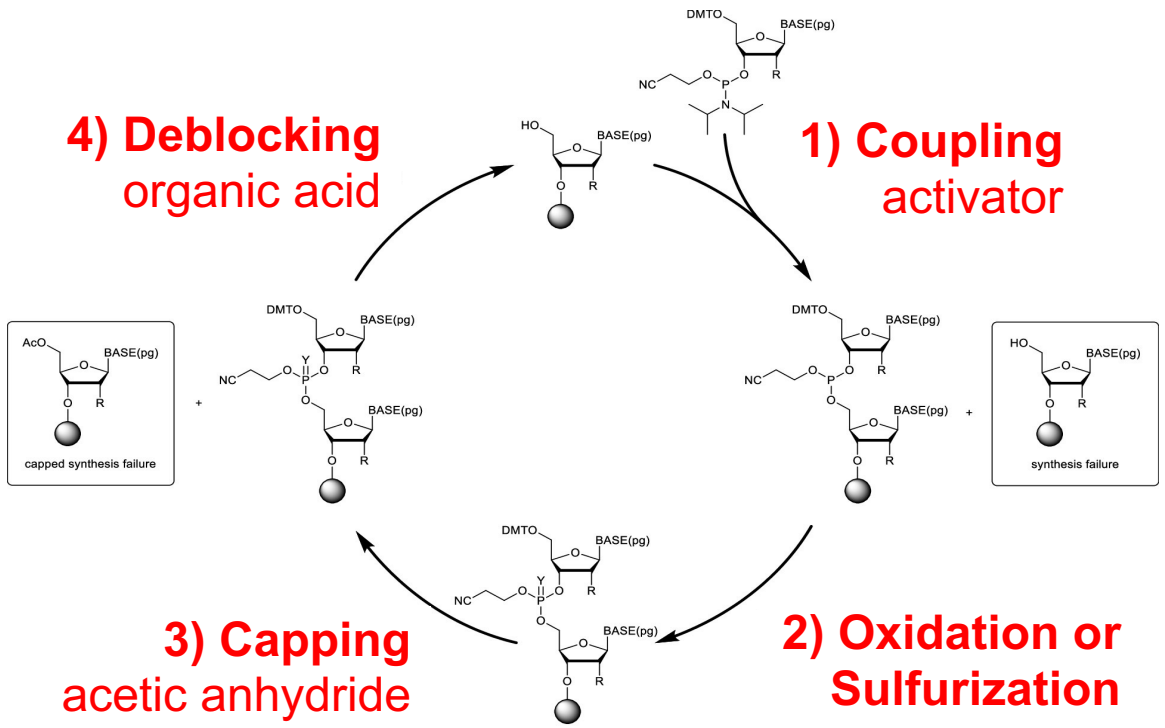
sgRNA Sequencing (NGS) Comments

- Not just for identity confirmation
- Orthogonal purity assay to LC/MS

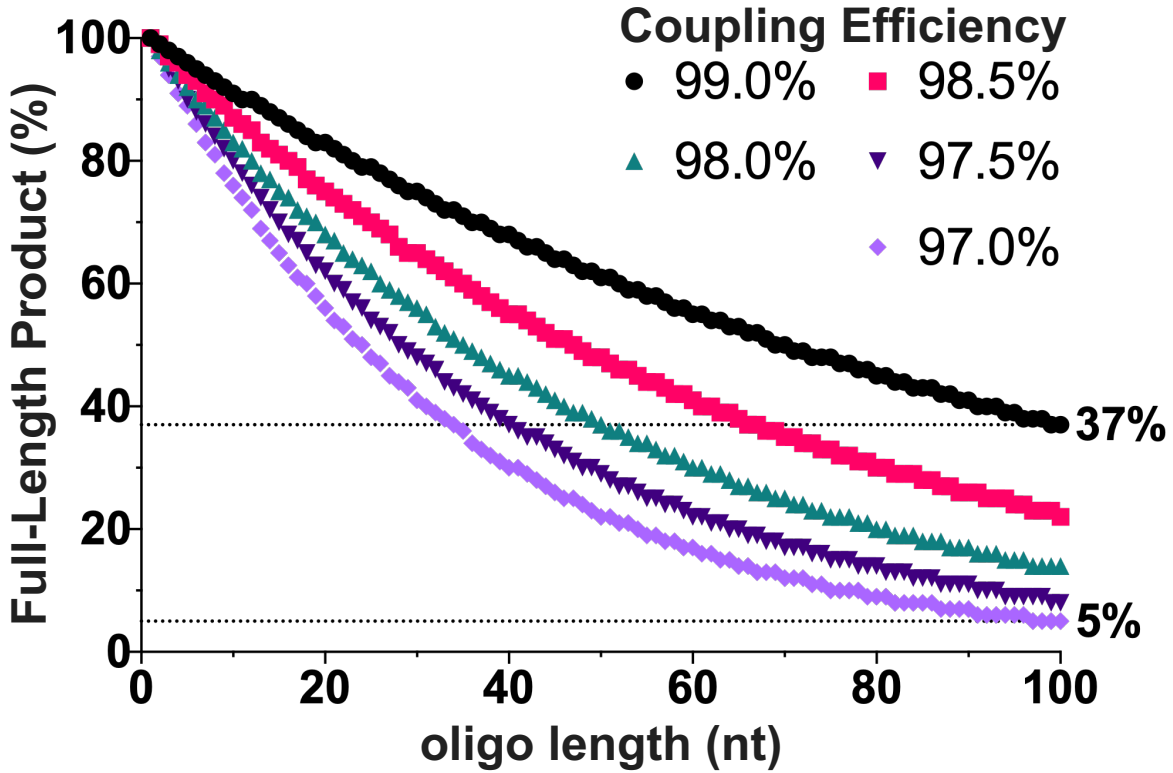
From: FDA CBER Webinar: Human Gene Therapy Products Incorporating Human Genome Editing, 29-Feb-2024

Synthesizing sgRNA at large scale with high purity is challenging

sgRNA synthesis cycle



Coupling efficiency impact on sgRNA full-length product yield

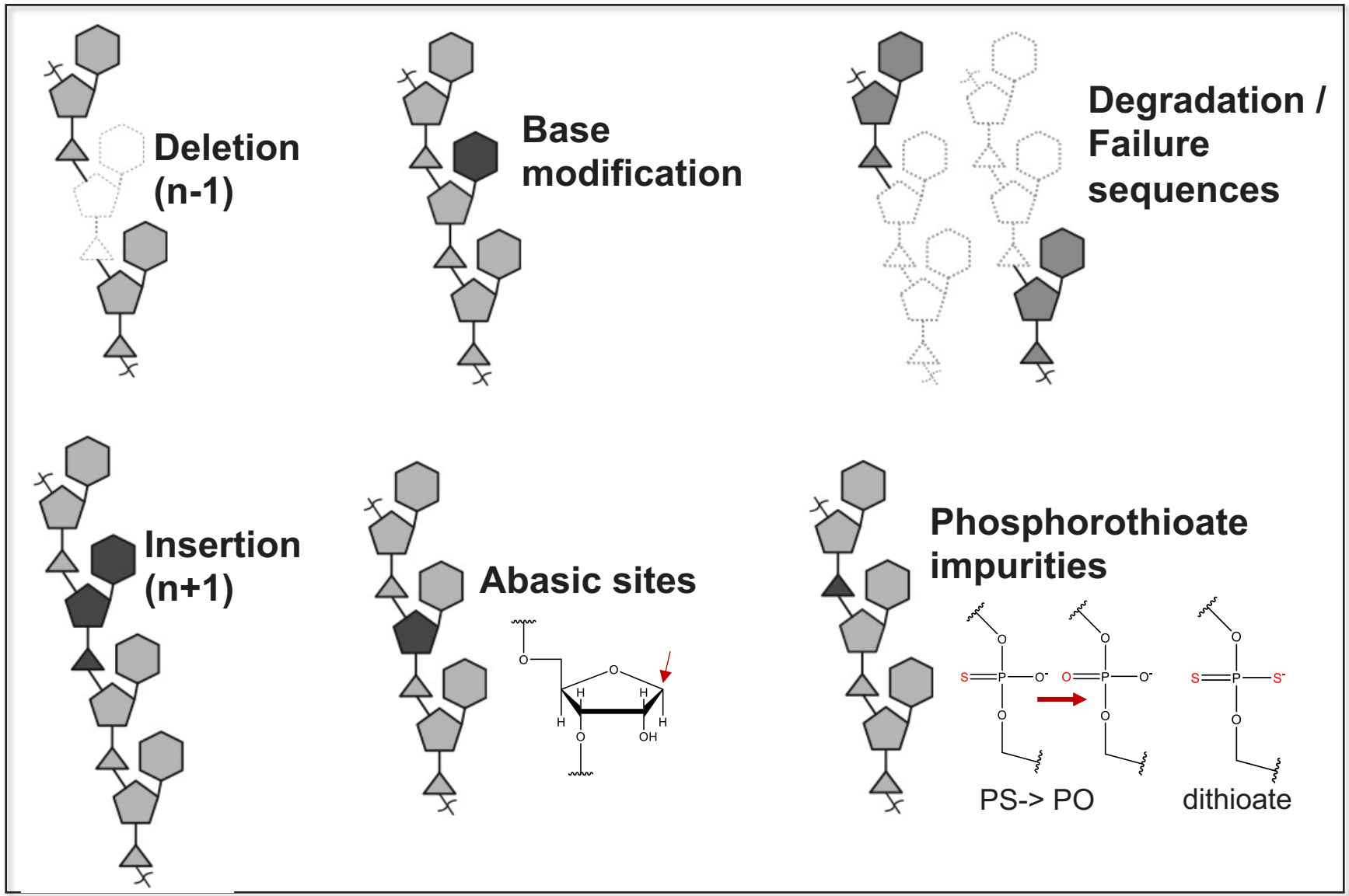


- sgRNA synthesis proceeds from 3′ to 5′ direction
- 100-mer gRNA ≈ 400+ sequential chemical reactions

- Theoretical % FLP = (efficiency)^(n-1)
- Greater oligo length, less full-length product

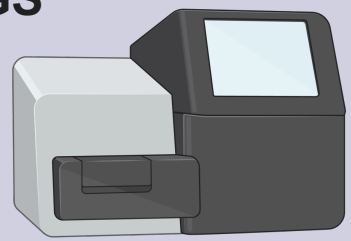
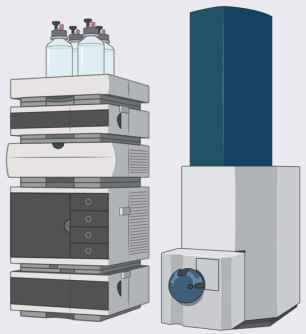
Figure adapted from: Andrews, B., *et al. J. Org. Chem.* 2021

Examples of product-related sgRNA impurities



Adapted from: D. Capaldi *et al.*, *Nucleic Acid Therapeutics*. (2017).

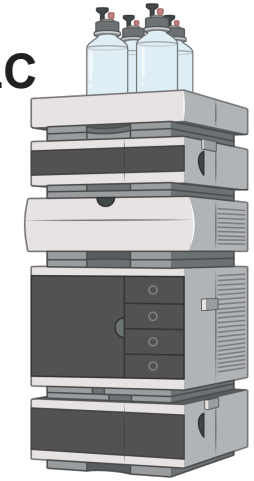
Using NGS and LC/MS as complementary methods for a comprehensive picture of sgRNA purity, identity and impurities

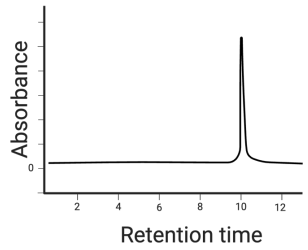
Method	Strengths	Weakness
NGS 	• Full length sequence coverage • Impurities sequence / position • High sensitivity (low LLOD) • High specificity	• Indirect method • Biases from library prep / sequencing errors • Modifications not captured / detected • Modifications interfere with library prep
LC-MS 	• Direct RNA detection • Intact mass of sgRNA • Impurities identification / quantification • Detection of modifications	• Intact mass alone not sufficient for identity • sgRNA sequence confirmation • Impurity sequence / position

Using NGS and LC/MS as complementary methods for a comprehensive picture of sgRNA purity, identity and impurities

Purity

HPLC

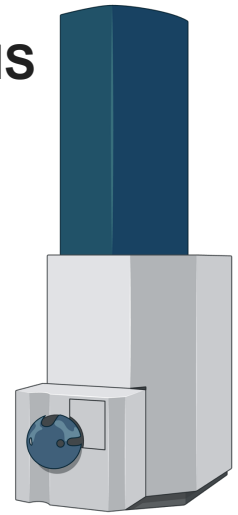


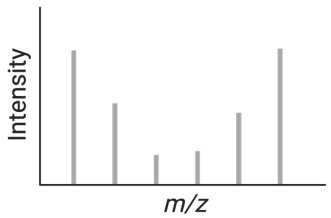


Purity as integrated peak area
Challenges:
Peak tailing / overlapping
Single-nucleotide resolution

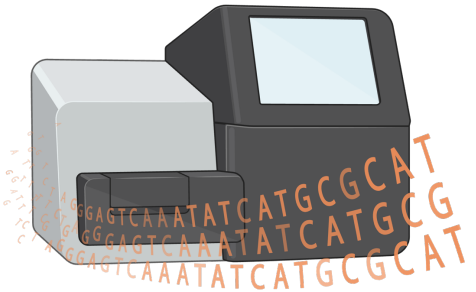
Identity

MS





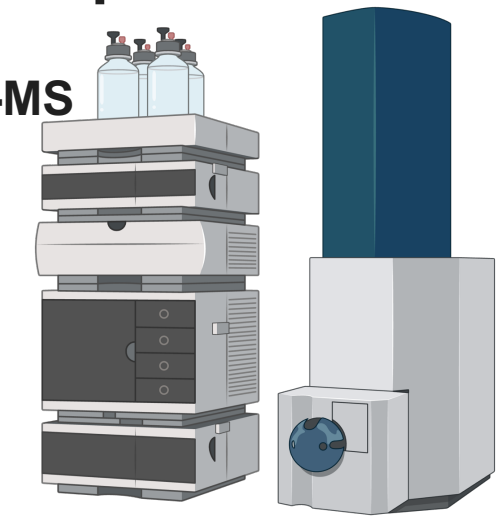
NGS



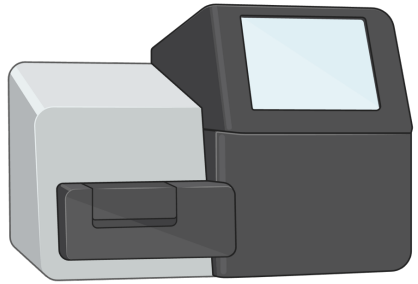
LC-MS: Intact mass
NGS: Full-length sequencing
LC-MS/MS: Full-length sequencing (challenging)

Impurities

LC-MS

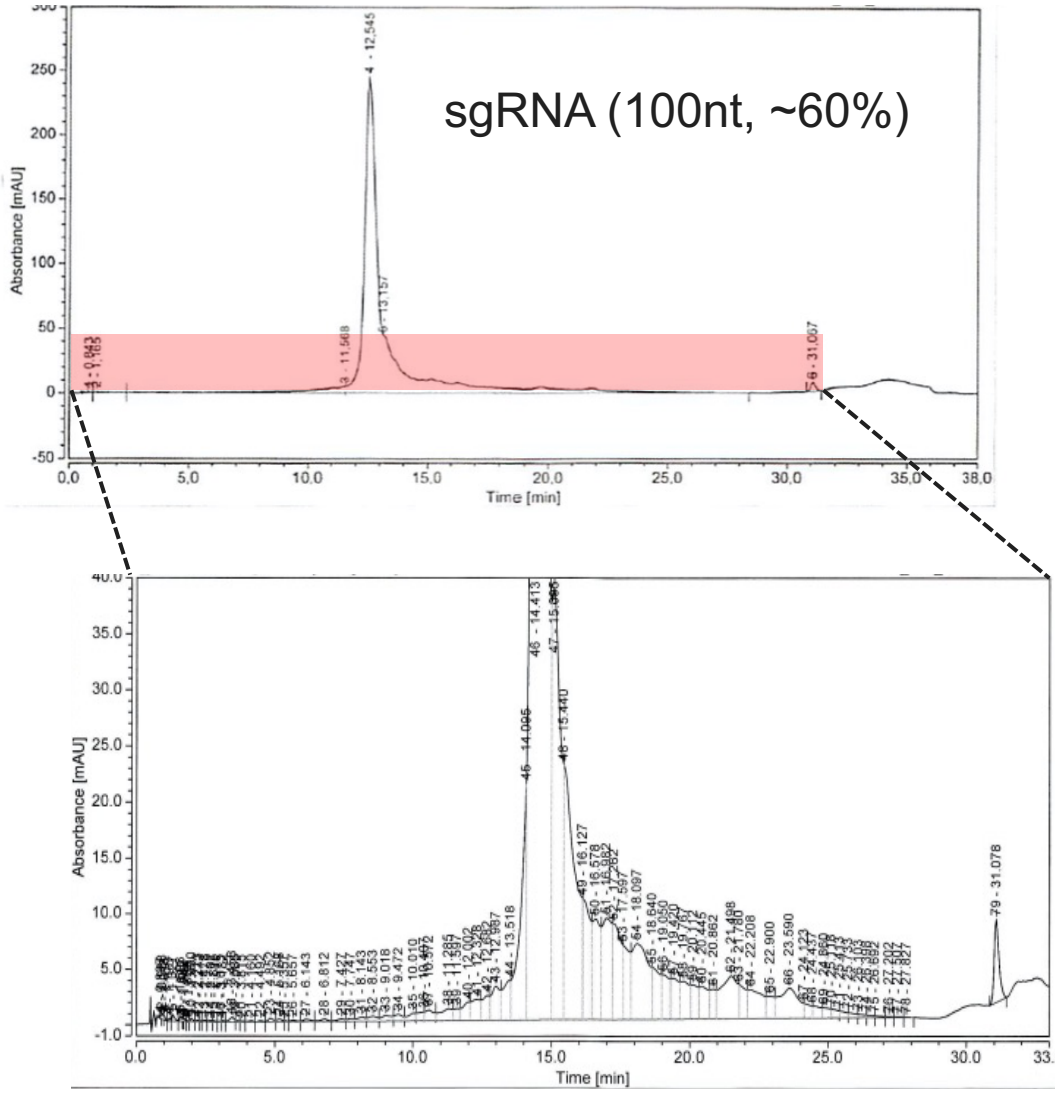


NGS



LC/MS: identification, quantification, chemical modifications / impurities
NGS: position of sequence impurities

HPLC is used for routine sgRNA purity assessment, but single-nucleotide resolution is challenging



100nt sgRNA with impurity spike ins

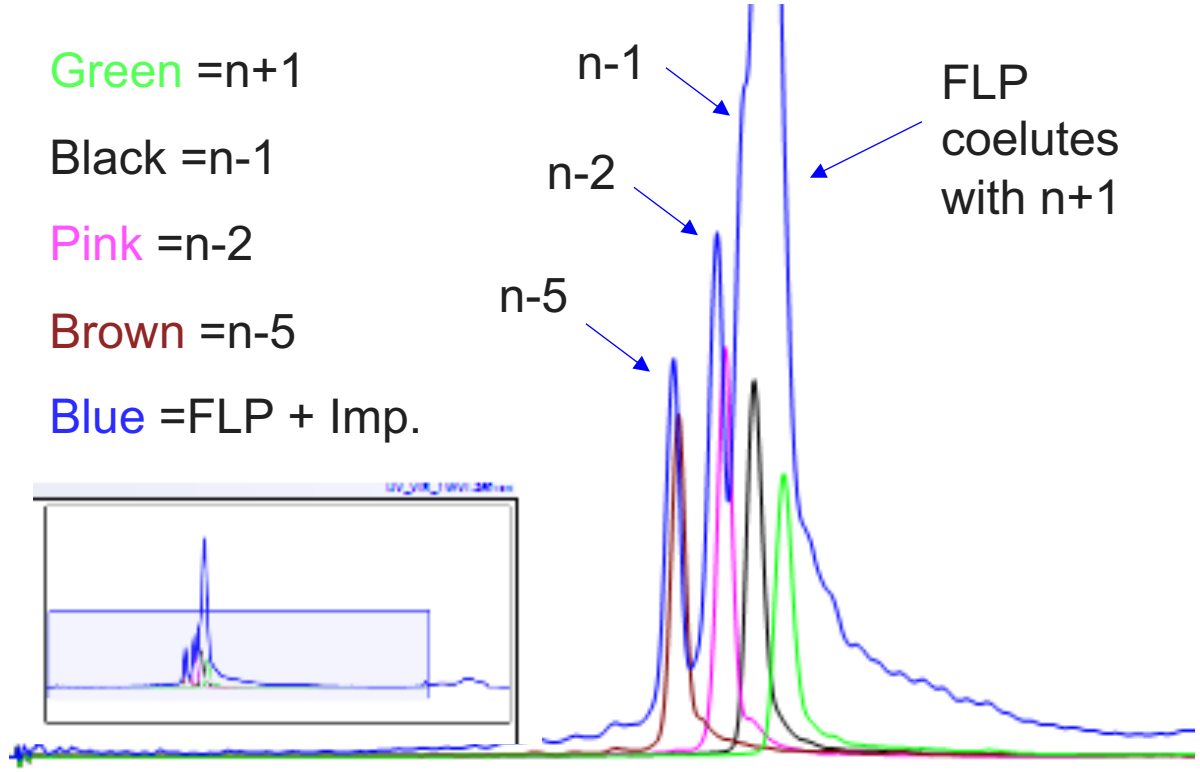
Green =n+1

Black =n-1

Pink =n-2

Brown =n-5

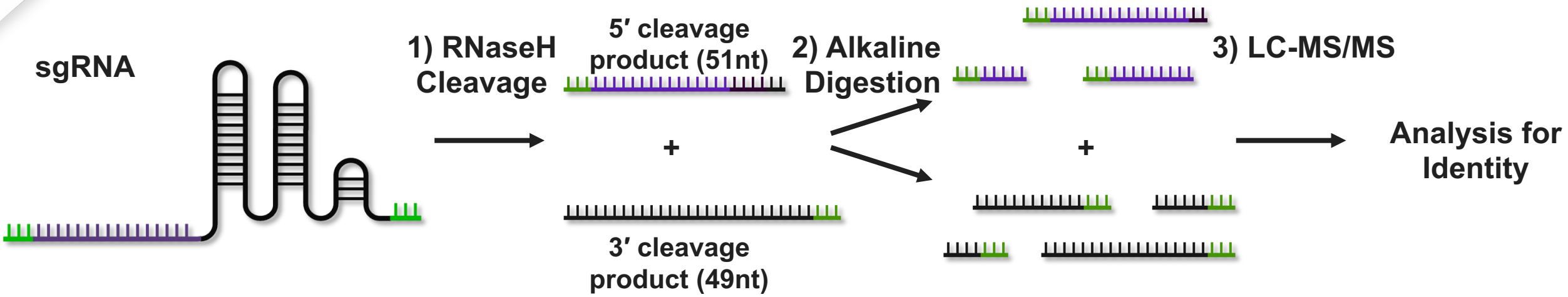
Blue =FLP + Imp.

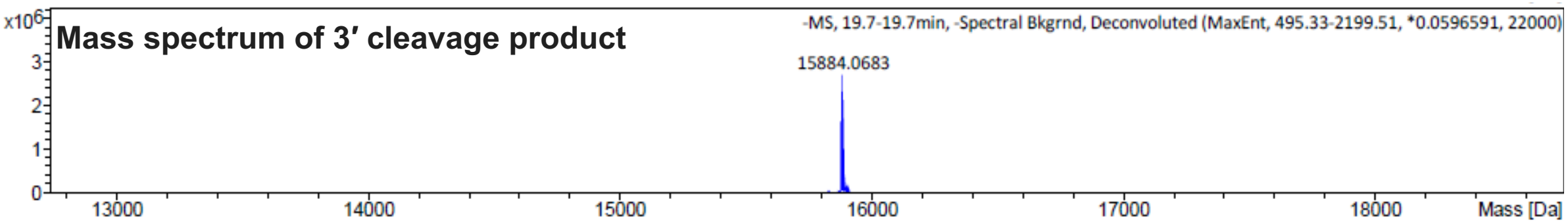


Challenges:

- Peak tailing leads to peak overlapping
- Resolving and collecting impurities
- Applying consistent peak integration

LC-MS/MS sequencing method provides complete coverage and sequence confirmation of modified sgRNA

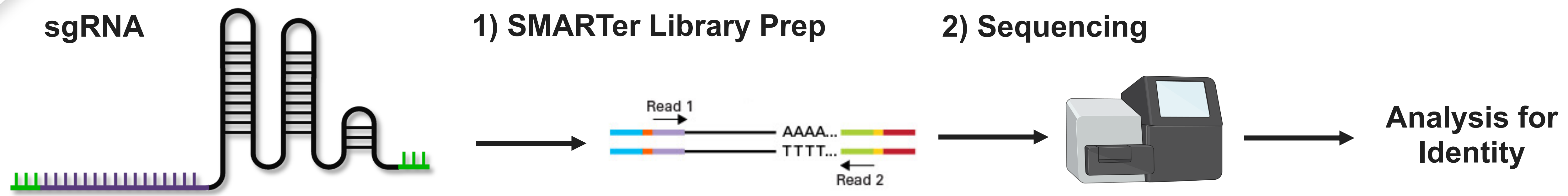




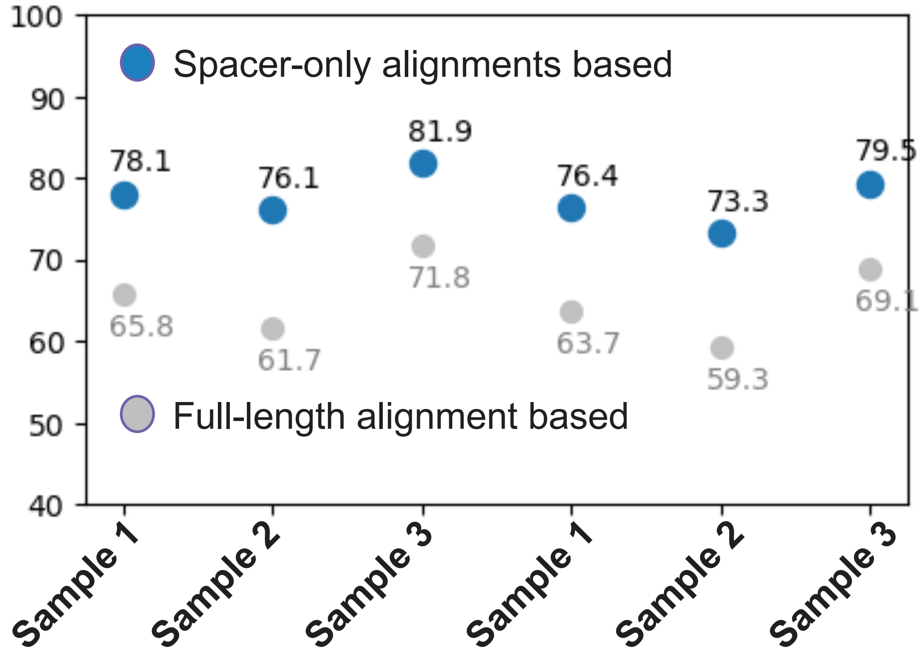
Full Sequence Confirmation

- LC/MS detected both full length and partial 5′ and 3′ cleavage products
- MS/MS sequenced chemically modified 5′ and 3′ ends
- Complete coverage provided by overlapping sequencing of 5′ and 3′ cleavage products
- Assay specific for test sgRNA – non-target sgRNA or n-1 fail sequence identity
- Submitted in VOR33/trem-cel IND

NGS confirms sequence identity of sgRNA



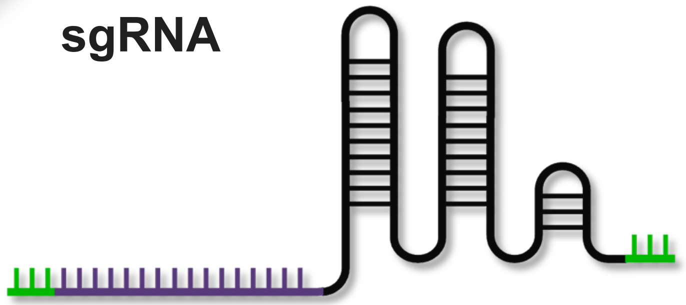
<table>
<tr><th rowspan="2">Alignment</th><th rowspan="2">Mismatch+gap</th><th colspan="3">Replicate 1</th><th colspan="3">Replicate 2</th><th rowspan="2"></th></tr>
<tr><th>Test Sample 1</th><th>Test Sample 2</th><th>Test Sample 3</th><th>Test Sample 1</th><th>Test Sample 2</th><th>Test Sample 3</th></tr>
<tr><td>XXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
XXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT</td><td>0</td><td>65.8</td><td>61.7</td><td>71.8</td><td>63.7</td><td>59.3</td><td>69.1</td><td>% reads aligned to reference sequence</td></tr>
</table>

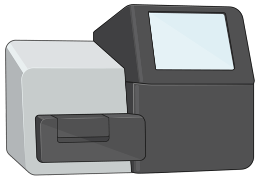


Full Sequence Confirmation

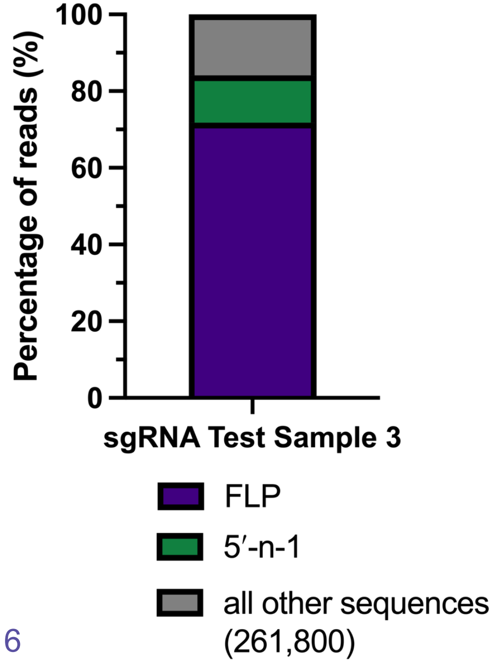
- same sgRNA sequence in 3 different batches, 2 replicates
- 3′ terminal UUUU trimmed for alignment
- most frequent sequence matches expected reference sequence
- >1M reads support full-length alignment
- sgRNA purity influences % reads aligned to reference sequence

Over 80% of NGS reads account for the full length and n-1 sgRNA sequences while all other sequences individually are <1%





Percentage of reads mapped to sgRNA full length and impurity sequences



<table>
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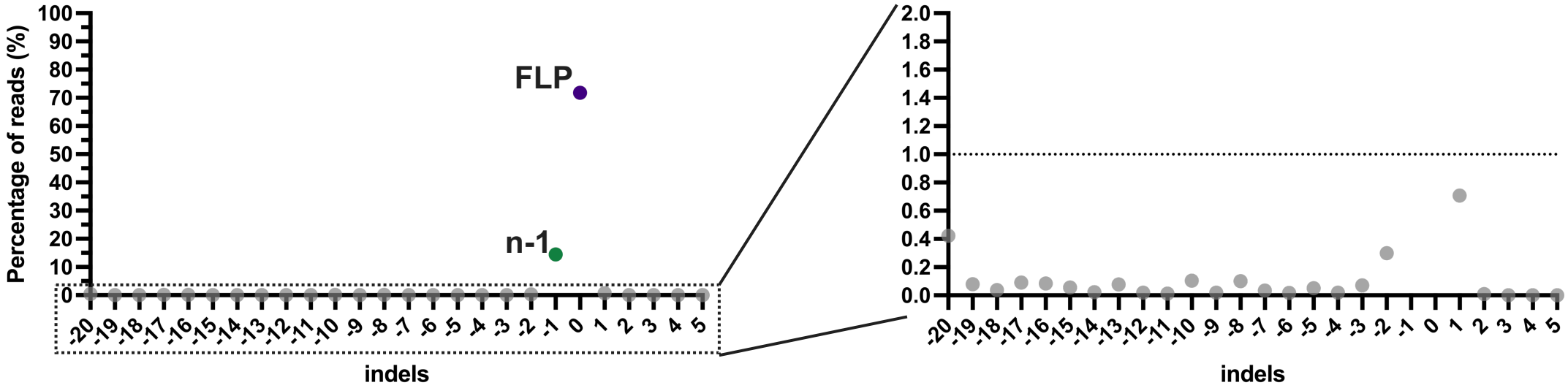
<tr><td>FLP</td><td>XXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
XXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT</td><td>0</td><td>1.77E+06</td><td>71.75</td></tr>
<tr><td>n-1</td><td>XXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT
~||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
-XXXXXXXXXXXXXXXXXXXGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT</td><td>1</td><td>3.05E+05</td><td>12.38</td></tr>
<tr><td>n-2</td><td>XXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT
~~|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
--XXXXXXXXXXXXXXXXXXGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT</td><td>2</td><td>4253</td><td>0.17</td></tr>
<tr><td>internal deletion</td><td>XXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||~|||||||||||||||||||||||
XXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTG-AAAAGTGGCACCGAGTCGGTGCT</td><td>1</td><td>10334</td><td>0.42</td></tr>
<tr><td>n+1</td><td>-XXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT
~|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
XXXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT</td><td>1</td><td>9873</td><td>0.40</td></tr>
<tr><td>internal deletion</td><td>XXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||~|||||
XXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTC-GTGCT</td><td>1</td><td>1578</td><td>0.06</td></tr>
</table>

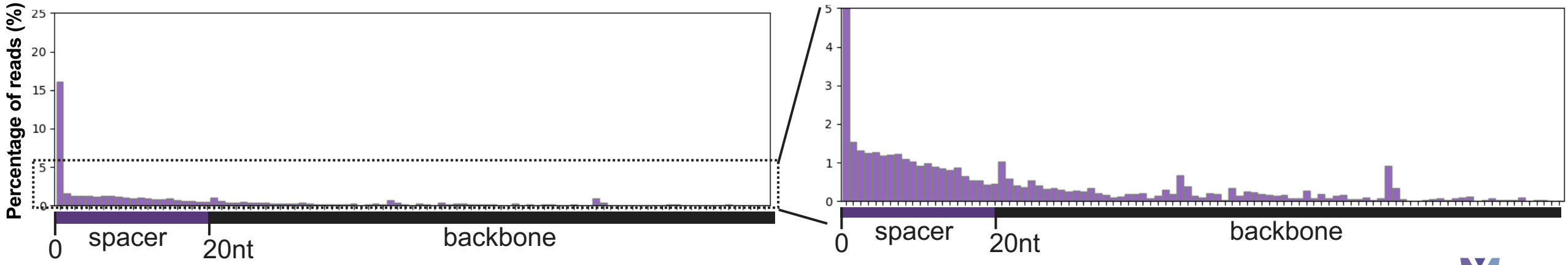
VOR

NGS reveals majority of insertions and deletions in sgRNA are <1% and tend to occur at the 5′ end

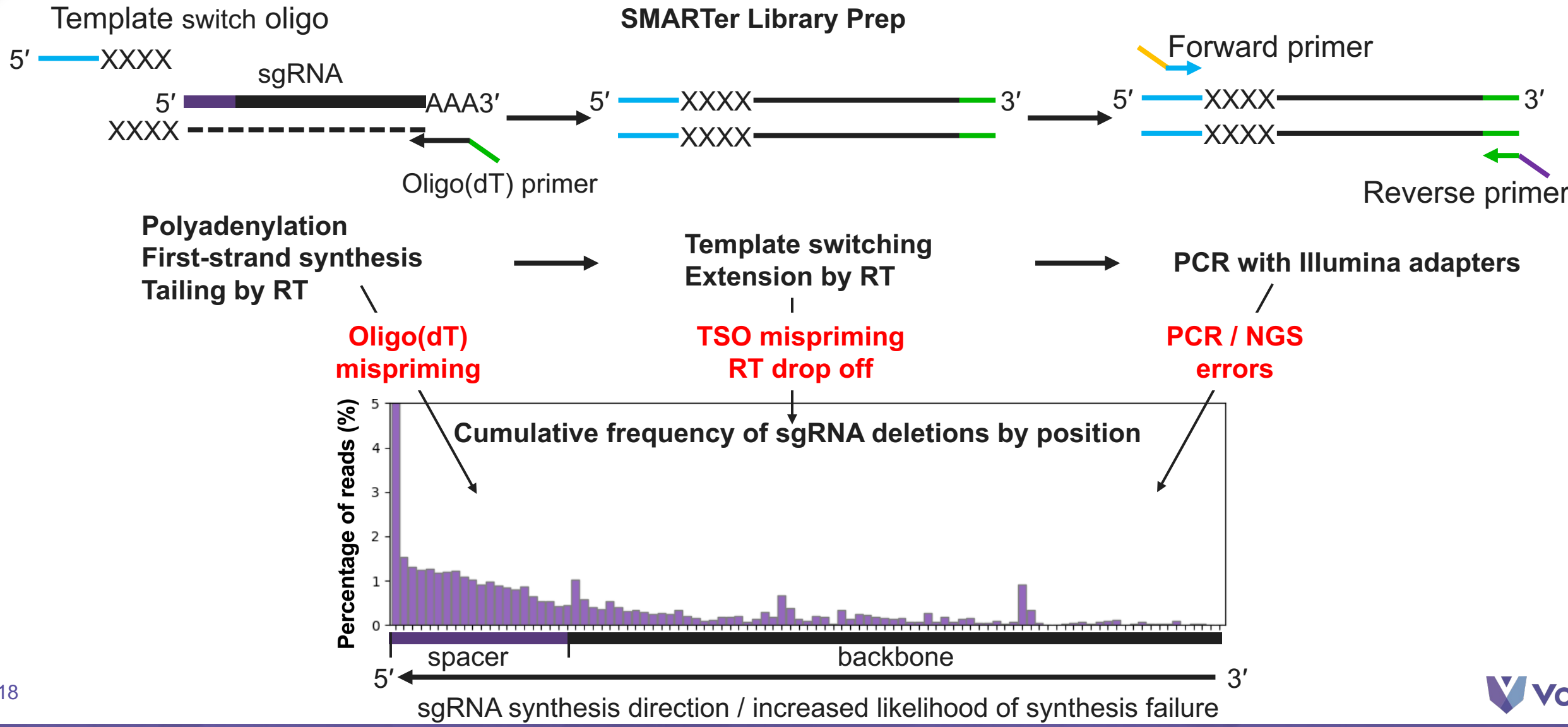
Insertions and deletions in the sgRNA sequence



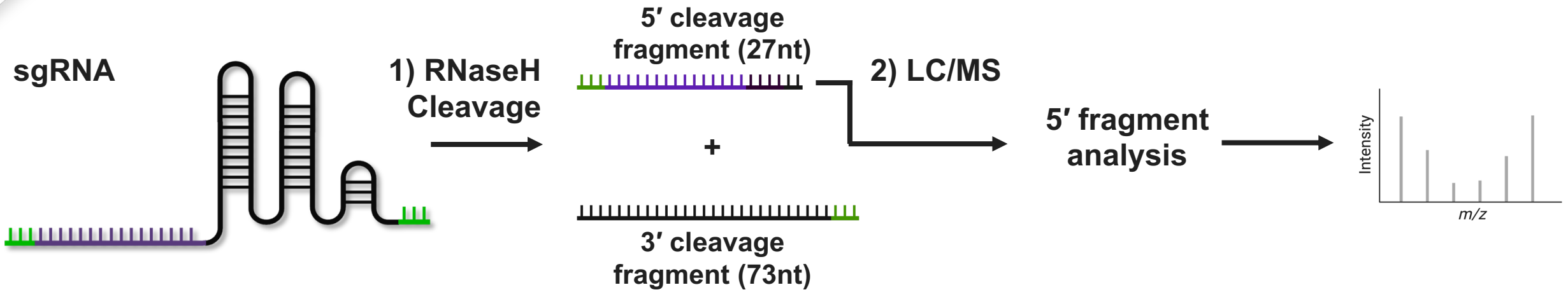
Cumulative frequency of sgRNA deletions by position



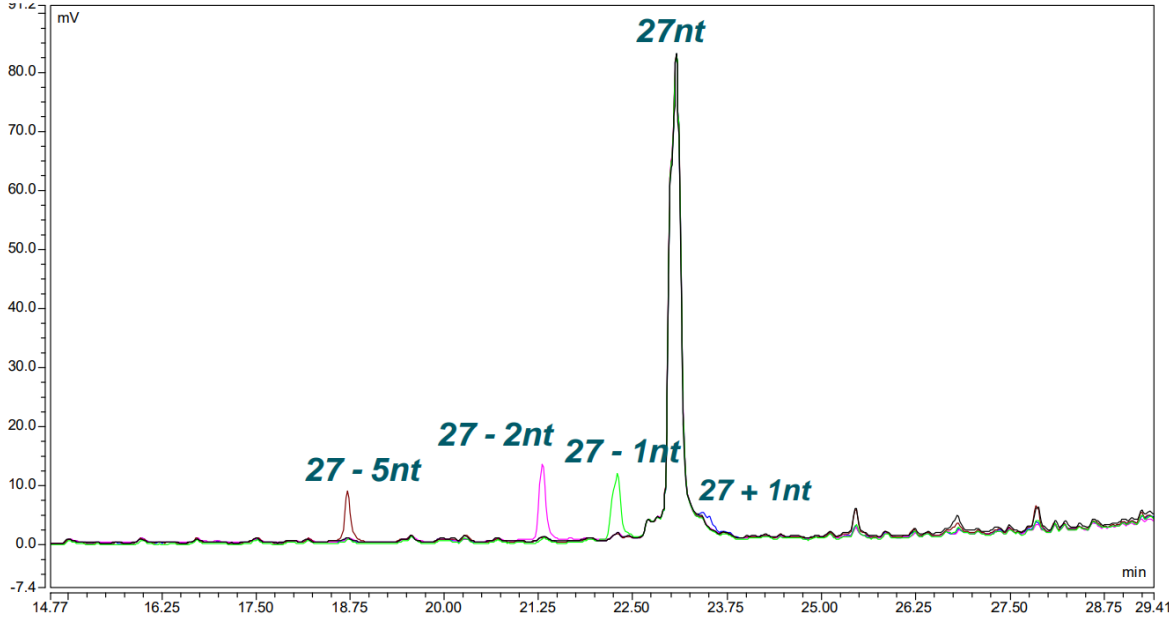
sgRNA sequence impurities identified by NGS are likely a combination of actual impurities and errors during library prep and sequencing



LC/MS fragment analysis allows for analysis of sgRNA chemical modifications and side reaction-derived impurities



5′ cleavage fragment (27nt) with impurity spike ins



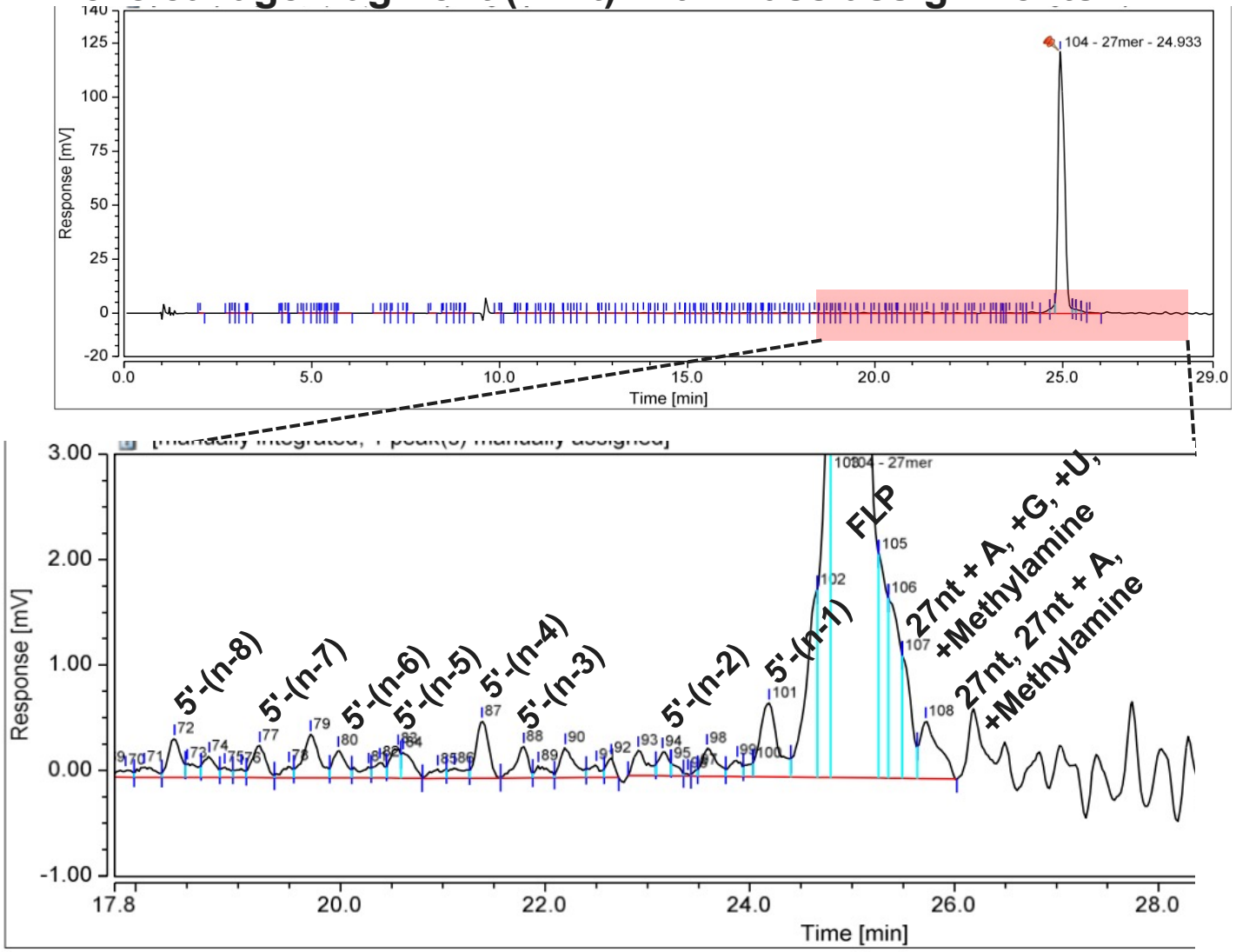
Assay design / optimization:

- Focused on LC/MS analysis of sgRNA spacer by generating short 5′ cleavage fragment (27nt)
- RNaseH / ASO design, conditions, activity, specificity
- IP-RP-HPLC method
  - no RNaseH control / blank subtraction
  - 10% truncated impurity spike ins
  - resolves n-1, n-2, n-5 truncations
  - note truncated series peaks (e.g., n-1) <<10%
  - does not fully resolve n+1

Method overview: Röhl, I., TIDES 2024

LC/MS fragment analysis confirms high purity and identifies impurities within the spacer region of sgRNA

5′ cleavage fragment (27nt) with mass assignments



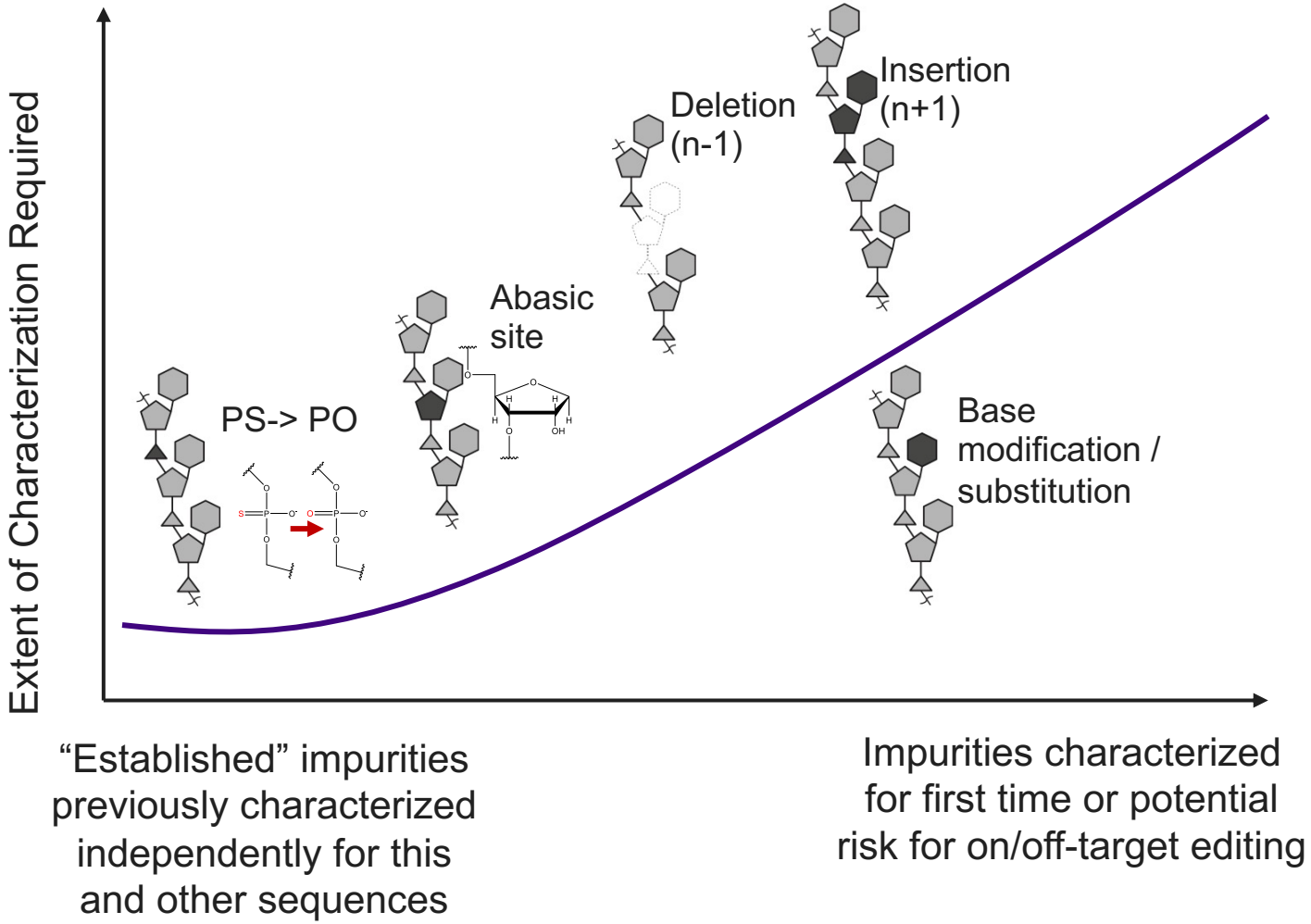
5′ cleavage fragment (27nt) analysis:

- precise cutting of 27nt, 5′ fragment
- main peak is 27nt (88%)
- Main peak observed MW matches theoretical MW of FLP

Impurities:

- Late-eluting peaks include n+1 and methylamine adducts
- truncation series n-1, n-2, etc.
- n-1 is the terminal 2′OMe base (<1%)
- abasic sites observed
- phosphorothioate impurities (PS->PO)
- degradation (e.g., 3′(n-1) +2′-3′-cyclic-P)

Overall sgRNA impurity strategy – how comprehensive is "comprehensive characterization"?



sgRNA Impurity Considerations

- What is the sgRNA purity?
- Can impurities be detected, identified and quantified?
- What type of impurities and where are they in the sgRNA sequence?
- What is the potential off-target risk and is this a novel off-target?
- What are the impacts on RNP formation, on-target editing and manufacturing process performance?
- Is isolation or synthesis of impurities needed for further characterization and functional testing?

Adapted from Capaldi, OPT Congress Conference (2021)

Summary

- sgRNA is a critical component required for genome engineering
- GMP manufacturing of sgRNA at scale is challenging, resulting in product-related impurities that may require identification
- sgRNA purity has the potential to impact both on and off-target editing activity
- sgRNA impurity identification is a regulatory requirement and can improve manufacturing processes
- HPLC, NGS and LC-MS/MS methods are complementary in assessing sgRNA identity, purity and impurity identification

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