Mastering and Accelerating sgRNA Synthesis: Unveiling Success Strategies

Yvonne Angell[1], Zhanlei Wei[2], Xi Chen[2], Yun Yang[2], Chenchen Hu[2], William Fang[2], Haiyang Hu[2]

[1]WuXi TIDES, WuXi AppTec, San Francisco, CA, USA

[2]WuXi TIDES, WuXi AppTec (HongKong) Limited

WuXi TIDES

Introduction

The popularity of CRISPR is largely due to its simplicity. The CRISPR-Cas system relies on two main components: a guide RNA (gRNA) and CRISPR-associated (Cas) nuclease. The guide RNA is a specific RNA sequence that recognizes the target DNA region of interest and directs the Cas nuclease there for editing. The CRISPR guide RNA sequence directly impacts the on-target DNA cleavage efficiency and unintentional off-target binding and cleavage. Therefore, an optimal guide with minimum off-target effects and maximum on-target efficiency is the goal, and obtaining the right guide RNA is a critical step for the success of CRISPR experiments.

We will present our advances in synthesis, purification and analytical characterization of sgRNA. Through several case studies we will demonstrate our success and experience in synthesis of 400+ sgRNA sequences in a scale from <1 mg to 1 g. We will also demonstrate the synthesis of a variety of complex gRNA (>101 mer) libraries, and show results from a study of how sgRNA purity affects editing efficiency. In addition, we will discuss our solutions from discovery to GMP with our completion of 4 GMP projects at 20g scale including GLP tox batches of high-quality sgRNA with impurity analysis.

Materials & Methods

Synthesis of sgRNA

Materials:

•Nucleotides: N-Acetyl-2'-deoxycytidine (dC), N-Acetyl-2'-deoxyadenosine (dA), N-Acetyl-2'-deoxyguanosine (dG), N-Acetyl-2'-deoxyuridine (dU)

•Phosphoramidites: Standard or modified nucleoside phosphoramidites for RNA synthesis

•Activating reagents: Tetrazole or other suitable coupling agents

•Solvents: Anhydrous acetonitrile, triethylamine

•Purification reagents: HPLC-grade water, ethanol, and suitable RP-HPLC columns

Synthesis Apparatus:

•Dr. Oligo 192 synthesizer, Mermade-12 synthesizer, OP-Synt synthesizer, or OP-Pilot synthesizer

Purification of sgRNA

The sgRNA were purified using phenol-chloroform extraction and ethanol precipitation to remove unincorporated nucleotides and other contaminants. The purity and yield of the sgRNA were assessed via spectrophotometric analysis (NanoDrop) and gel electrophoresis.

Analytical Characterization

To evaluate the quality of the synthesized sgRNA, we conducted several analyses:

1. **Gel Electrophoresis:** To confirm the size and integrity of the sgRNA.
2. **Mass Spectrometry:** To determine the molecular weight and detect potential impurities.
3. **High-Performance Liquid Chromatography (HPLC):** For further purification and quantification of sgRNA.

Case Studies on sgRNA Efficiency

We synthesized 3 different regular and highly modified sgRNA sequences, and compared our purified products with both regular and highly modified sgRNA sequences from 3 different competitive vendors to evaluate product quality. In addition, we compared 24 sgRNA samples from in-house synthesized material to those from a competitive vendor to evaluate on-target and off-target editing efficiency in various cell lines. Editing efficiency was measured using a combination of targeted deep sequencing and T7 endonuclease I assay.

Production of Complex sgRNA Libraries

For the synthesis of complex sgRNA libraries (greater than 101 mer), we employed a modified IVT protocol that included multiplexed DNA templates. The libraries were generated by pooling multiple sgRNA sequences prior to transcription, followed by purification and characterization as described above.

GMP Compliance and Scale-Up Projects

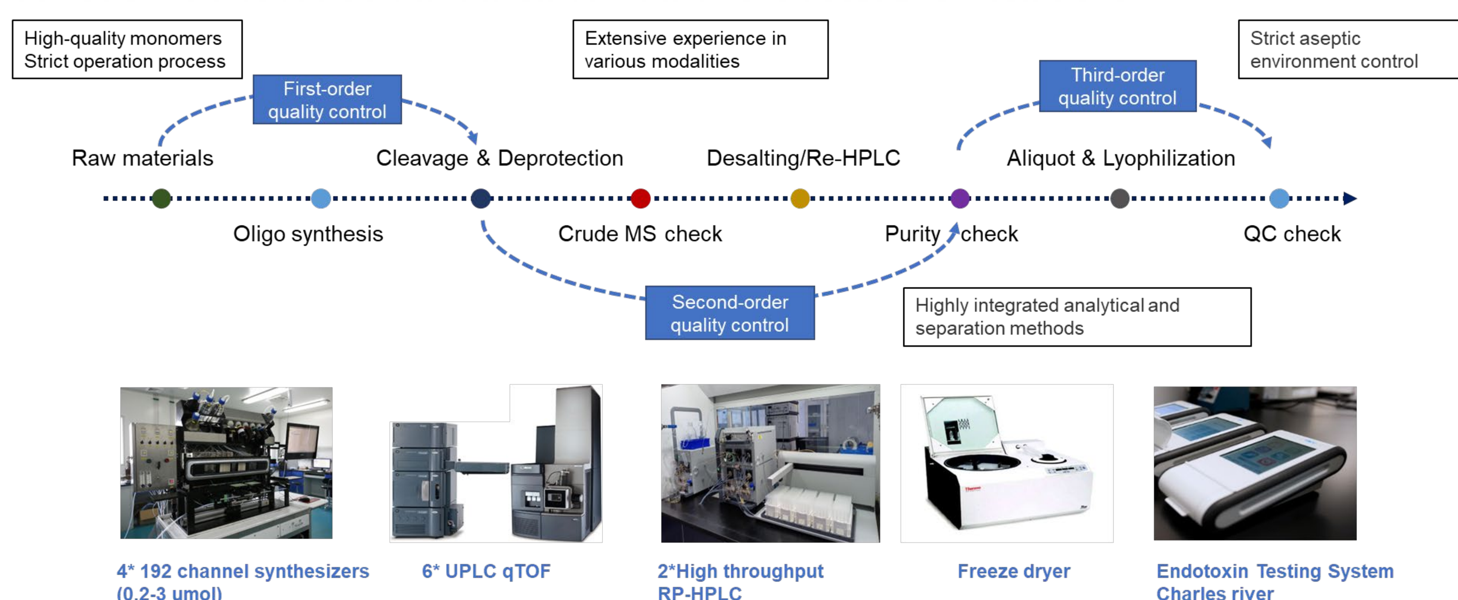
We successfully completed four GMP projects at a 20 g scale, adhering to Good Laboratory Practice (GLP) guidelines. Each batch underwent thorough impurity analysis and characterization to ensure high-quality sgRNA suitable for preclinical studies. Key metrics such as purity, yield, and functional integrity were rigorously assessed to meet regulatory standards.

Statistical Analysis

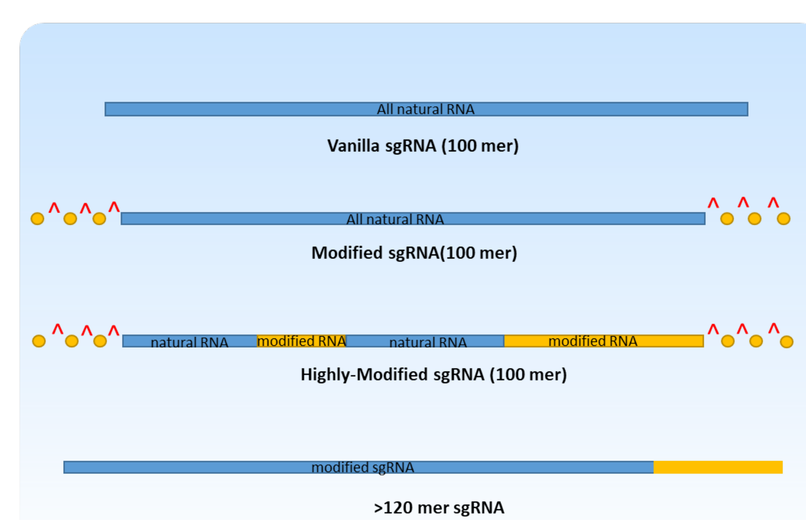
Data were analyzed using appropriate statistical methods to evaluate the correlation between sgRNA purity and editing efficiency, employing software such as GraphPad Prism for statistical significance testing.

This comprehensive approach to sgRNA synthesis, purification, and characterization highlights our commitment to advancing CRISPR technology while ensuring high-quality, reproducible results.

Quality control for high throughput sgRNA synthesis

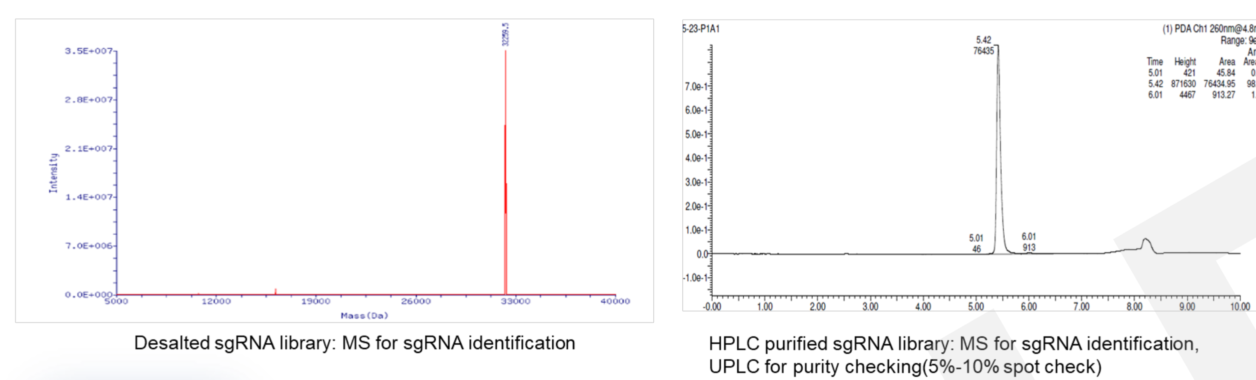


Typical sgRNA types



- Experienced in solid phase synthesis of Up to 128 mer sgRNA (for >128 mer sgRNA, we suggest fragment synthesis + ligation strategy).
- Multiple modifications including 2'F 2'OMe, phosphorothioate, LNA, cET, MsPA, DNA etc.
- Can achieve >80% gene editing efficiency.

Screening test – 2-3 weeks lead time for a sgRNA library

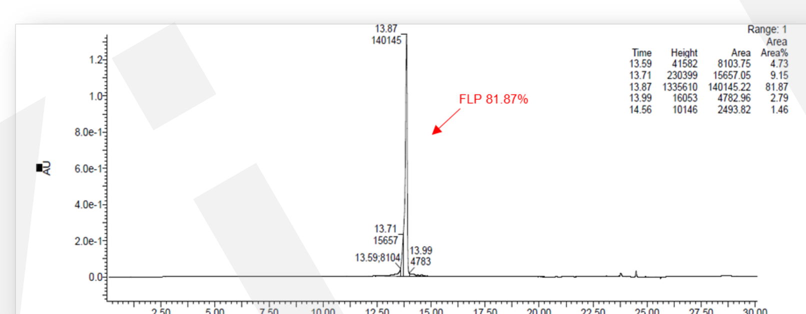


Desalted sgRNA library: MS for sgRNA identification

HPLC purified sgRNA library: MS for sgRNA identification, UPLC for purity checking(5%-10% spot check)

High through-put synthesis	Dr. Oligo 192 synthesizer + 96-well plate package; Mermade-12
Fast turn around time	2-3 weeks for desalted sample and 4 weeks for HPLC purified sample
Multiple modifications tolerant	Up to 128 mer, 2'F 2'Ome, LNA, phosphorothioate, DNA modification etc.

In vivo test – strict endotoxin and environmental control



120 mer gRNA purified by RP-HPLC purification method using 30 min analytical methods

- **Synthesis:** Mermade-12; OP-Synt
- **Purification:** RP-HPLC and IEX HPLC
- **Analysis:** Developed analytical method for sgRNA (30 min)
- **Lead time:** 3~4 weeks

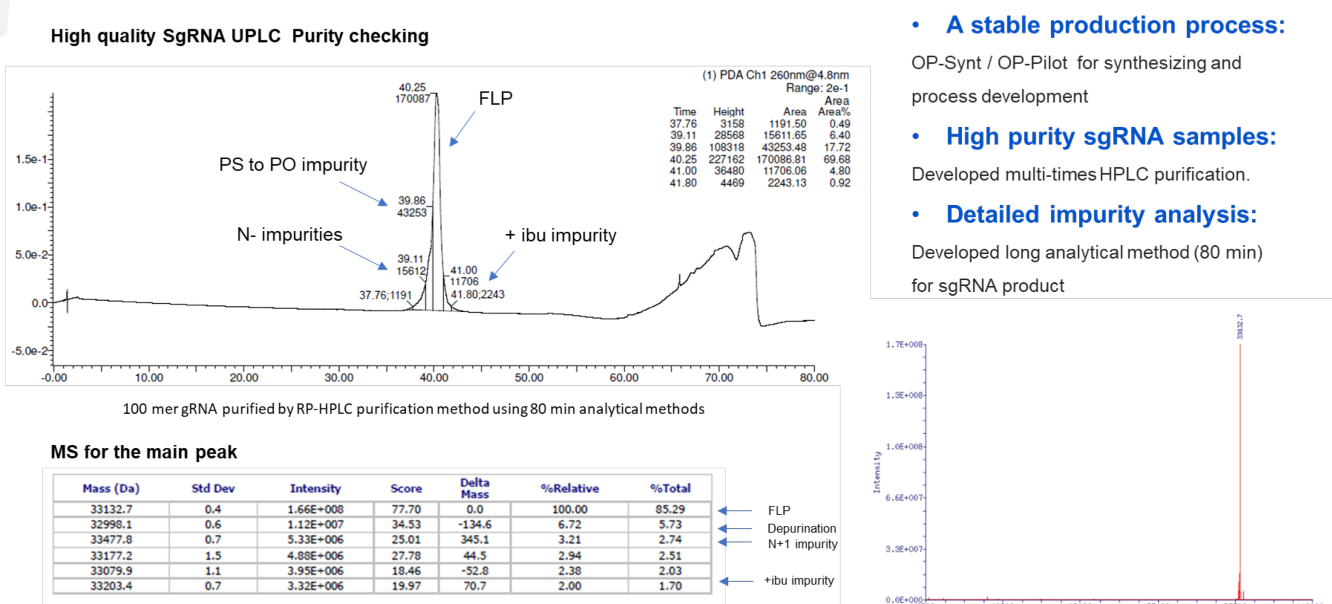
Environmental control
- All post processing are carried out in biosafety cabinet.

Water control
- Milli-Q water (endotoxin free) is used during purification and post processing

Endotoxin control
- LAL Kinetic Chromogenic method for endotoxin test (0.05 EU/mg endotoxin level can be achieved for sgRNA)

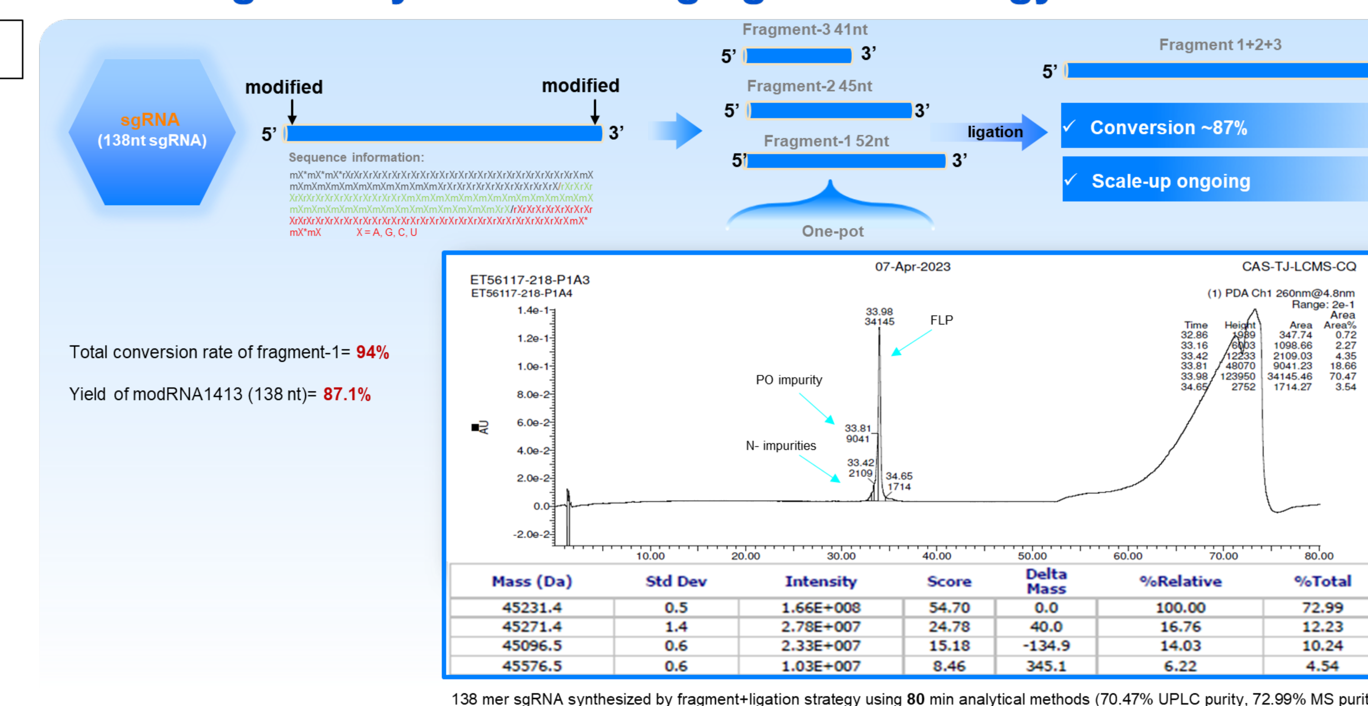
Bioburden test
- microbial limit report can be provided

[Case study] GLP Tox/GMP – High-quality sgRNA sample with impurity analysis



Mass (Da)	Std Dev	Intensity	Score	Delta Mass	%Relative	%Total
33132.7	0.4	1.66E+008	77.70	0.0	100.00	85.29
32990.1	0.6	1.12E+007	34.53	-134.6	6.72	5.73
33477.8	0.7	5.33E+006	25.01	345.1	3.21	2.74
33177.2	1.5	4.00E+006	27.78	44.5	2.94	2.51
33079.9	1.1	3.95E+006	18.46	-52.8	2.38	2.03
33203.4	0.7	3.32E+006	19.97	70.7	2.00	1.70

[Case study] 138-mer sgRNA synthesis using ligation strategy



Mass (Da)	Std Dev	Intensity	Score	Delta Mass	%Relative	%Total
45231.4	0.5	1.66E+008	54.70	0.0	100.00	72.99
45271.4	1.4	2.78E+007	24.78	40.0	16.76	12.23
45096.5	0.6	2.33E+007	15.18	-134.9	14.03	10.24
45576.5	0.6	1.03E+007	8.46	345.1	6.22	4.54

138 mer sgRNA synthesized by fragment+ligation strategy using 80 min analytical methods (70.47% UPLC purity, 72.99% MS purity)

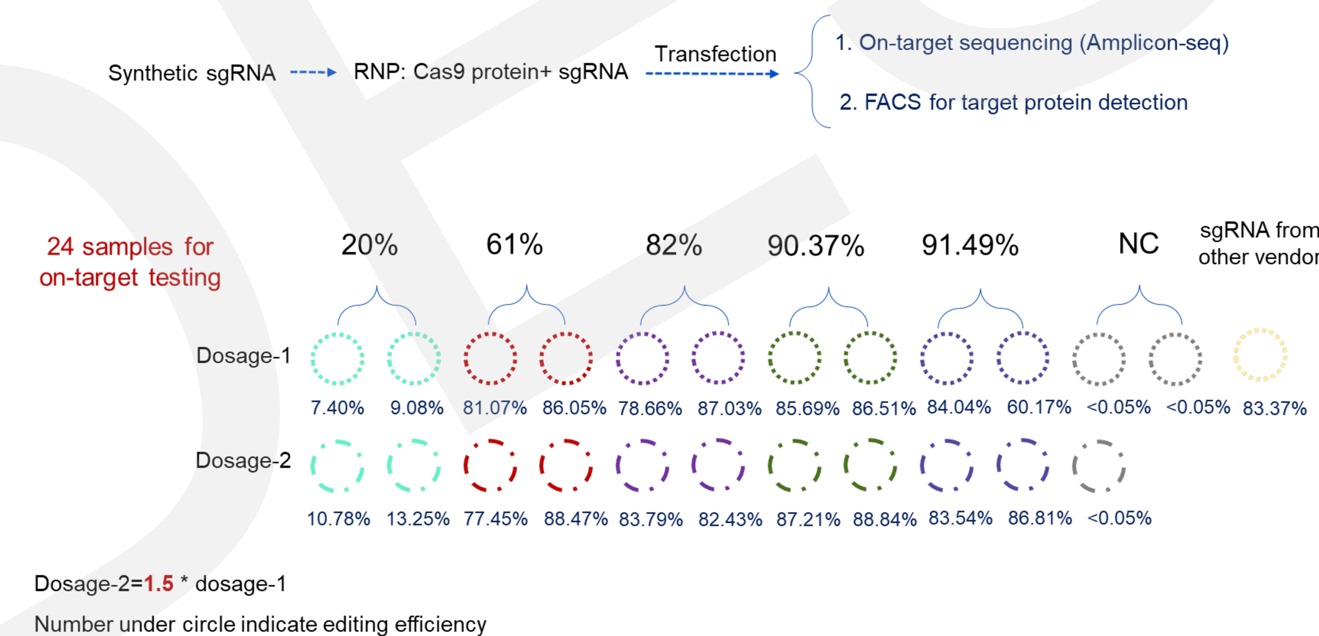
sgRNA products with higher quality

Vendor #	Purification Method	Purity provided by vendor	UPLC Purity Checked by WuXi	Cycle time
Vendor 1 (highly modified sgRNA)	HPLC purification	85%	36.68%	>6 weeks
Vendor 1 (regular sgRNA)	HPLC purification	90%	50.53%	>6 weeks
Vendor 2 (regular sgRNA)	HPLC purification	90%	54.04%	>6 weeks
Vendor 3 (regular sgRNA)	SPE	90%	21.96%	3-4 weeks
WuXi (regular/highly modified)	HPLC purification	-	50% - 60%	3-4 weeks
WuXi (regular/highly modified)	PAGE purification	-	50% - 60%	2-4 weeks
WuXi (regular/highly modified)	HPLC (multi-times) purification	-	70% - 80%	5-6 weeks

80 min method was used for sgRNA analysis

- Faster turnaround time
- Higher quality sgRNA product
- Comprehensive analytical platform and dedicated analytical methods

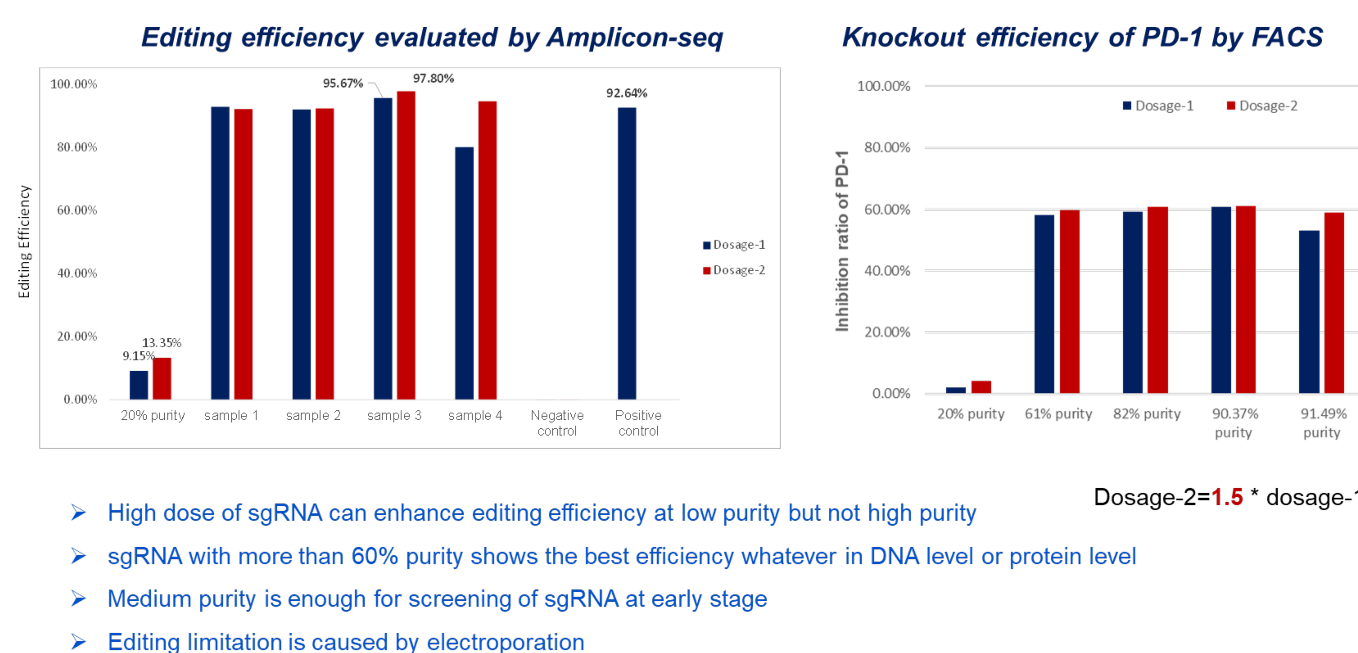
How sgRNA purity affects editing efficiency



Dosage-2=1.5 * dosage-1

Number under circle indicate editing efficiency

How sgRNA purity affects editing efficiency

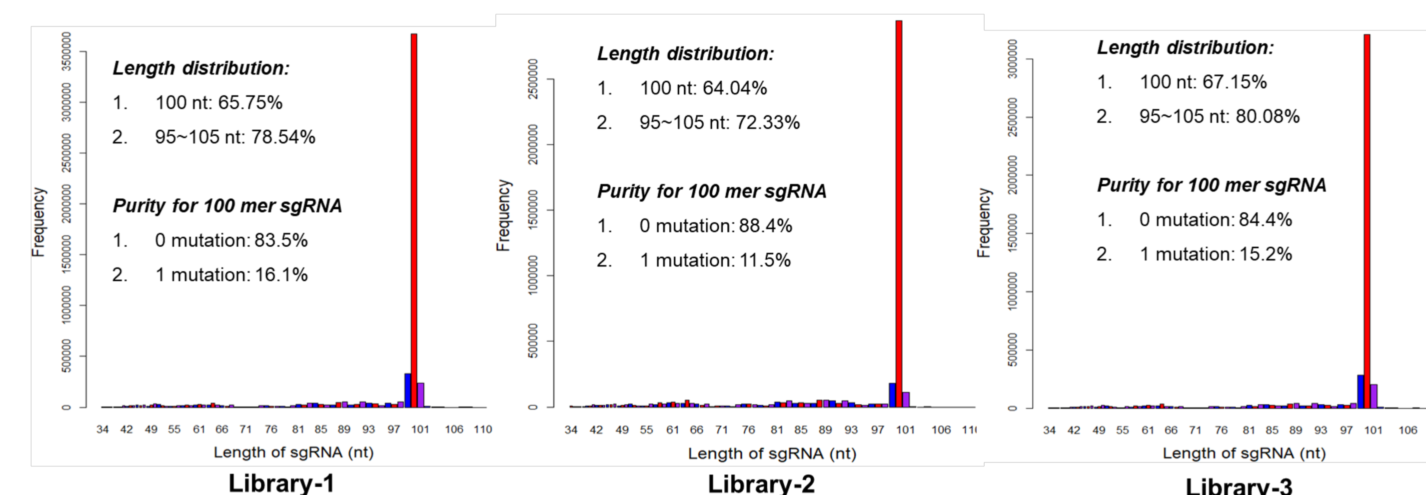


Dosage-2=1.5 * dosage-1

- High dose of sgRNA can enhance editing efficiency at low purity but not high purity
- sgRNA with more than 60% purity shows the best efficiency whatever in DNA level or protein level
- Medium purity is enough for screening of sgRNA at early stage
- Editing limitation is caused by electroporation

[Case study] NGS sequencing for a HPLC purified sgRNA

NGS Sequencing:



- NGS analysis results for a highly modified sgRNA sequence purified by HPLC
- NGS sequencing with HPLC purification for sgRNA impurity identification available

Conclusion

In summary, our research highlights the pivotal role of high-quality guide RNA in the success of CRISPR-based gene editing. By advancing the synthesis, purification, and analytical characterization of over 400 sgRNA sequences, we demonstrate that optimizing sgRNA not only enhances on-target efficiency but also minimizes off-target effects. Our findings, including the successful creation of complex gRNA libraries and insights from GMP projects, underscore the importance of rigorous quality control in the development of effective CRISPR applications. As we continue to refine these processes, we pave the way for more reliable and impactful gene editing solutions in diverse fields, from basic research to therapeutic applications. Thank you for your interest in our work!

Contact Us

Email: DiscoveryTides@wuxiapptec.com
Website: https://tides.wuxiapptec.com/
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