Hongene Biotech

Chemoenzymatic Ligation for siRNA and sgRNA Manufacturing: Recent Advances and Development Strategies

David Butler, Chief Technology Officer

TIDES USA: Bridging Research to Reality. San Diego – May 22nd 2025

Disclosures

- Dr. Butler is an employee of Hongene Biotech Corporation and serves on the board of directors of Akte Therapeutics

Presentation overview

- Recap of OTS conference presentation
- Chemoenzymatic ligation for siRNA synthesis
  - Inclisiran case study
  - Divalent siRNA case study
  - siRNA summary
- Chemoenzymatic ligation for sgRNA synthesis
  - 100mer G211 sgRNA case study
  - Hongene/ReciBioPharm partnership
- Engineered thermostable T4 RNA ligase
- Control of stereochemistry
- Conclusions

Recap: Presentation at OTS conference, Montreal, October 2024

Title: The Evolving Landscape of Oligonucleotide Manufacturing: Meeting Future Demand with Ligation Technologies

Topics covered:

- The evolving market for sgRNA and siRNA therapeutics
- Three generations of oligonucleotide synthesis technology
- Mechanism, properties, and immediate applications of ligation technology
- Examples of products synthesized by ligation at Hongene
- CMC features of ligation
- Current and future oligonucleotide fragment manufacturing processes

Email me - david.butler@hongene.com - for a copy of the OTS deck, which can also be viewed here

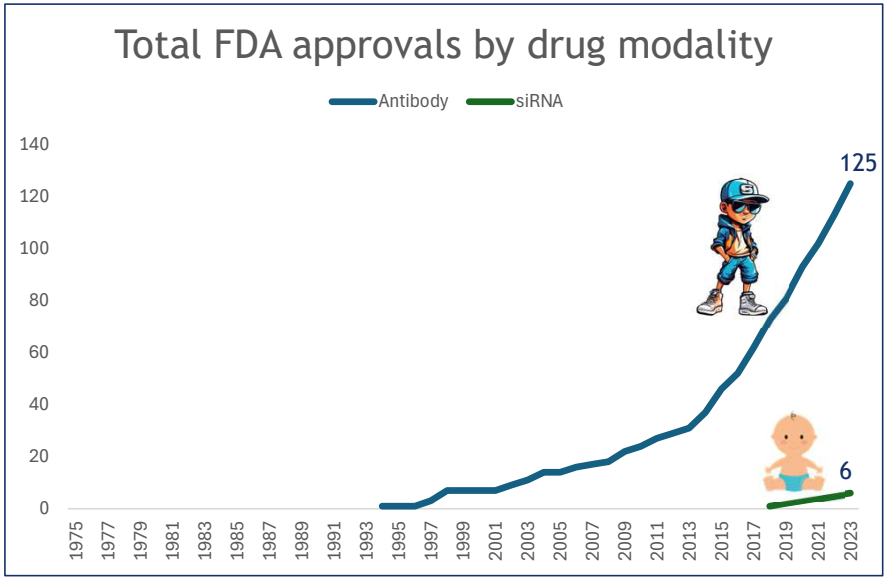
Market potential of siRNA drugs

Market demand for siRNA drugs and CMC challenges expected to grow significantly over the next decade

Cardiovascular disease area	Lead siRNA drug	Target	Phase	Possible approval
Hypercholesterolemia	Inclisiran	PCSK9	4	Approved
Hypertriglyceridemia	Plozasiran	APOC3	3	~2025
Elevated Lp(a)	Olpasiran	LPA	3	~2027
Hypertension	Zilebesiran	AGT	2	~2030
NASH/MASH	Rapirosiran	HSD	2	~2030
Obesity	*Multiple*	INHBE	1	2030s

- Several disease areas have potential to reach >10M US patients
- inclisiran + 4 could be approved by 2030
- Research suggests >10 t API required by early 2030s

siRNA is in its infancy in its life trajectory as a therapeutic drug modality



The Antibody Society. Therapeutic monoclonal antibodies approved or in regulatory review. (Aug 2024); Antibody therapeutics product data - The Antibody Society

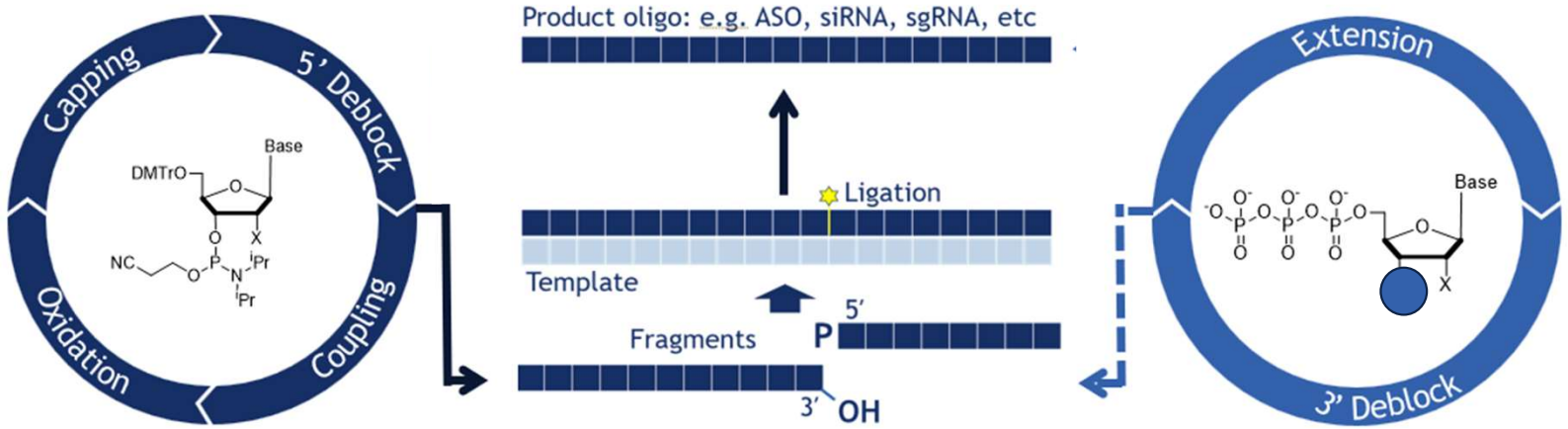
Three generations of oligonucleotide synthesis technology

	GENERATION 1	
Raw materials	Phosphoramidites	
Development status	>40 years, current paradigm	
Product purity	Lower	
Product yield	Lower	
Sustainability	>3,000 kg RM/kg API[1]	

GENERATION 3	
NTPs, 3'-protected NTPs, enzymes	
Very early[2,3,4,5]	
Enzymes need to be engineered	
Enzymes need to be engineered	
Best (aqueous)	

1. B. Andrews et al, *JOC*, 2021; 2. E.R. Moody et al, *Science*, 2023; 3. N. Sabat et al, *Front Chem*, 2023; 4. D. Wiegand et al, *Nat Biotechnol*, 2024; 5. S. Forget et al, *NAR*, 2025.

Three generations of oligonucleotide synthesis technology

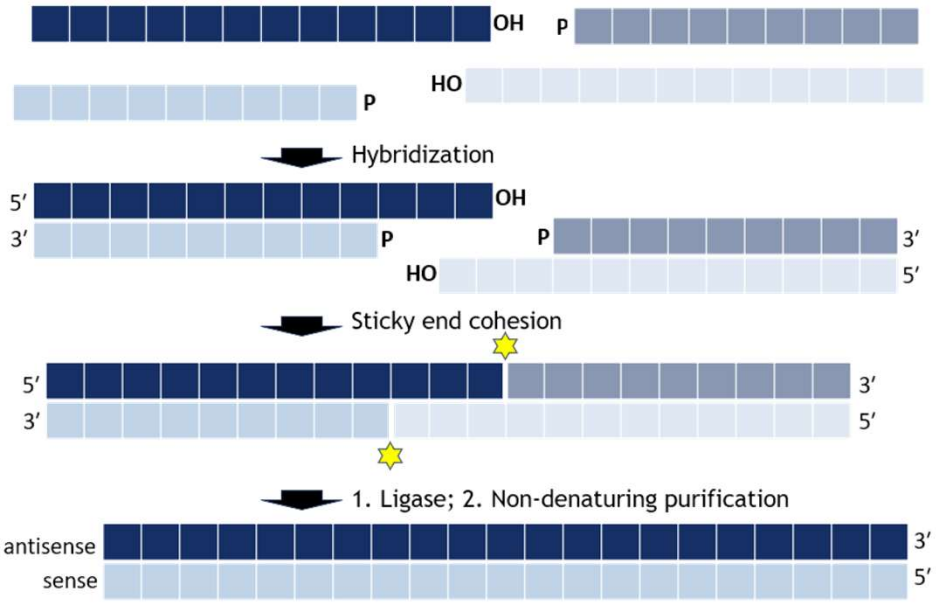


	GENERATION 1		GENERATION 2		GENERATION 3	
Raw materials	Phosphoramidites		Oligonucleotide fragments		NTPs, 3'-protected NTPs, enzymes	
Development status	>40 years, current paradigm		Ready for manufacturing		Very early[2,3,4,5]	
Product purity	Lower		Higher		Enzymes need to be engineered	
Product yield	Lower		Higher		Enzymes need to be engineered	
Sustainability	>3,000 kg RM/kg API[1]		Better (partly aqueous)		Best (aqueous)	

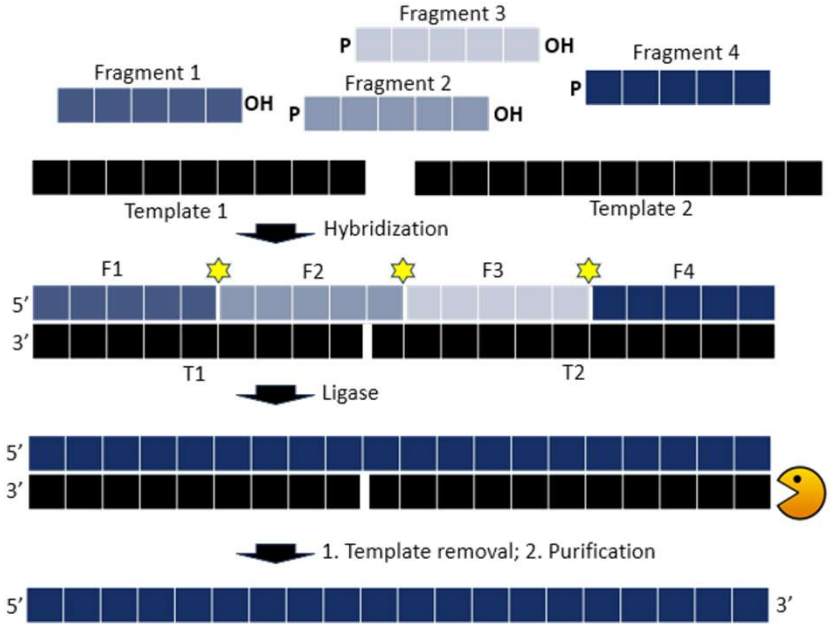
1. B. Andrews et al, *JOC*, 2021; 2. E.R. Moody et al, *Science*, 2023; 3. N. Sabat et al, *Front Chem*, 2023; 4. D. Wiegand et al, *Nat Biotechnol*, 2024; 5. S. Forget et al, *NAR*, 2025.

Hongene's chemoenzymatic ligation processes for siRNA and sgRNA

1. siRNA
Sticky end ligation[1,2,3,4]



2. sgRNA
Splinted (template) ligation[5,6]



1. H.G. Khorana, *Pure Appl Chem*, 1968; 2. A. Sosic et al, *Bioconj Chem*, 2014; 3. S. Paul et al, *ACS Chem Biol*, 2023; 4. S. Kajimoto et al, *AEM*, 2022;
5. M. Moore et al, *Methods Enzymol*, 2000; 6. N.Sabat et al, Nat Commun, 2024.

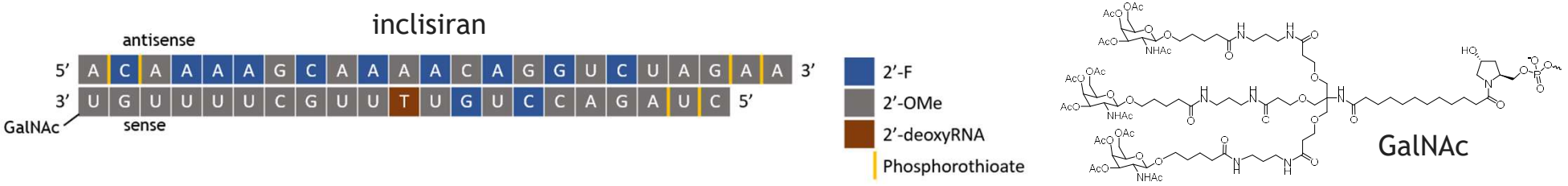
Chemoenzymatic ligation for siRNA synthesis

siRNA ligation case study inclisiran

siRNA ligation case study - inclisiran

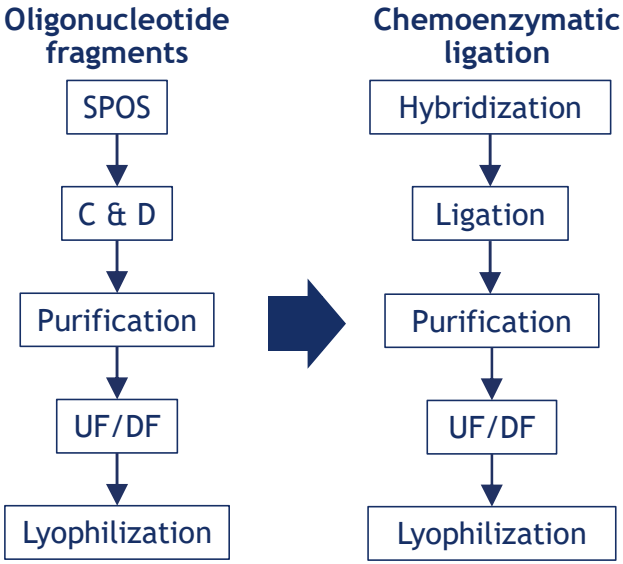
Background

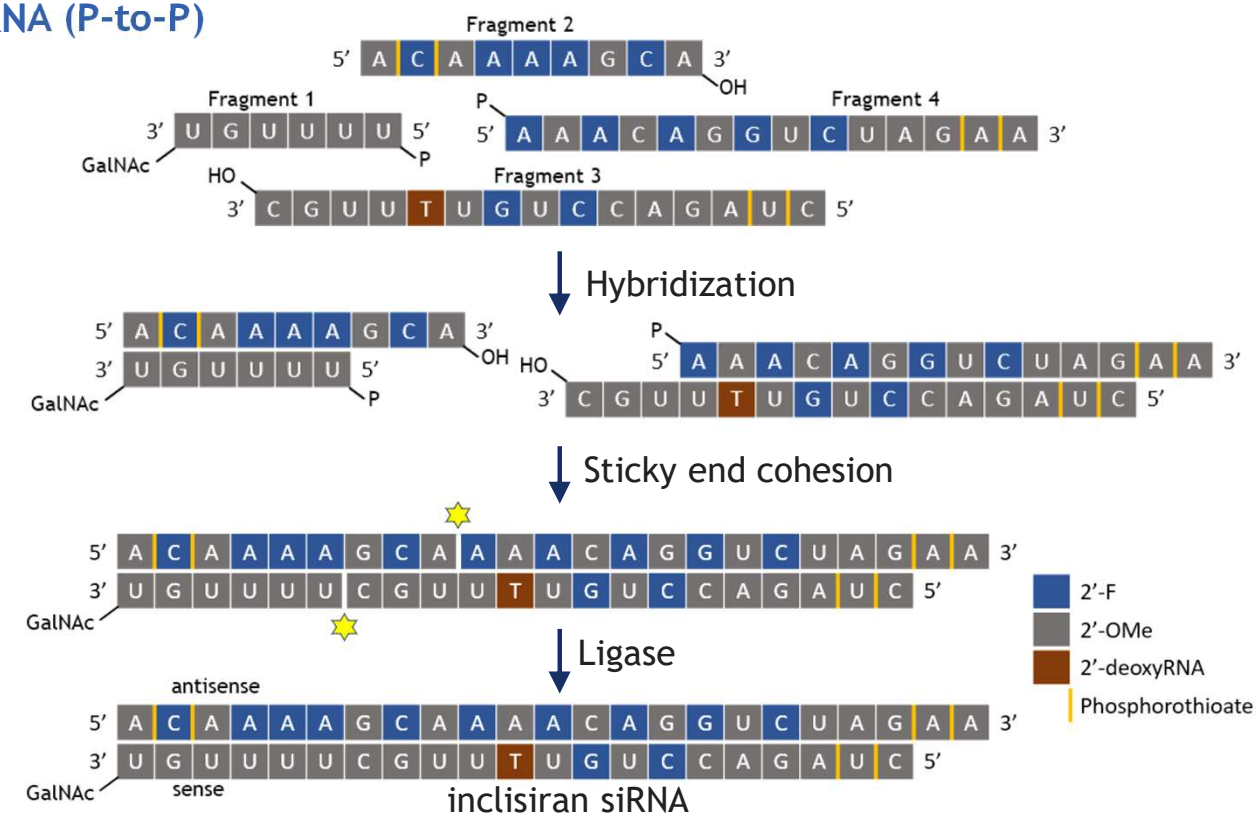
- inclisiran (LEQVIO®)
  - siRNA targeting PCSK9
  - Adjunct to diet and statin therapy for adults with primary hyperlipidemia, including HeFH, to reduce LDL-C
- Contains chemistries typically found in siRNA therapeutics
  - Ribose: 2′-F, 2′-OMe, 2′-deoxy
  - Backbone: PO with PS on ends
  - Targeting: Triantennary GalNAc
- Excellent model siRNA for chemoenzymatic ligation PoC studies



Inclisiran: Chemoenzymatic process design

Purified fragments and purified siRNA (P-to-P)

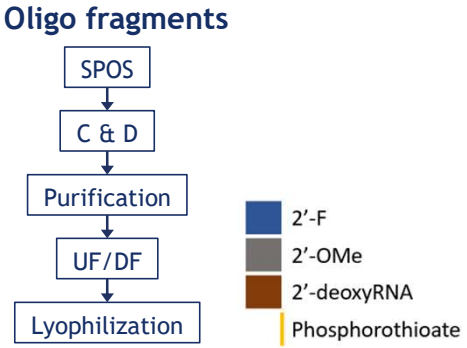




Inclisiran: Control of oligonucleotide fragment purity

IPRP-HPLC/MS

- Oligo fragment and siRNA purified by HPLC (P-to-P strategy)
- Most conservative approach - Clean oligo fragments for ligation
- Oligo fragment purity and impurities are well-controlled



Fragment	Sequence and chemistry	Length	Purity	Yield
1		6	98.9%	39.2%
2		9	98.4%	45.2%
3		15	97.9%	55.5%
4		14	96.3%	35.8%

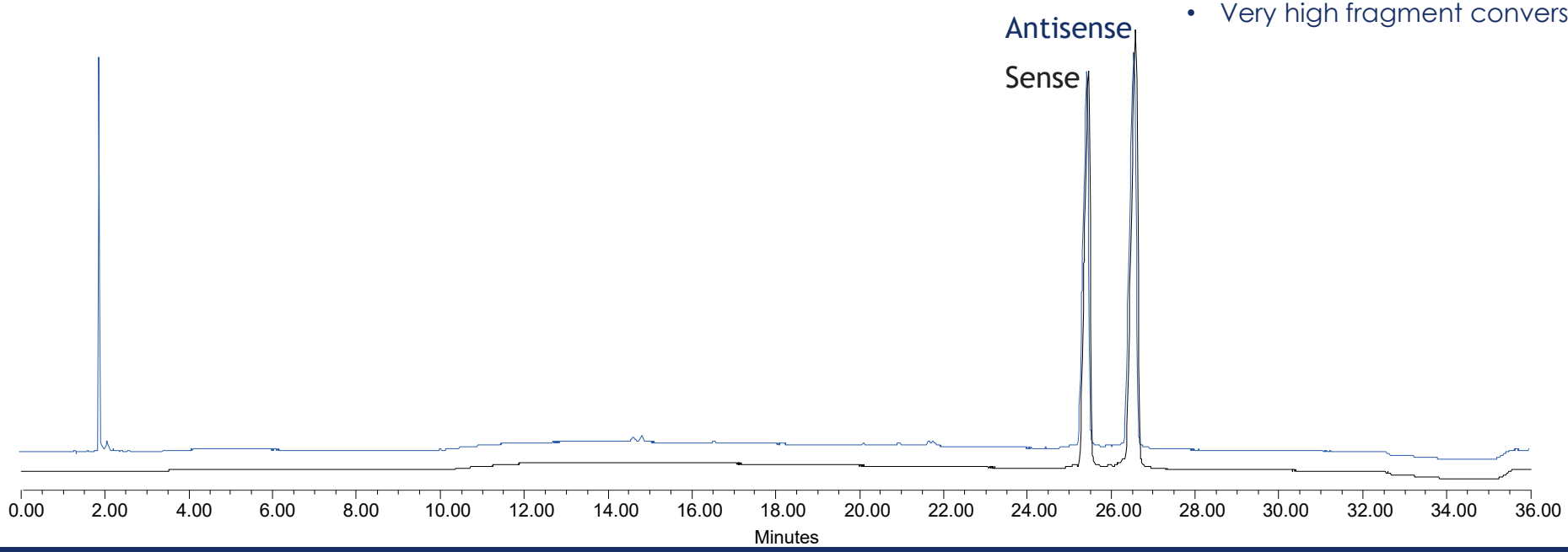
Unoptimized yields based on calculated molar extinction coefficients. Purity measured by IPRP-HPLC

Inclisiran: Control of product purity and impurities

IPRP-HPLC/MS

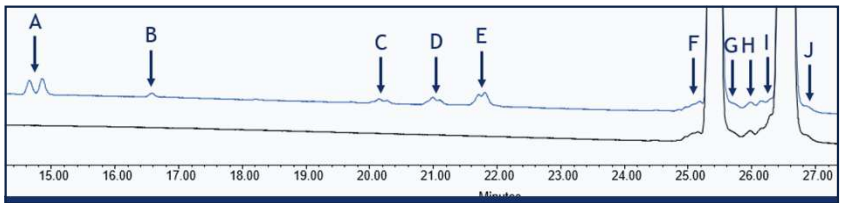
Crude: 95.1%
Purified: 96.8%

- Very high fragment conversion



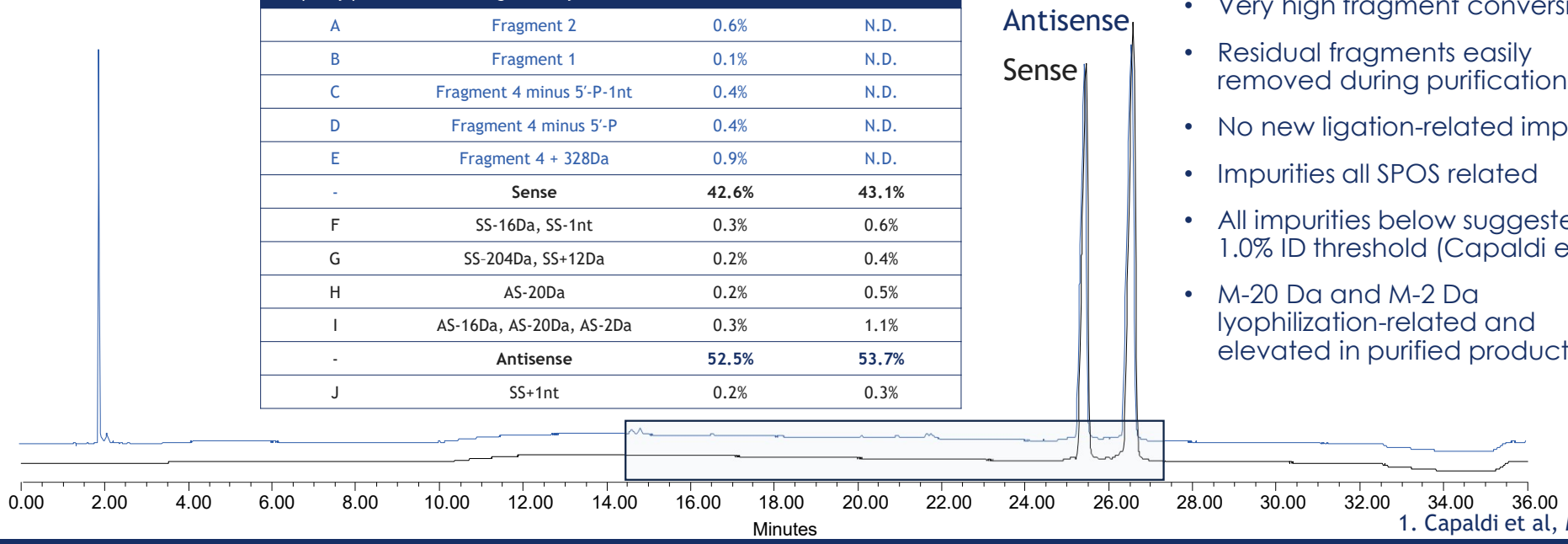
Inclisiran: Control of product purity and impurities

IPRP-HPLC/MS



Impurity peak	Assignment by MS	Crude area %	Purified area %
A	Fragment 2	0.6%	N.D.
B	Fragment 1	0.1%	N.D.
C	Fragment 4 minus 5′-P-1nt	0.4%	N.D.
D	Fragment 4 minus 5′-P	0.4%	N.D.
E	Fragment 4 + 328Da	0.9%	N.D.
-	**Sense**	**42.6%**	**43.1%**
F	SS-16Da, SS-1nt	0.3%	0.6%
G	SS-204Da, SS+12Da	0.2%	0.4%
H	AS-20Da	0.2%	0.5%
I	AS-16Da, AS-20Da, AS-2Da	0.3%	1.1%
-	**Antisense**	**52.5%**	**53.7%**
J	SS+1nt	0.2%	0.3%

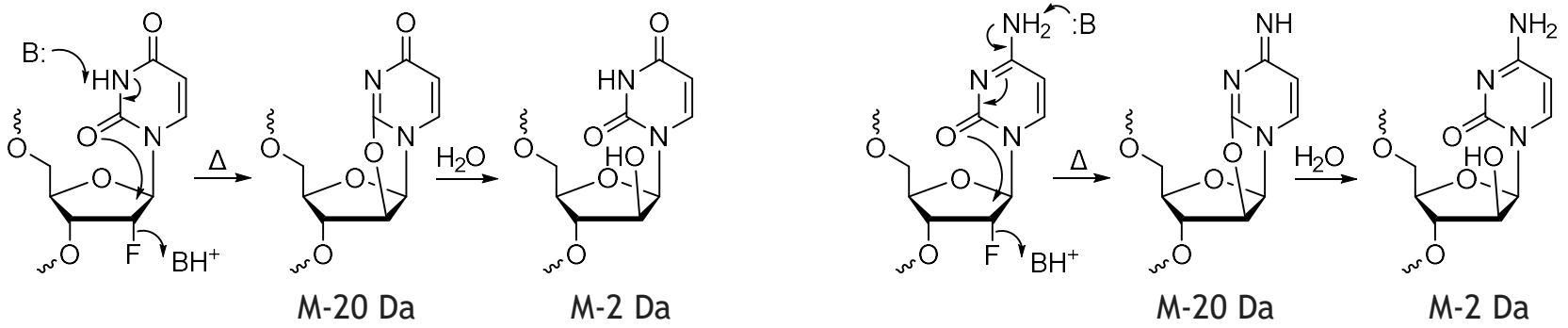
Crude: 95.1%
Purified: 96.8%



- Very high fragment conversion
- Residual fragments easily removed during purification
- No new ligation-related impurities
- Impurities all SPOS related
- All impurities below suggested 1.0% ID threshold (Capaldi et al[1])
- M-20 Da and M-2 Da lyophilization-related and elevated in purified product

1. Capaldi et al, *NAT*, 2017.

Degradation of siRNA containing 2'-F pyrimidines



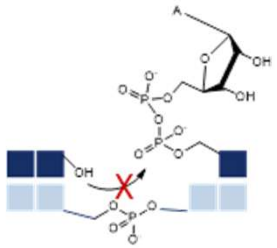
- Pathway previously reported for siRNA duplexes containing 2′-F pyrimidines on storage at elevated temperatures
  - 2,2′-anhydropyrmidines (M-20 Da) were observed predominantly during storage as solid[1]
  - 2,2′-anhydropyrimidines hydrolysis products (arabinosyl pyrmidines, M-2 Da) observed predominantly during storage in solution[1,2]
  - Conversion of 2,2′-anhydro to arabinosyl pyrimidines increases with neutralization of liberated HF[3]
- Aligned with our observations of elevation of these impurities following lyophilization

1. C.J. Calvitt et al, *Oligonucleotides*, 2010; 2. S. Seiffert et al, *Anal Biochem*, 2011; I.L. Doerr et al, *JOC*, 1967.

Oligonucleotide fragments are a focus of impurity control

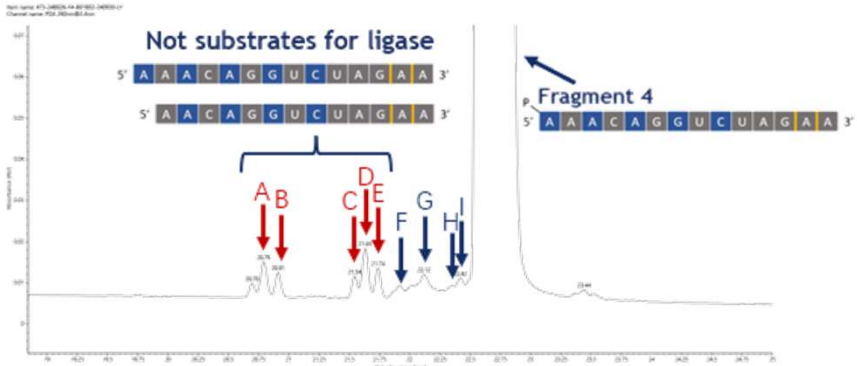
Two reasons for improved purity for Gen 2 vs Gen 1

1. SPOS-related impurities such as N-1nt, N+1nt, PO, M-20Da, M-2Da, CNET, iBu, and GalNAc degradants reduced compared to full-length synthesis
   - Less opportunity to compound and accumulate
2. Some oligonucleotide fragment impurities aren't good substrates for ligase
   - These don't persist in drug because they are purged during siRNA column chromatography



Many impurities in oligonucleotide fragments are purged
- Not ligase substrates (e.g. 5'(N-1nt) or
- Inefficient hybridization

Fragment 4 impurities - IPRP-HPLC/MS



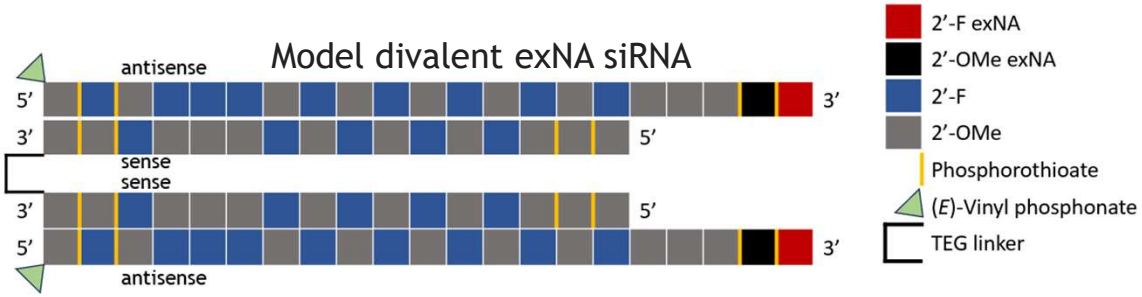
Impurity peak	RT (min)	Assignment	UV area%	In crude siRNA?
A	20.8	N-P-1nt	0.3	N
B	20.9	N-P-1nt	0.2	N
C	21.5	N-P	0.2	N
D	21.6	N-P	0.5	N
E	21.7	N-P	0.3	N
F	21.9	M-16Da	0.2	Y
G	22.1	M-16Da	0.5	Y
H	22.4	M-16Da	0.2	Y
I	22.4	M-2Da	0.2	Y
-	**22.6**	**Fragment 4**	**96.3**	**Y**

Ligation case study
Divalent exNA siRNA

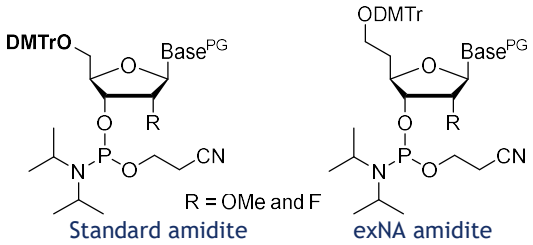
Ligation case study: Divalent exNA siRNA

Background

- UMass has invented divalent siRNAs with enhanced efficacy. These contain exNA chemistry[1]
- Scientists at Broad, UMass and Harvard recently identified a divalent siRNA clinical candidate for Prion disease[2]
- Hongene manufactured this clinical candidate using SPOS for the clinical trial
- We were curious as to whether divalent siRNA chemistry was amenable to chemoenzymatic ligation
- A model divalent siRNA construct was selected for PoC studies



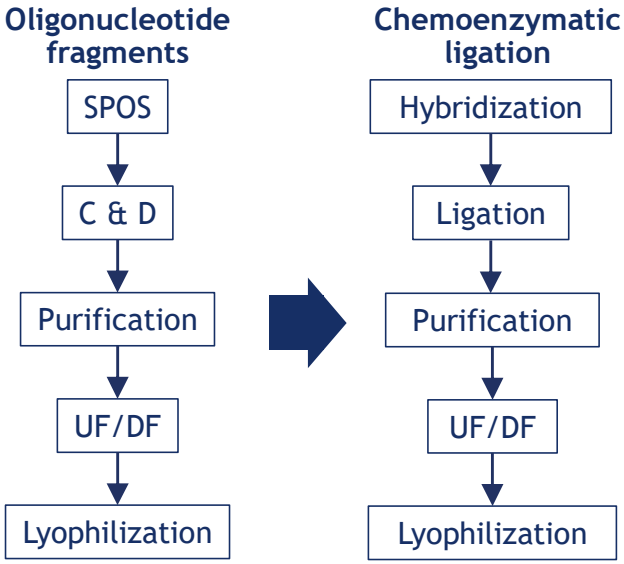
Novel exNA phosphoramidite Starting Materials scaled for GMP oligonucleotide CDMO services

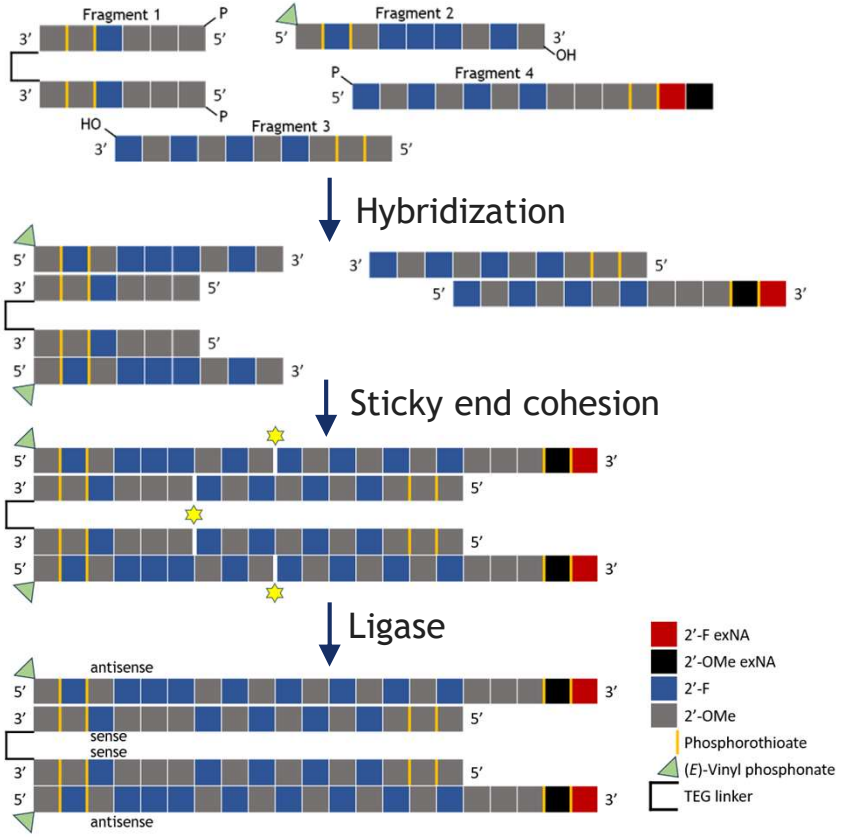


1. K. Yamada et al, *Nat Biotech*, 2024; J.E. Gentile et al, *bioXriv*, 2024.

Divalent siRNA: Chemoenzymatic process design

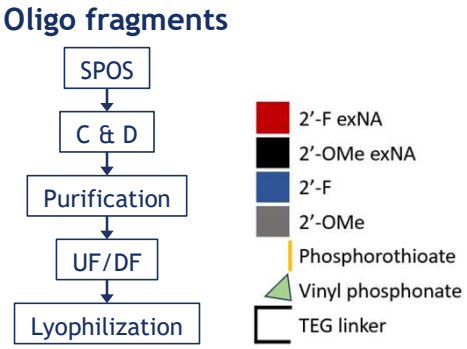
Purified fragments and purified siRNA (P-to-P)

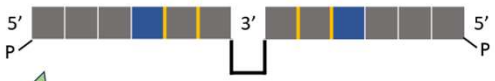




Divalent siRNA: Control of oligonucleotide fragment purity

IPRP-HPLC/MS



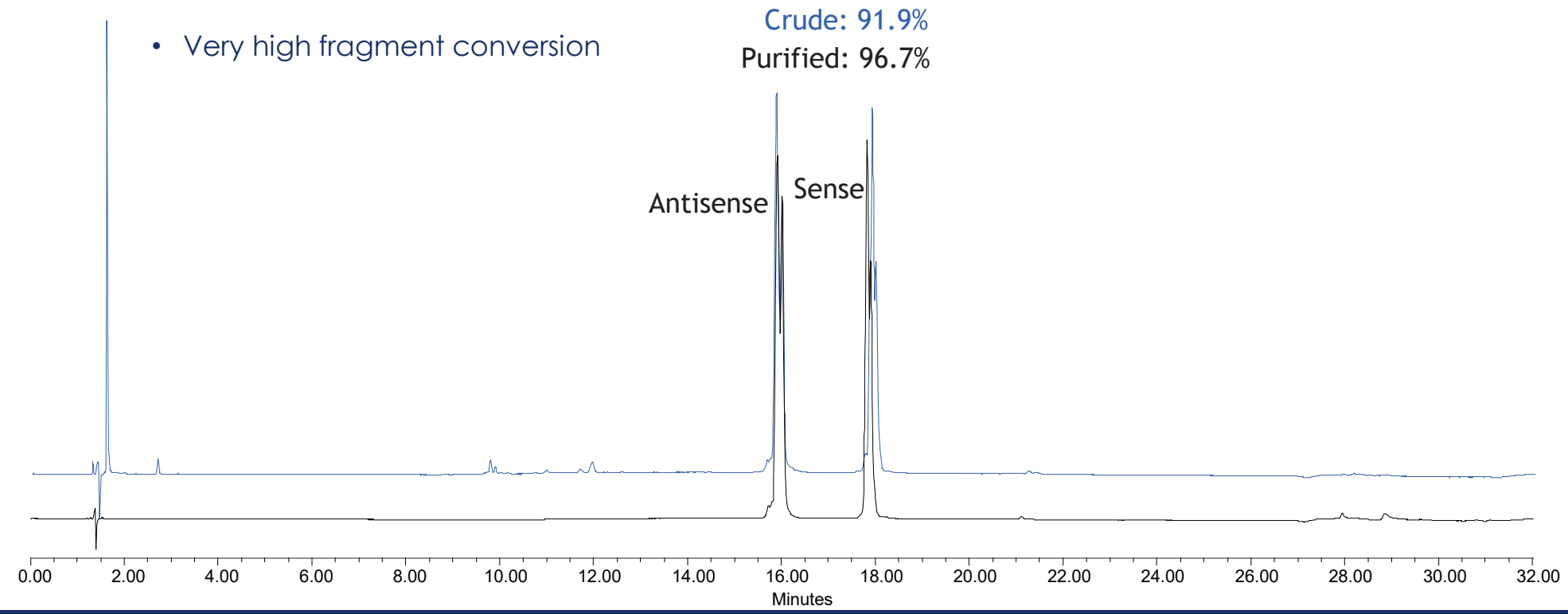
Fragment		Length	Purity	Yield
1		12	96.2%	26.3%
2		9	95.4%	48.2%
3		10	96.0%	42.5%
4		12	93.6%	30.2%

Unoptimized yields based on theoretical molar extinction coefficients. Purity measured by IPRP-HPLC

Divalent siRNA: Control of purity and impurities

IPRP-HPLC/MS

- Very high fragment conversion



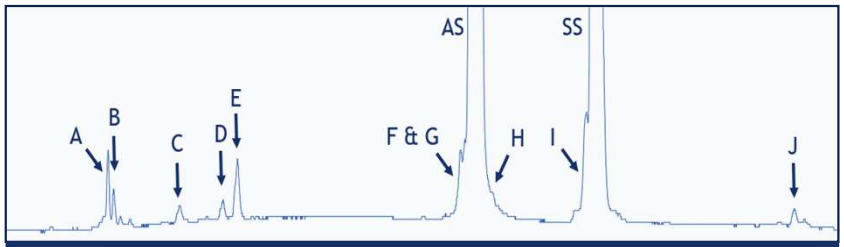
Divalent siRNA: Control of purity and impurities

IPRP-HPLC/MS

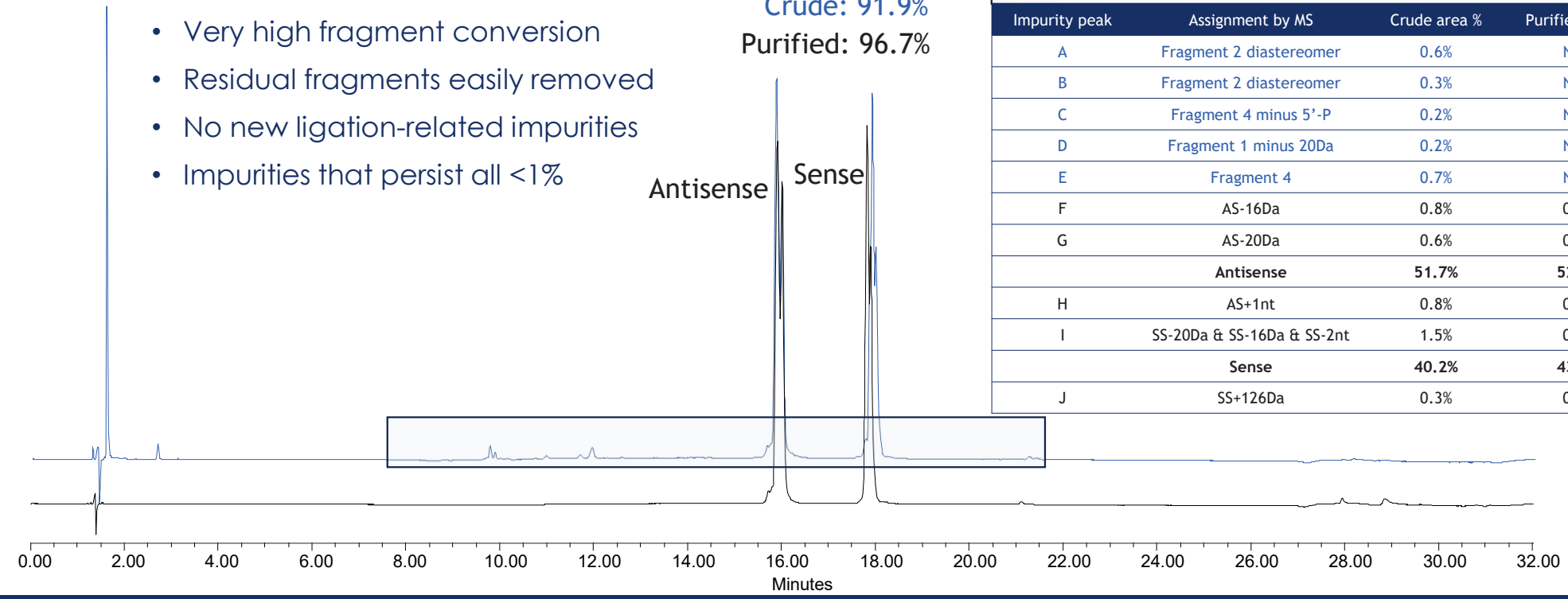
- Very high fragment conversion
- Residual fragments easily removed
- No new ligation-related impurities
- Impurities that persist all <1%

Crude: 91.9%

Purified: 96.7%



Impurity peak	Assignment by MS	Crude area %	Purified area %
A	Fragment 2 diastereomer	0.6%	N.D.
B	Fragment 2 diastereomer	0.3%	N.D.
C	Fragment 4 minus 5'-P	0.2%	N.D.
D	Fragment 1 minus 20Da	0.2%	N.D.
E	Fragment 4	0.7%	N.D.
F	AS-16Da	0.8%	0.8%
G	AS-20Da	0.6%	0.6%
	Antisense	**51.7%**	**52.9%**
H	AS+1nt	0.8%	0.5%
I	SS-20Da & SS-16Da & SS-2nt	1.5%	0.2%
	Sense	**40.2%**	**43.8%**
J	SS+126Da	0.3%	0.3%



siRNA summary

Summary of exemplary siRNAs synthesized by ligation

Molecule	GMP	Oligonucleotide chemistry				Synthesis strategy[1]	Yield[2]	Purity[3]
		SS/AS	2′-Ribose mods	Backbone	GalNAc			
Inclisiran siRNA	-	21/23	2′-OMe, 2′-F, 2′-deoxy	PS/PO	✓	P-to-P	26%	97%
Divalent siRNA	-	16/21	2′-OMe, 2′-F, exNA, (*E*)-VP	PS/PO/TEG	x	P-to-P	19%	97%
C-to-P siRNA	-	21/23	2′-OMe, 2′-F	PS/PO	✓	C-to-P	43%	96%
CDMO siRNA	No	ND	2′-OMe, 2′-F	PS/PO	✓	C-to-P	~960 g	95%
CDMO siRNA	Yes	ND	2′-OMe, 2′-F	PS/PO	✓	P-to-P	~1,020 g	97%

1. P-to-P = HPLC purified fragments and purified siRNA; C-to-P = UF/DF processed fragments and HPLC purified siRNA; 2. % Yields based on theoretical MEC, calculated from lowest yielding fragment; 3. Denaturing IPRP-UPLC method

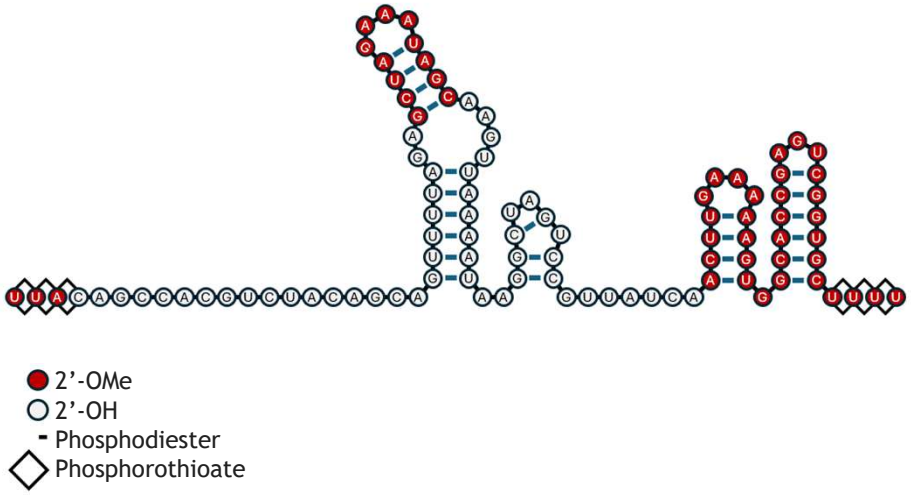
- Managing quality and cost of production
  - P-to-P generally adopted as the most conservative for PoC studies and FIH GMP batch
  - C-to-P gives similar purity as P-to-P, but with higher yield
  - C-to-C will be most effective strategy to manage costs for high volume CVD indications
  - Batch mode and single use bioreactors (scalable); at least 100 g siRNA per L is feasible
  - Plenty of room to optimize oligonucleotide fragments processes for scalability and yield

Chemoenzymatic ligation for sgRNA synthesis

Ligation case study
G211 sgRNA

Ligation case study: G211 100mer sgRNA

Highly modified 100mer G211 sequence



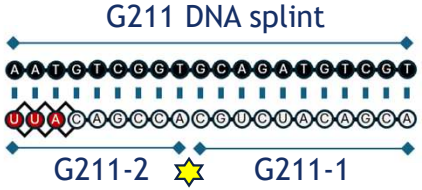
Background

- We sought out a relevant sgRNA sequence for PoC ligation study
- *In vivo* TTR gene editing with LNP delivery paper published by Intellia[1]
- Highly modified 100mer G211 sequence selected
- Highest editing of all guides tested
- Contains sgRNA chemistry typically found in non-virally delivered gene editing drugs
  - Ribose: 2'-OH, 2'-OMe
  - Backbone: PO, PS on ends

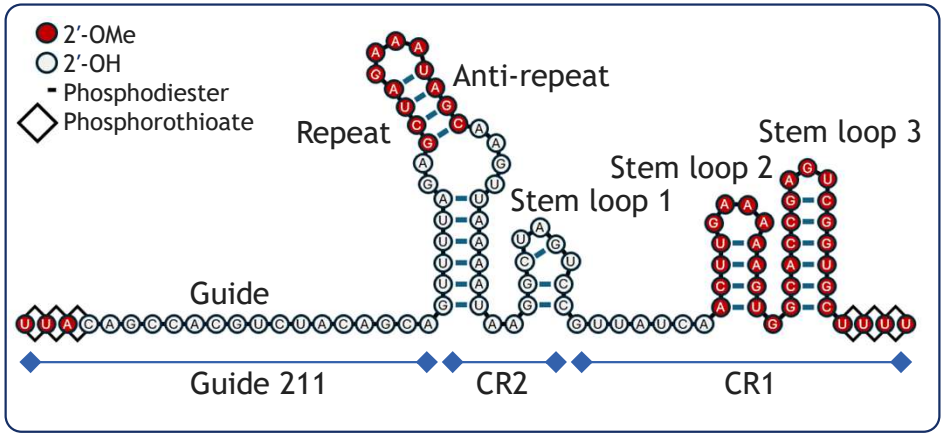
1. Finn et al, *Cell Reports*, 2018.

Two step splinted ligation strategy for ultra-high purity guide

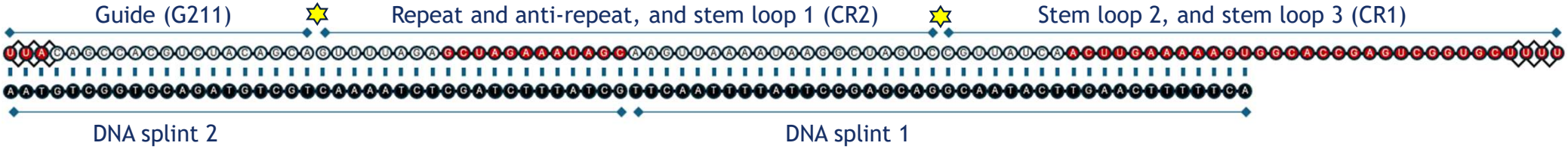
Step 1: G211 guide ligation



Pre-assembly of G211 20mer from two short fragments assures excellent guide sequence fidelity

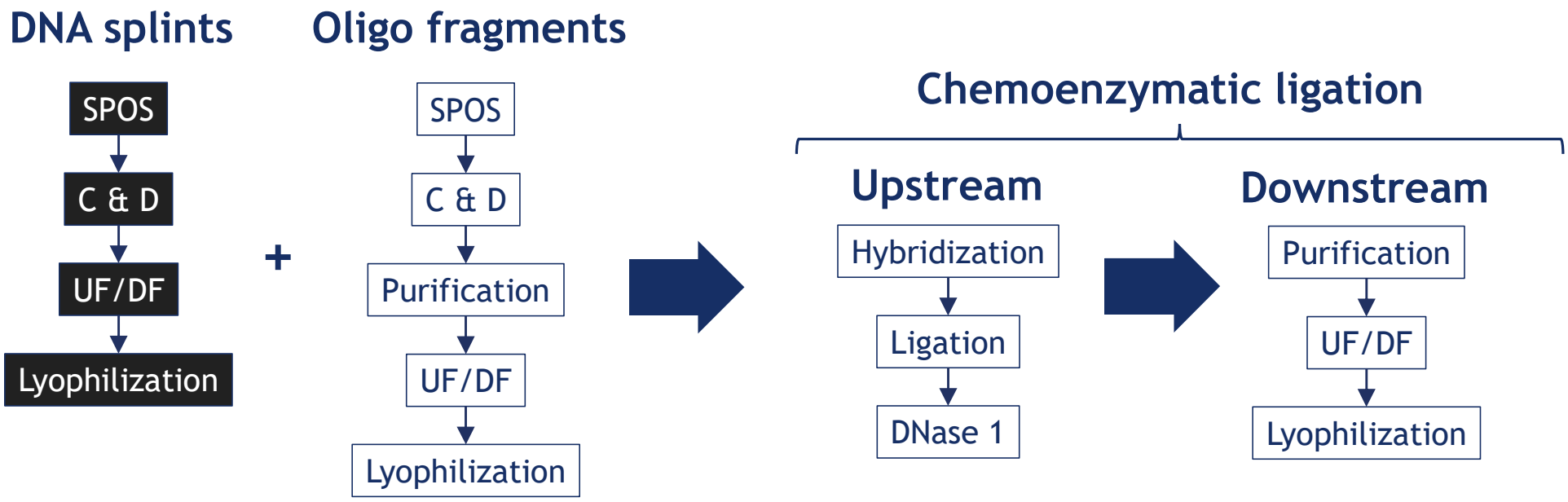


Step 2: Assembly of 100mer sgRNA by ligation of G211 guide, CR1 and CR2 oligo fragments

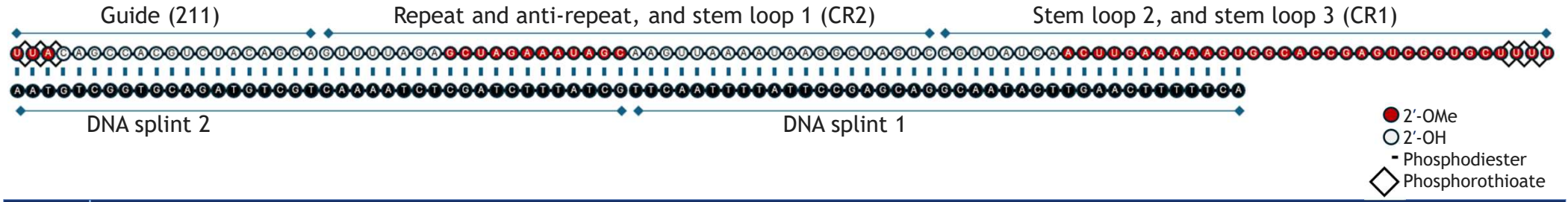


Chemoenzymatic process flow for step 1 and step 2

Purified oligonucleotide fragments and crude splints



Components for assembly of ultra-high purity sgRNA



Step	Sequence	Length	5′-P	Purif[n]	Purity[1]	Sequence information (5′ to 3′ fragments, 3′ to 5′ splints)
1	G211-1	9	N	HPLC	98.4%	UUACAGCCA
	G211-2	11	Y	HPLC	98.9%	CGUCUACAGCA
	G211 splint	20	-	UF/DF	82.6%	AATGTCGGTGCAGATGTCGT
2	G211	20	N	HPLC	98.1%	UUACAGCCACGUCUACAGCA
	CR1	40	Y	HPLC	90.4%	CGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
	CR2	40	Y	HPLC	81.4%	GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUC
	DNA splint1	40	-	UF/DF	70.4%	TTCAATTTTATTCCGAGCAGGCAATACTTGAACTTTTTCA
	DNA splint2	40	-	UF/DF	77.5%	AATGTCGGTGCAGATGTCGTCAAAATCTCGATCTTTATCG

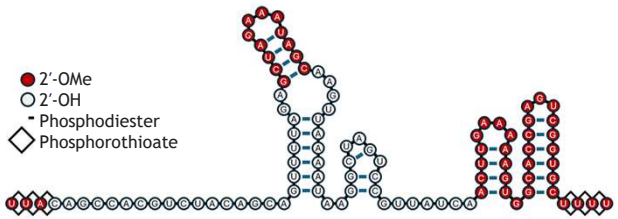
1. Purity measured by IPRP-HPLC/MS

sgRNA before and after HPLC purification

IPRP-HPLC/MS

Crude after ligation, before DNase 1: 39.0%

HPLC purified: 96.8%



sgRNA before and after HPLC purification

IPRP-HPLC/MS

Crude after ligation, before DNase 1: 39.0%

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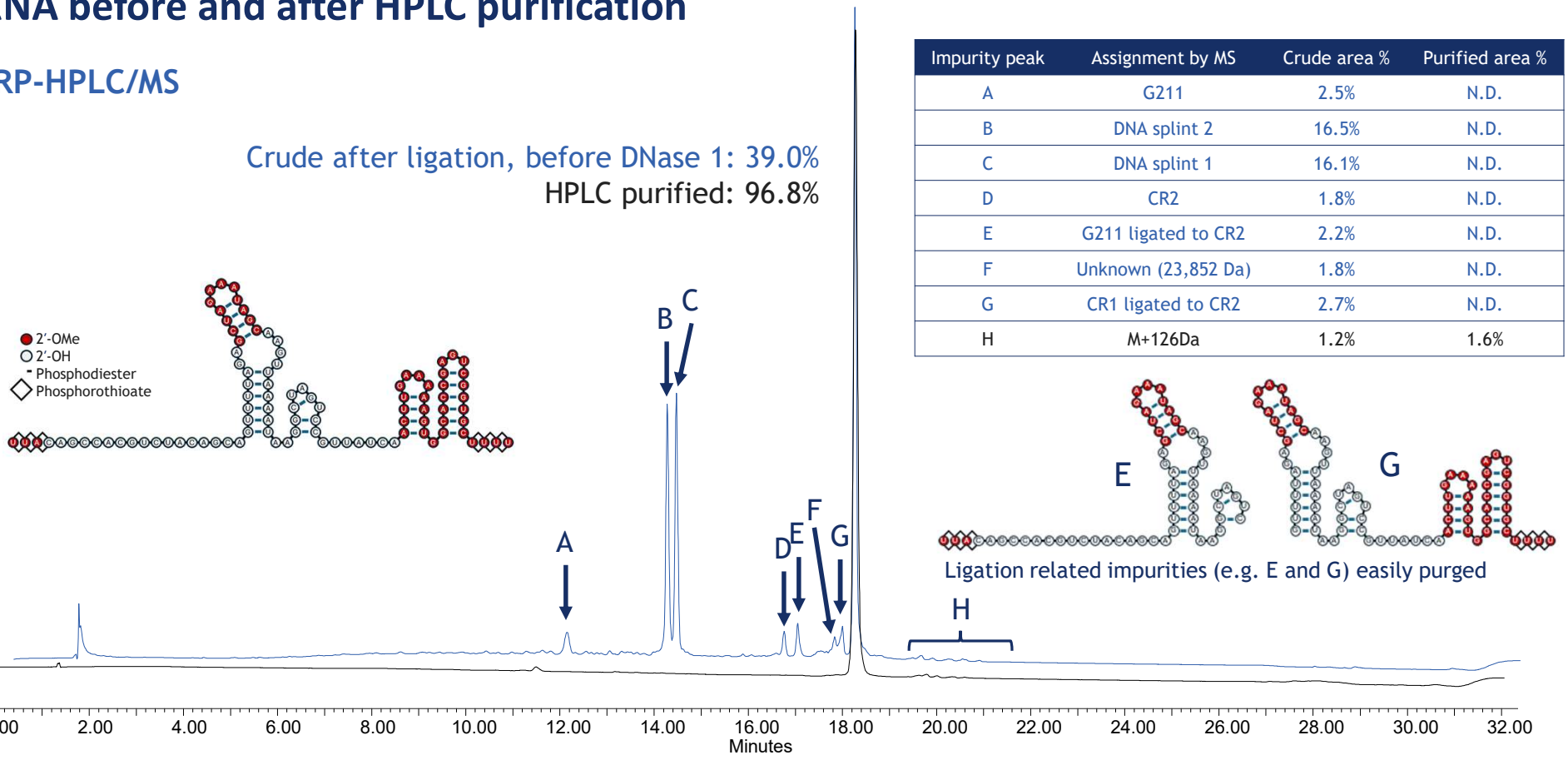


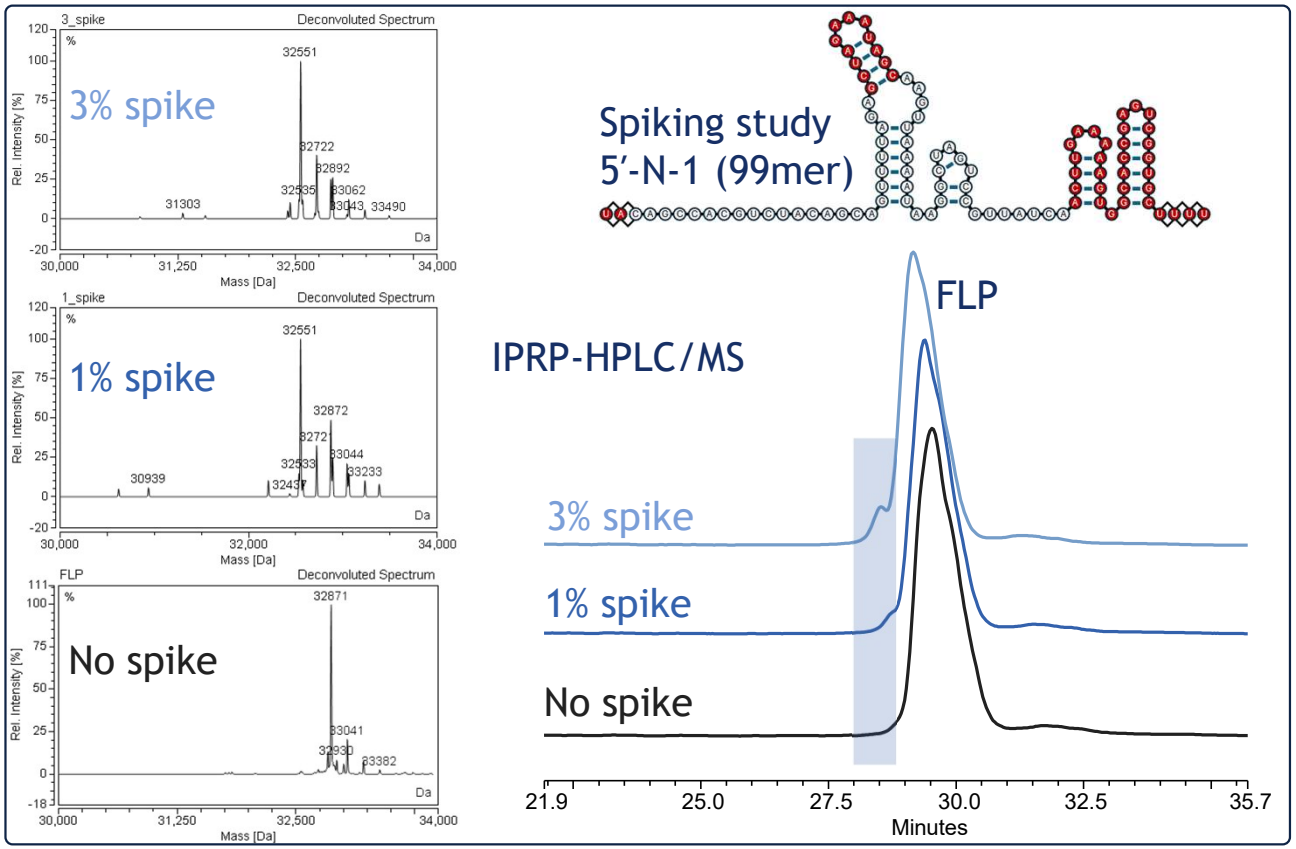
Impurity peak	Assignment by MS	Crude area %	Purified area %
A	G211	2.5%	N.D.
B	DNA splint 2	16.5%	N.D.
C	DNA splint 1	16.1%	N.D.
D	CR2	1.8%	N.D.
E	G211 ligated to CR2	2.2%	N.D.
F	Unknown (23,852 Da)	1.8%	N.D.
G	CR1 ligated to CR2	2.7%	N.D.
H	M+126Da	1.2%	1.6%

Ligation related impurities (e.g. E and G) easily purged

sgRNA purity and impurity control

Heavy reliance on HPLC and MS

- HPLC methods for separation of closely-related impurities (e.g. N-1nt)
- Required MS capabilities
  - Intact mass identification
  - Impurity profiling
    - 1Da resolution…?
  - Sequence confirmation



Gene editing drug CDMO services under one roof in MA, USA

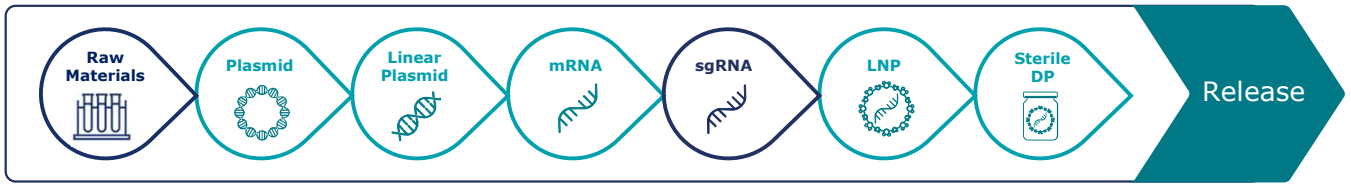
ReciBioPharm Hongene Biotech partnership

The value proposition

Hongene Biotech
- High-quality, competitively priced raw materials
- Chemoenzymatic ligation technology for sgRNA

ReciBio Pharm
- State-of-the-art US facilities and top technical expertise
- GMP plasmid, mRNA, LNP formulation and DP manufacture

One facility, one point of contact, one master services agreement, one quality agreement

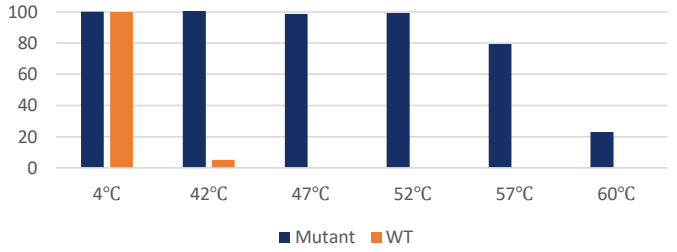


Engineered thermostable T4 RNA ligase

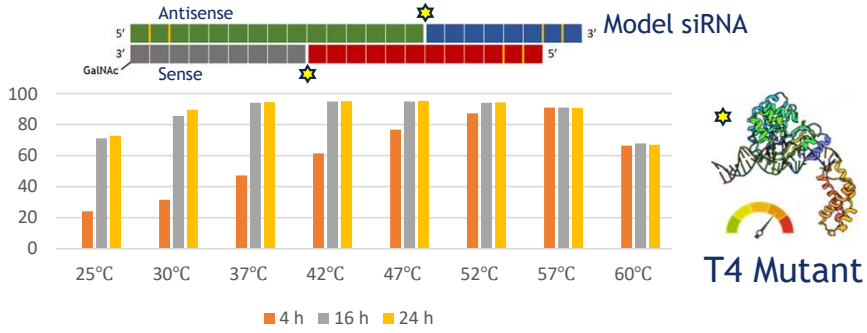
Engineered thermostable T4 RNA ligase is highly active at 52°C

- Rationale for thermostable enzyme
  - Facilitates ligation at higher temperature
  - Reduced secondary structures
  - Improved selectivity for fragments with optimal hybridization
  - Mitigation of incorrectly ligated byproducts
  - Leverage C-to-C strategy further driving down cost

T4 mutant retains activity after incubation at 52°C



T4 mutant is maximally active between 52-57°C



Activity study: 20ul enzyme solution (0.1mg/mL in 50mM tris, 10mM $Mg(Ac)_2$ 5mM ATP) was incubated at the indicated temperature for 8h. This solution was then added to 20ul volume of 2.5 mM each of four oligo fragments and ligation was allowed to proceed at 33°C for 2h. Ligation activity calculation: Percentage of siRNA product (Temp)/siRNA product (4°C).

Control of stereochemistry

Collaboration with Bruker: Control of stereochemistry using NMR

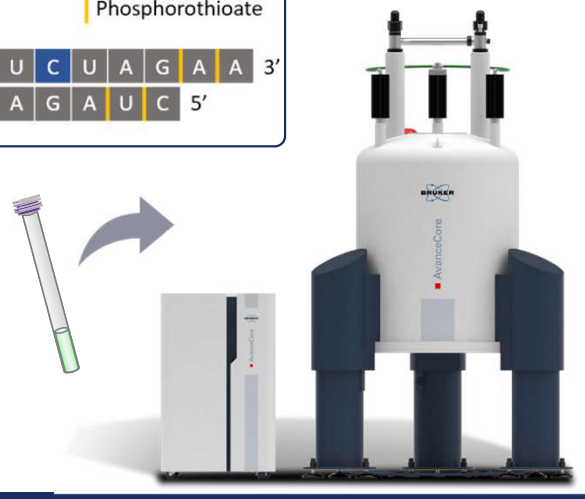
Hongene Biotech

BRUKER

Structural Analysis by NMR:

- Nondestructive
- In formulation
- High-resolution (atomic)
- Sensitive to changes
- Selective

Booth #32
Harbor Foyer



Current Phase

- NMR method development to distinguish stereochemistry of oligonucleotide fragments, single strands, & duplex

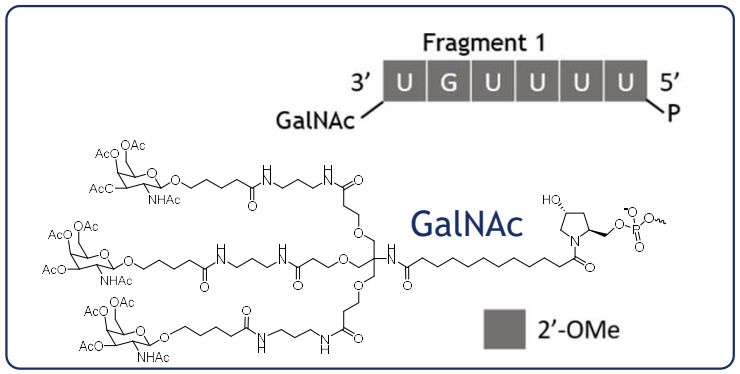
Phase 2

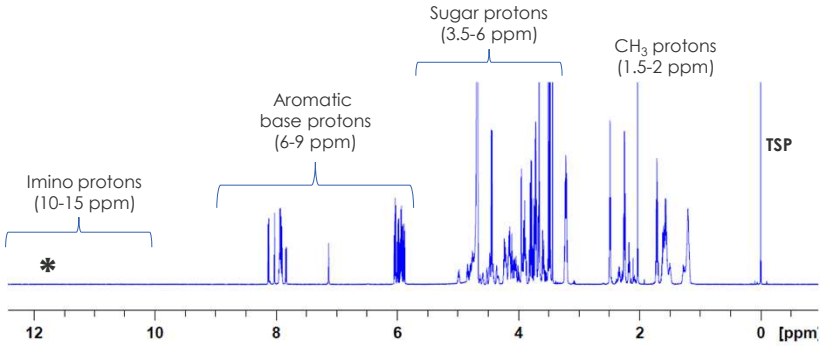
- Compare the effects of synthesis conditions (e.g. activators) on the stereochemistry by NMR

Phase 3

- Study stereochemical control of stereo-random oligos by NMR, compare with stereo-pure reference materials

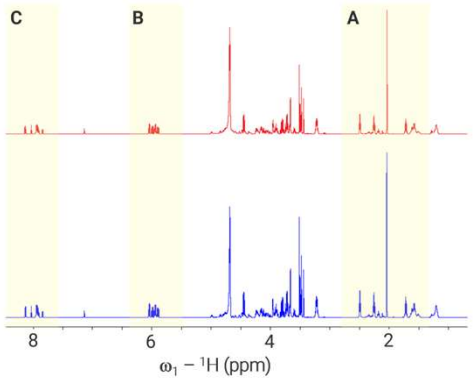
Batch to batch comparison of inclisiran Fragment 1 (crude)



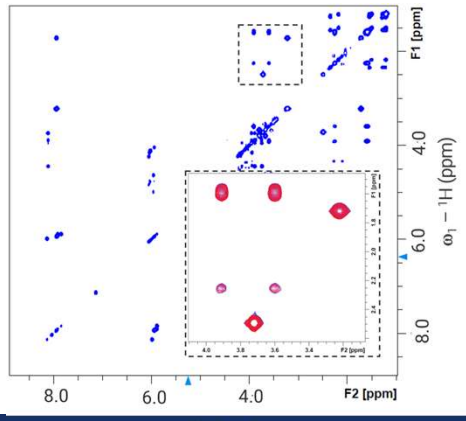


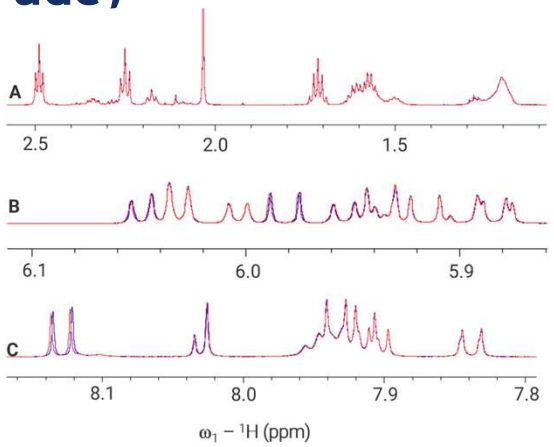
1mM Fragment1 in 90%H_2O/10%D_2O 45 mM Phosphate Buffer (0.01%TSP, 0.06mM NaN3)

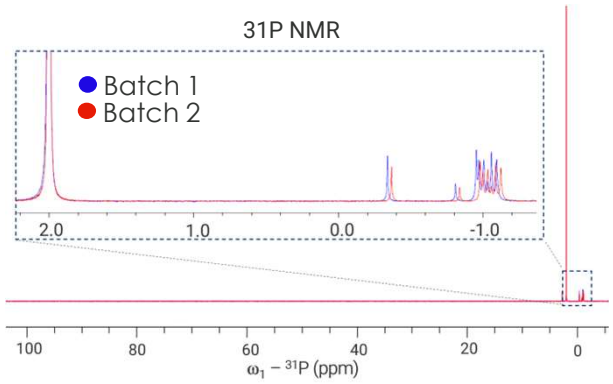
*imino protons not observed in ss oligo fragment



2D-TOCSY







Conclusions

Conclusions

- Market demand for siRNA drugs is anticipated to grow significantly over the next decade
- This demand will be met by adoption of oligonucleotide fragment ligation technology
- Advantages are improved quality, yield, environmental sustainability and scalability
- We have manufactured 1 kg of siRNA GMP drug substance using ligation technology
- We envision no significant regulatory hurdles to wider adoption of the technology
- C-to-P and C-to-C purification strategies can be leveraged for large scale manufacturing
- Engineered T4 RNA ligases will provide future efficiencies and cost improvements
- Oligonucleotide fragments are manufactured today using scale-limited SPOS
  - Future demand will require scalable processes adapted to batch reactors
- High purity sgRNA for CRISPR based gene editing drugs is accessible by splinted ligation

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Hongene Biocatalysis Team

- Minzhi Wei
- Min Guo
- Zaiji Xiong
- Juan Xia
- Zhaoyue Fan
- Yanxing Liu
- Yumai Lu
- Liang Jiang
- Jianxiong Liu

Hongene Biotech

Thank you!