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Contract Testing Services

sgRNA sequencing

Leveraging an established NGS assay to sequence single-guide RNAs (sgRNA)

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June 2024

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Cell and Gene Editing

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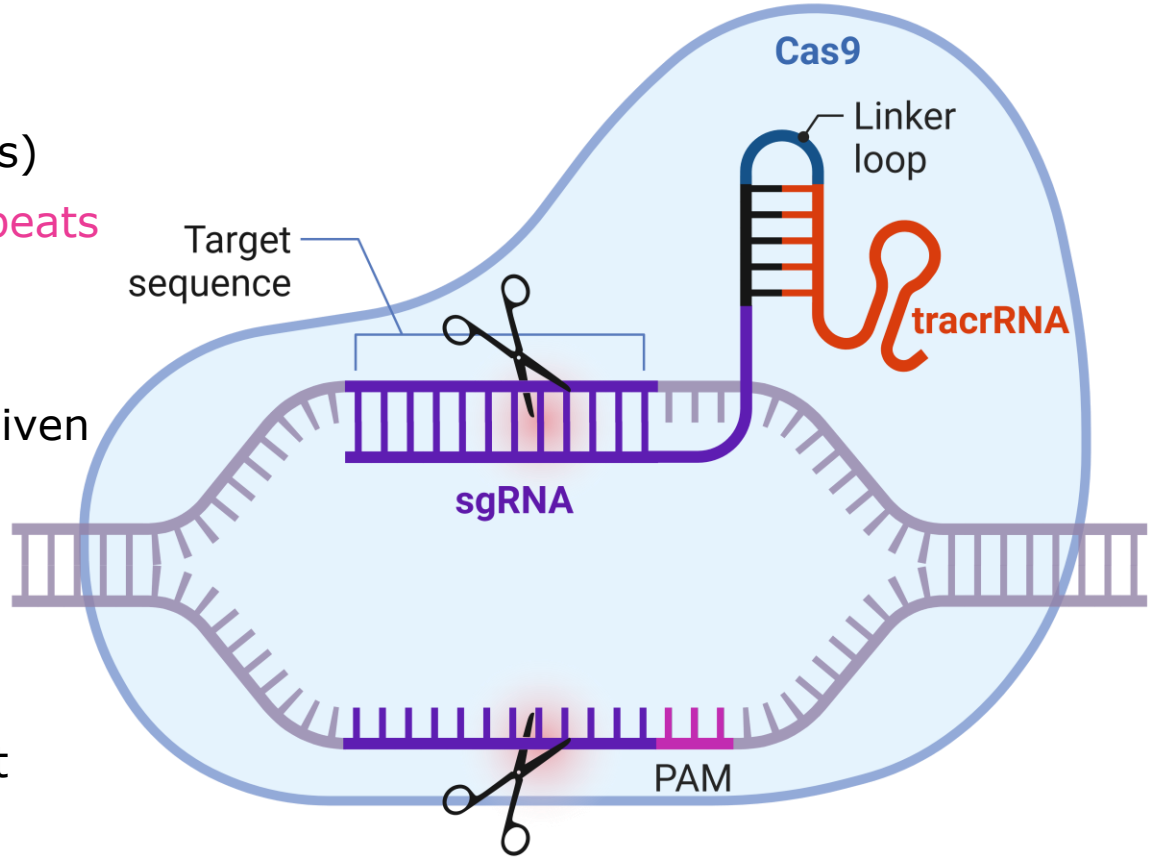
- Current Gene editing technologies:
  - Zinc-finger nucleases
  - Transcription Activator-Like Effector Nucleases (TALENs)
  - Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) complexes (e.g., CRISPR-Cas9)

- CRISPR has opened the door for gene editing that has given more accessibility to researchers due to its simplicity

- CRISPR-Cas system is composed of:
  - A (CRISPR-associated) Cas nuclease
  - The Cas nuclease is shuttled/guided to intended target sequence by a single-guide RNA (sgRNA)



Created with BioRender.com

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What is a sgRNA?

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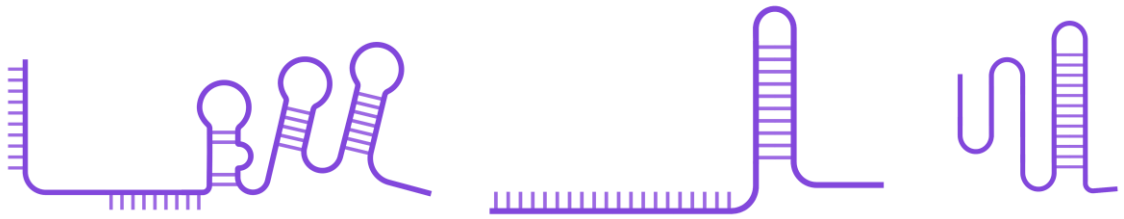
- The sgRNA is composed of a CRISPR RNA (crRNA) and a trans-activating CRISPR RNA (tracrRNA) that is compatible with the specific endonuclease

- The customizable part of a sgRNA is the crRNA:
  - 17-24bp sequence complementary to the "on-target" site where the dsDNA break and edit should occur
- Unintentional binding of the sgRNA to any other areas → "off-target" event
- The design of the sgRNA is a critical step for gene editing experiments



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Current QC for sgRNAs and gaps

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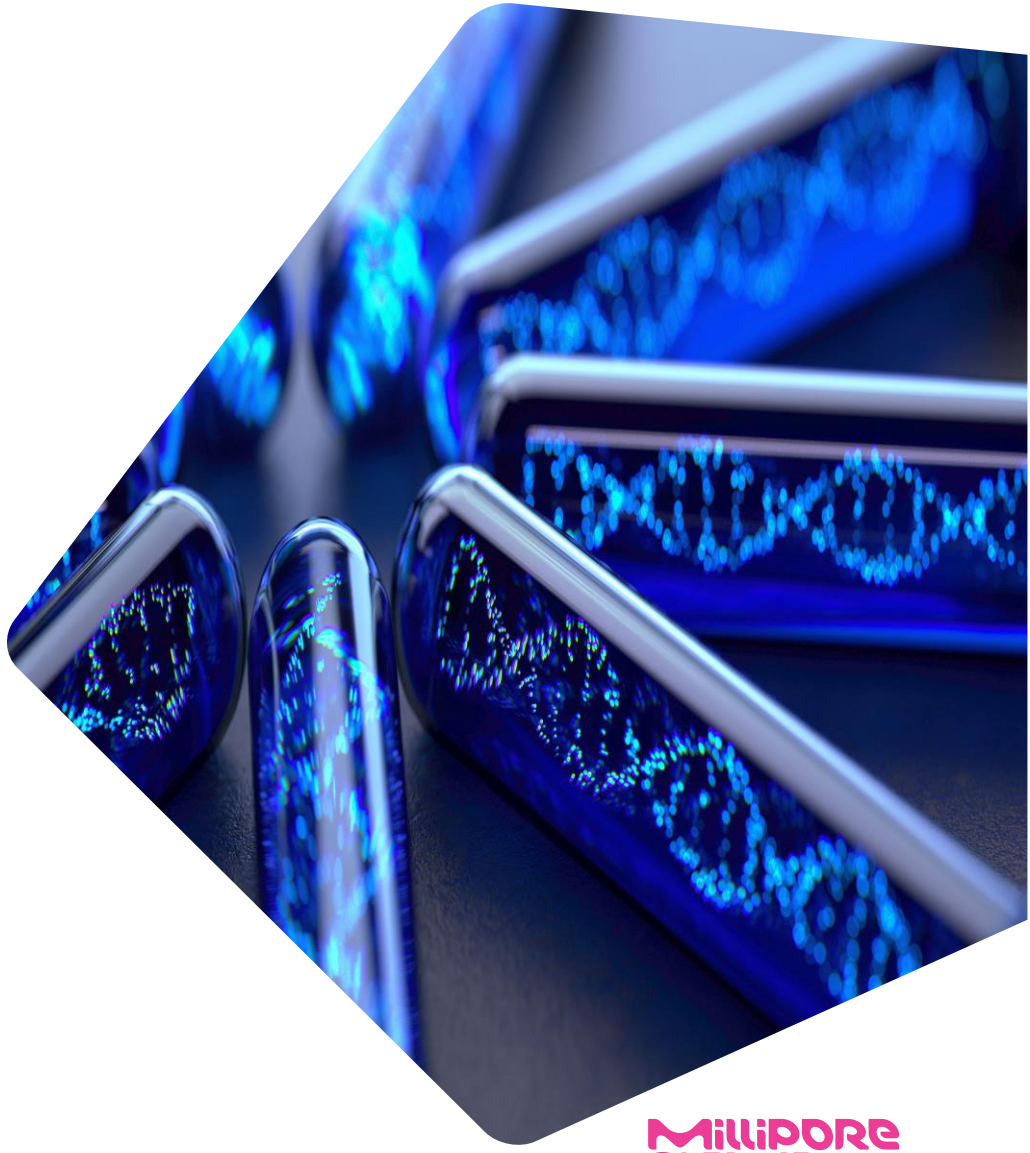
- Chemical synthesis of sgRNAs can introduce low level contamination → need to measure guide sequence fidelity and purity
- Current QC Method:
  - Mass Spec technology
    - Lacks specificity
    - Can miss subtle variations that could contribute to off-target events

  No longer sufficient for regulatory agencies!
- Need ultrasensitive techniques to assess quality, safety and efficacy
- What about Sanger sequencing?
  - Low sensitivity for variant detection, not high-throughput
- NGS
  - Identify variants down to a 1% presence in the population



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Existing assay

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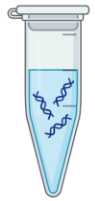
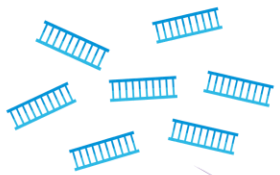
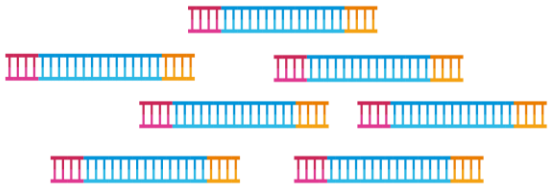
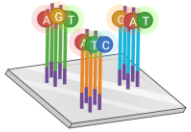


Nucleic Acid Extraction



DNA Fragmentation
Library Preparation





DNA Sequencing

Data Analysis
Bioinformatics Algorithm specific to each assay

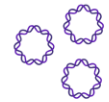
Each assay is specifically developed and validated to answer a specific question

Genetically modified cells

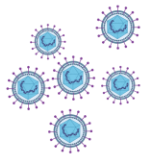
Bacteria

Virus Stock

Virus-based vaccines



Plasmids



Gene Therapy Vectors

RNA

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Methods

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- Identify a kit to generate a compatible library for these short RNA sequences
- Two leading contenders for library preparation kits

- Tested various sgRNA with different modifications to assess and optimize these kits to fit into our current methodology
  - Many sgRNAs have termini modifications to decrease degradation and improve gene editing efficiency
  - Successful libraries can be sequenced using the Illumina Sequencers

- Bioinformatics analysis
  - Sequence Identity, and Variant calling
  - Customization of existing workflow used for identity testing assays

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Kit A

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- Modified Protocol to capture larger RNAs (~100nt)
- Sequential Ligation Methodology
- Unique Molecular Indexes (UMIs)
- No gel purification

- Possible limitations:
  - Requires 3’ hydroxyl and 5’ phosphate for ligation

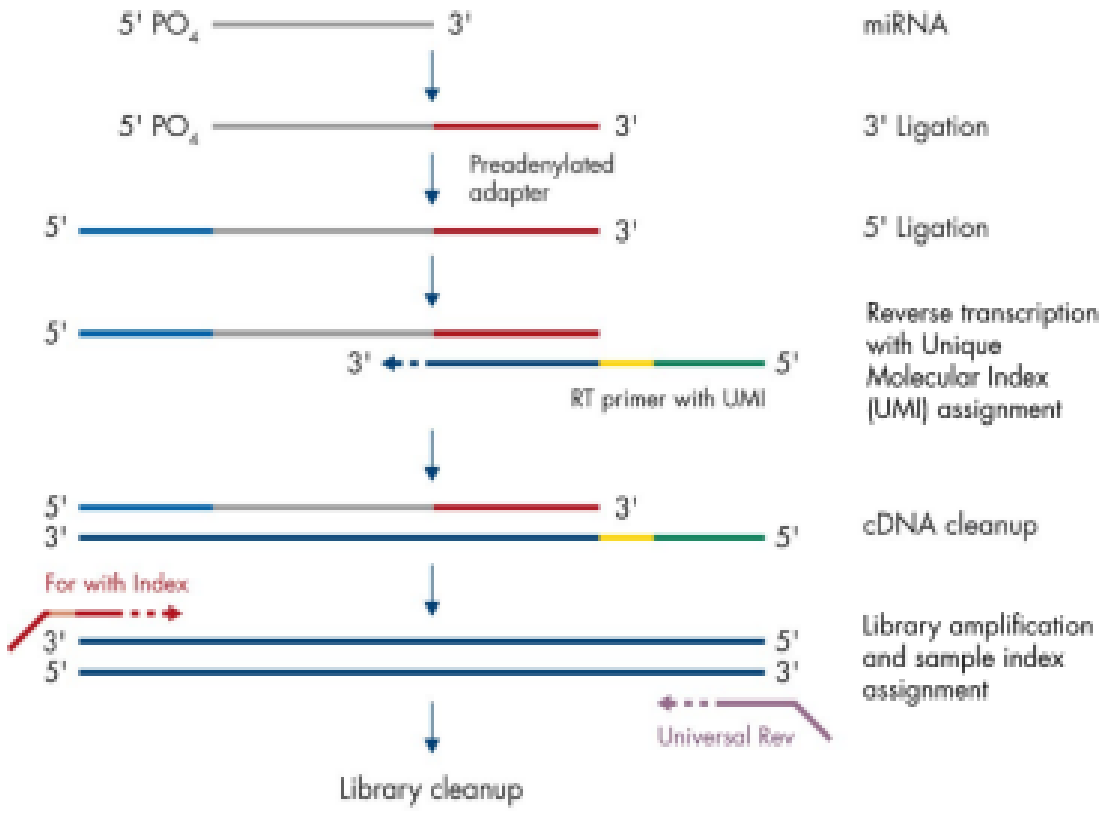

Figure: Qiagen

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Kit B

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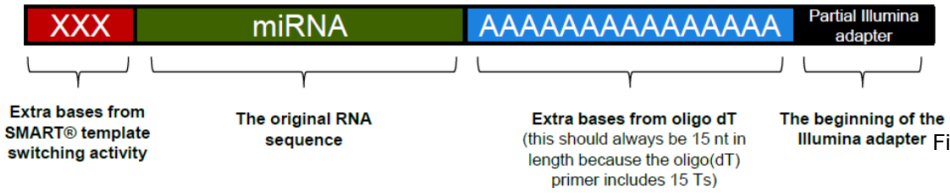
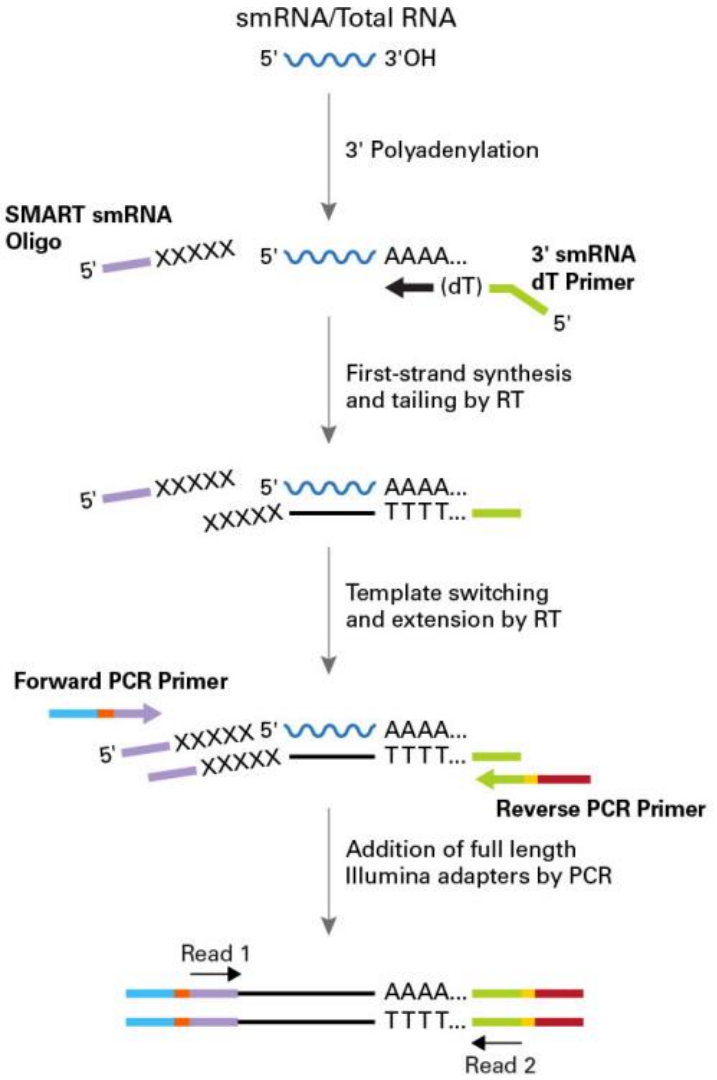
- Methodology:
  - Ligation free
  - 3’ Polyadenylation
  - 1st strand synthesis followed by template-switching and extension by RT

- Possible limitations - unambiguous determination of RNA termini not possible due to:
  - poly A tail addition
  - at low frequencies, template switching can add more than 3 nt to cDNA 5’ ends and make identifying the beginning of the sequence with 100% confidence not possible





Figures: Takara Bio Inc.

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sgRNAs that have been tested

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- Purchased sgRNAs from 3 different manufacturers
  - All had similar end modifications:
    - Phosphorothioate bonds on 3’ and 5’ ends (terminal nucleotides)
      - Protects ends of molecules from degradation → increases editing efficiency
    - 2’Ome
      - Protects from hydrolysis

  - Two Client drivers to aid in development
    - Company 1 tested 3 different samples
    - Company 2 tested 8 different samples of varying lengths and modifications

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Results – success!

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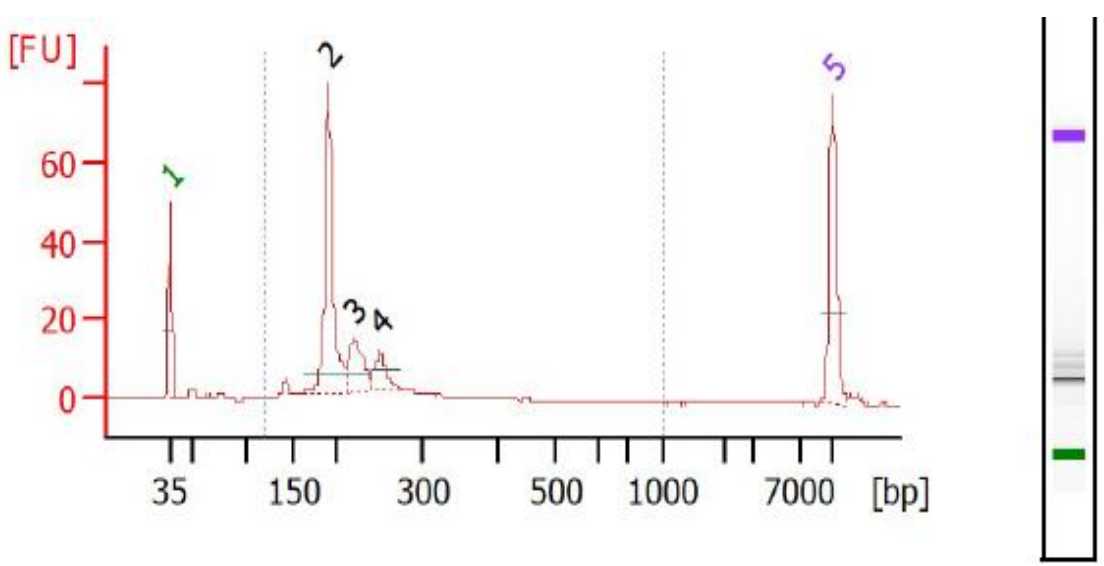
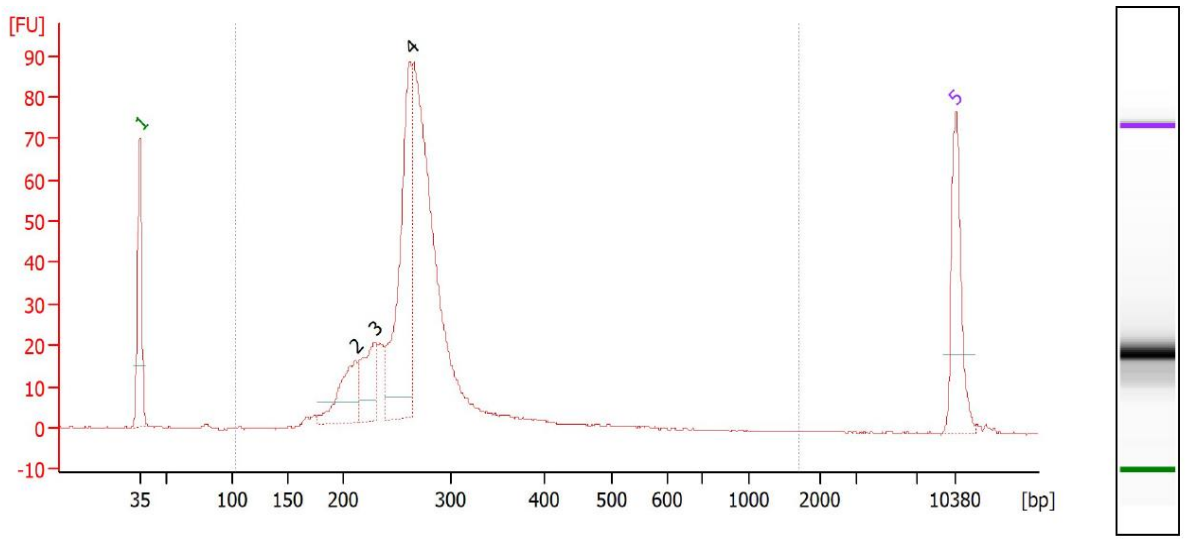
- CNAT was able to successfully generate libraries with both kits





- **Kit A**
- Dilution 1:20
- Average Size: 216 bp

- **Kit B**
- Dilution 1:5
- Average Size: 279 bp

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Data Analysis
Bioinformatics

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- Data analysis was performed with our proprietary Variant Caller Algorithm (VCA) 2.0
  - No changes made to the algorithm itself

	Kit A	Kit B
Reference Coverage (%)	100	100
Consensus Similarity to Reference (%)	100	100
Total # reads used for mapping	39 million	35 million
% population mapped	30.13	94.5

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Read Distribution and Depth – Side by Side

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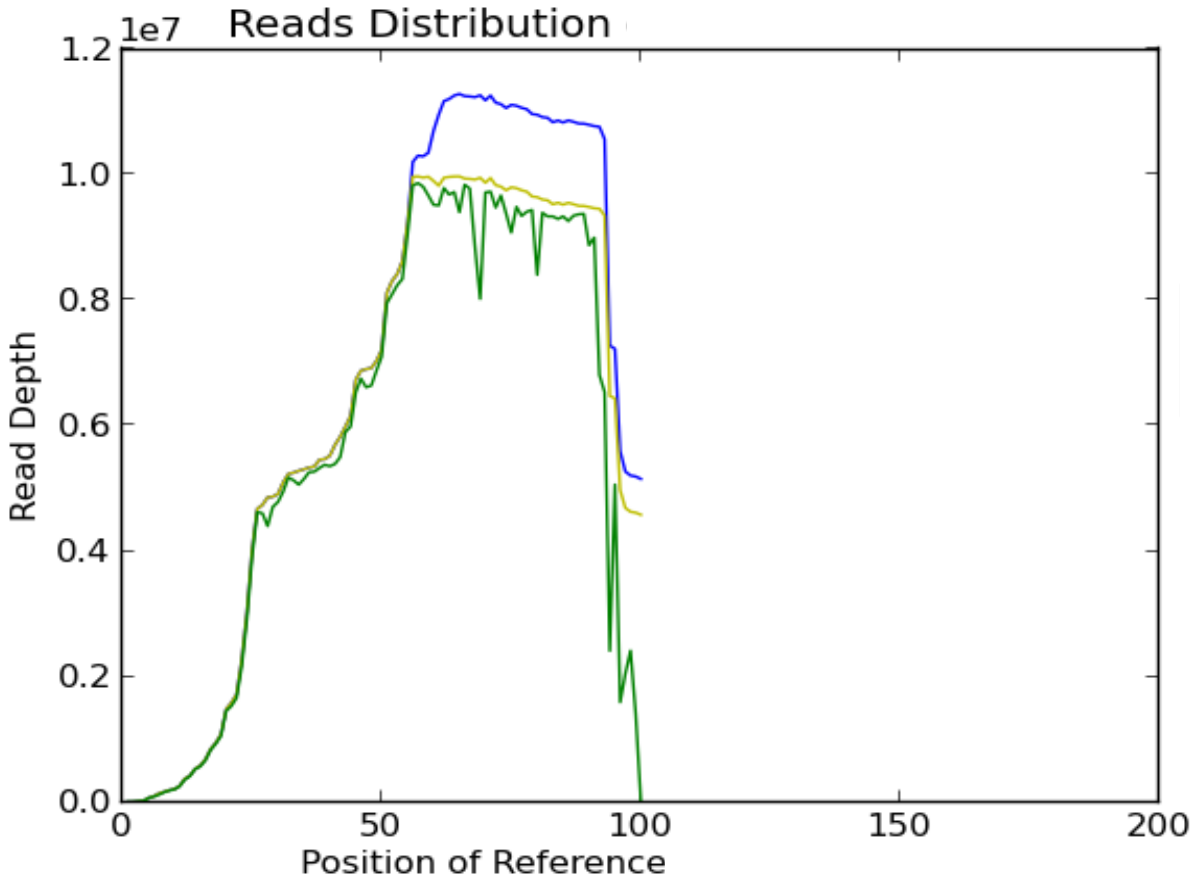

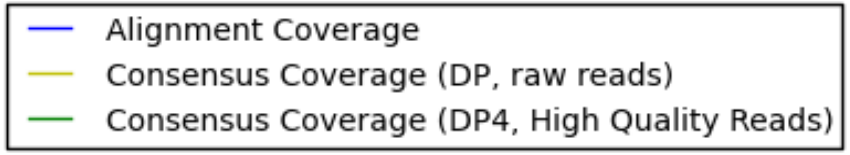

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Kit A vs. Kit B Overview

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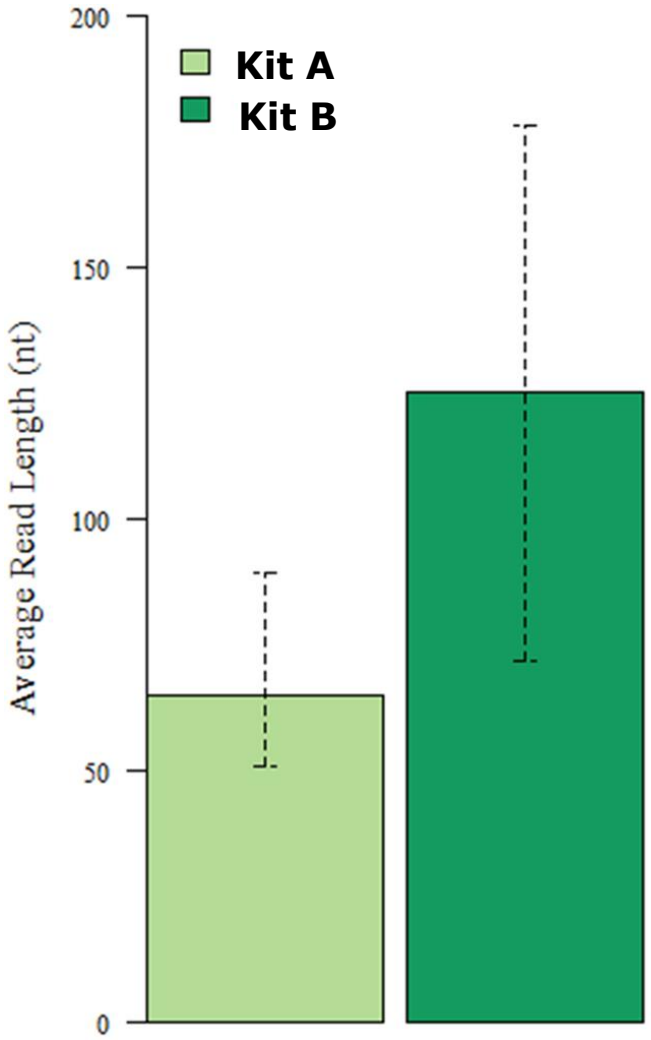

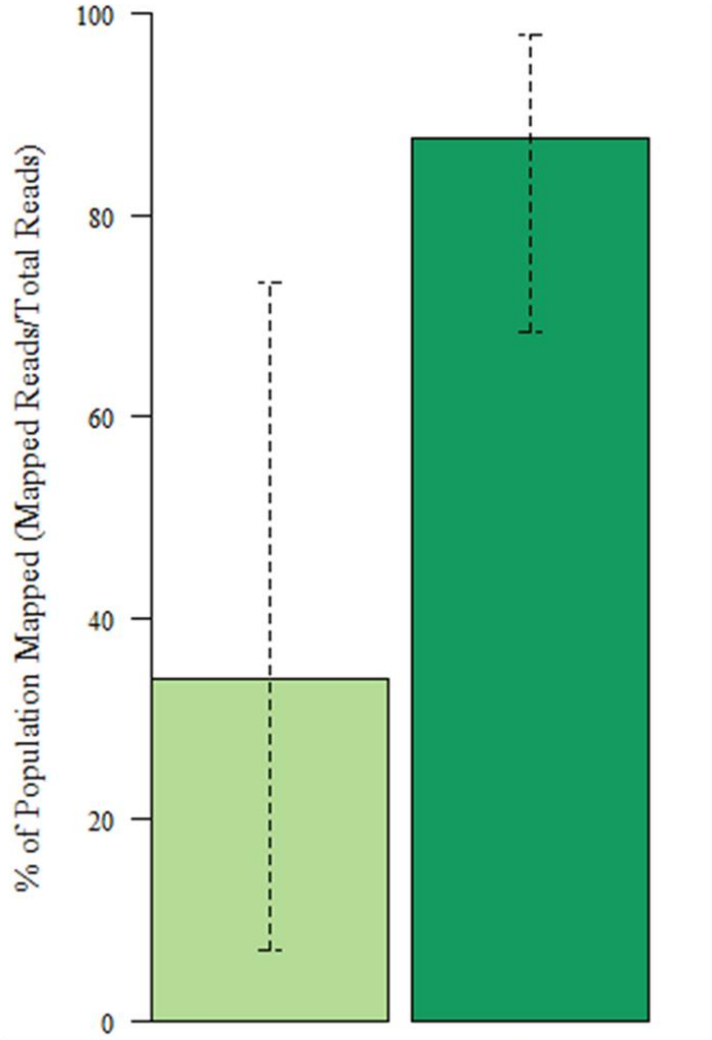

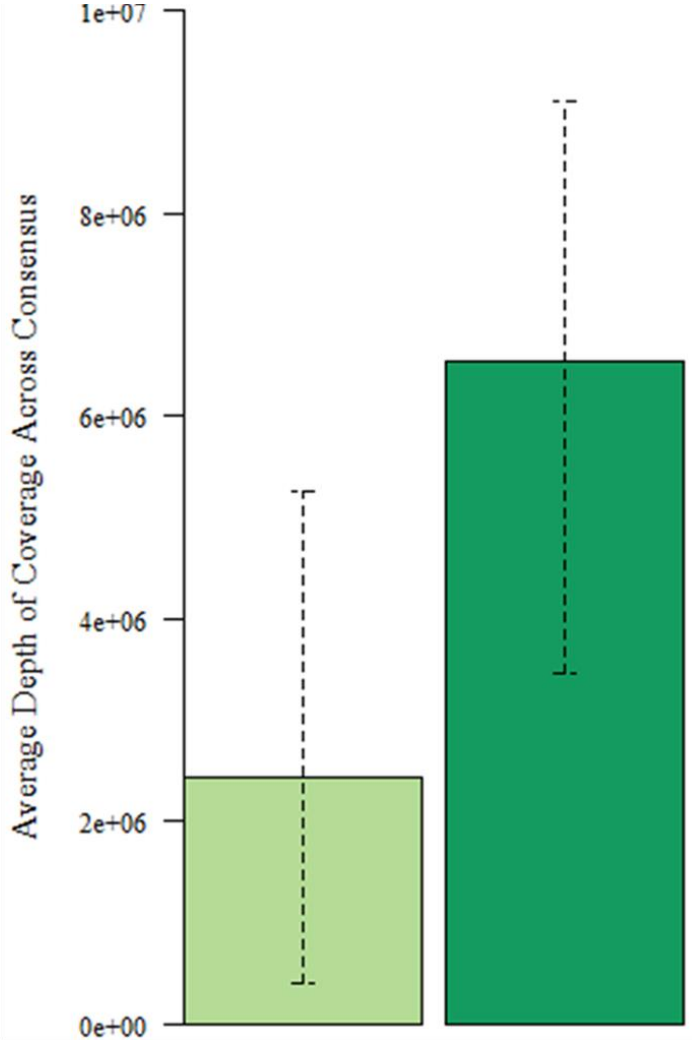

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Key Takeaways

1. sgRNA ID by NGS can be performed using Kit B
2. NGS provides the level of sensitivity and depth of coverage to accompany current QC technologies
3. MilliporeSigma will have a GMP offering starting July 2024!

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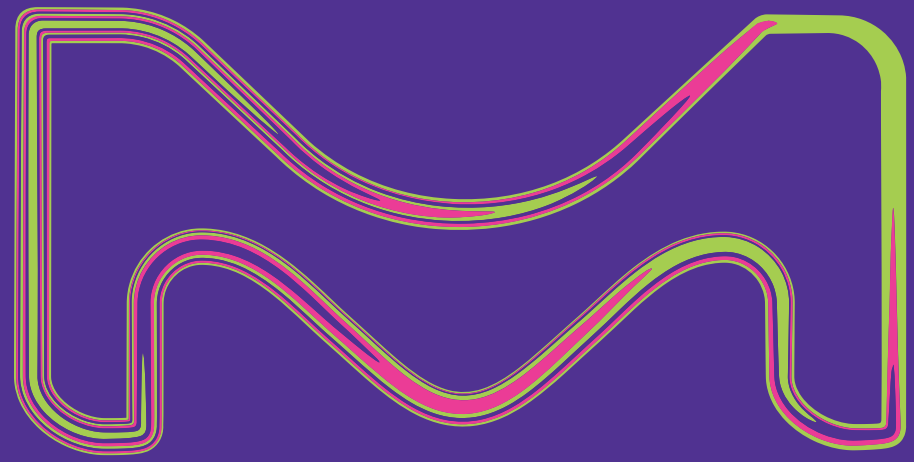
Contract Testing Services

Learn more:
SigmaAldrich.com/NGS



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