Use of Capillary Electrophoresis Methodologies to Guide mRNA CMC Development

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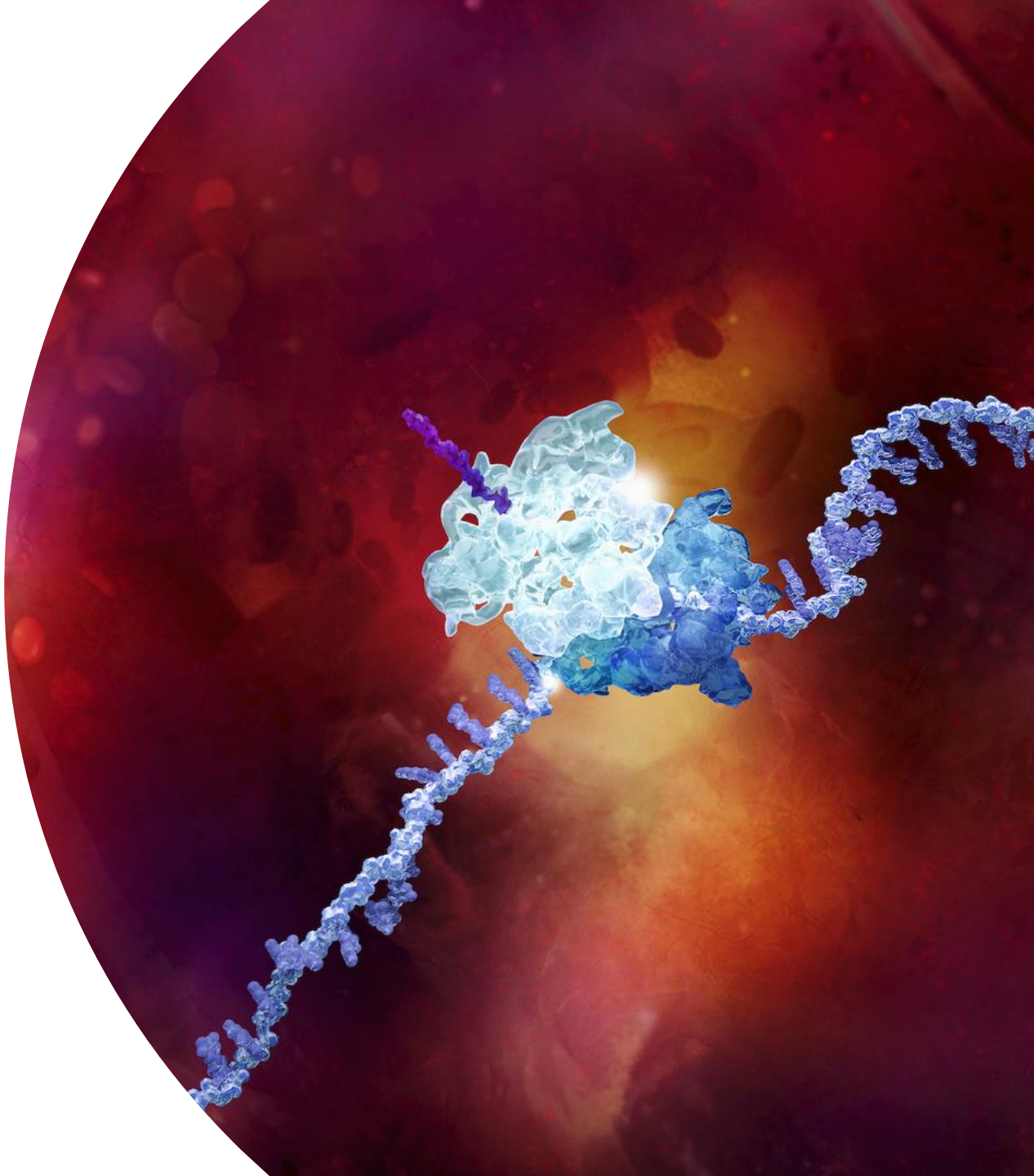

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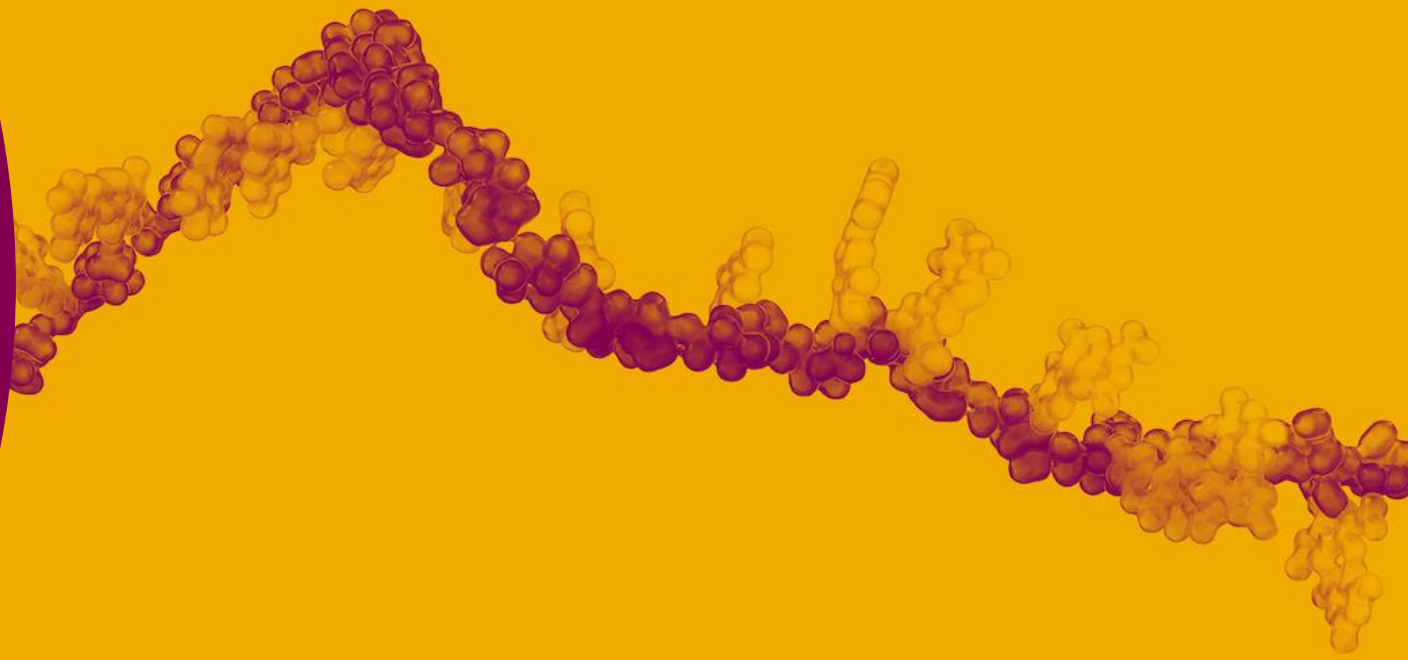
Contents

1

Introduction

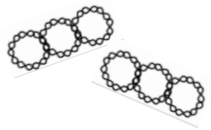


Example Manufacturing Process

Starting Materials

HQ pDNA

HC pDNA
LC pDNA



Drug Substances

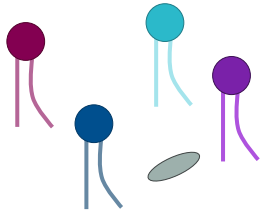
mRNA (DS)

HC mRNA
LC mRNA

Lipids

Proprietary 3rd party lipid

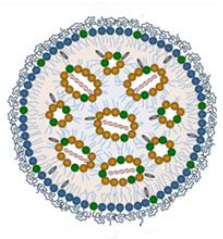
+



Commercially available lipids

Bulk Drug Product

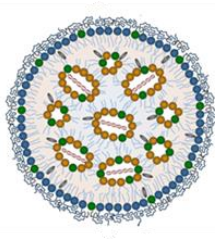
LNP encapsulated mRNA



[excipients]

Formulated Drug Product

Formulated, LNP-encapsulated mRNA



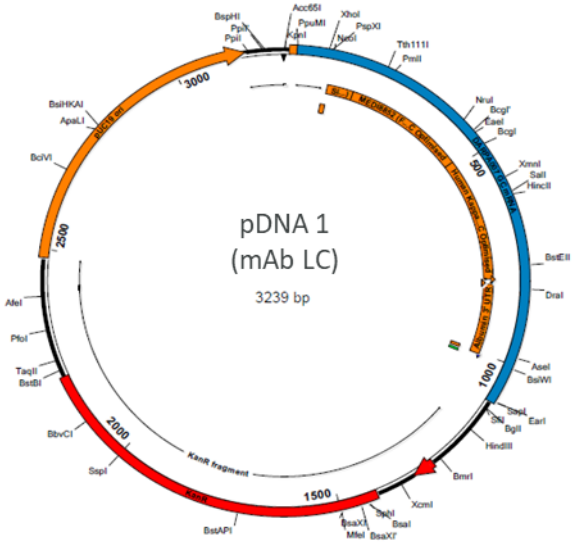
CMO

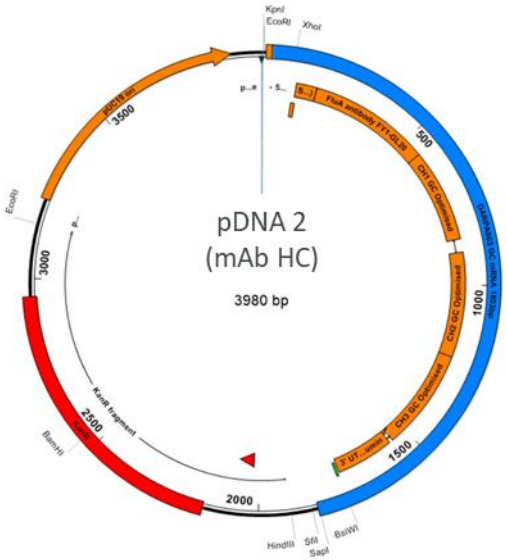
mRNA HC & mRNA LC
- released as **two separate Drug Substances**
- **mixed** together with lipids for encapsulation

Formulated LNP-Encapsulated mRNA DP
- 1.0 mg/mL
- Liquid
- -90°C to -70 °C

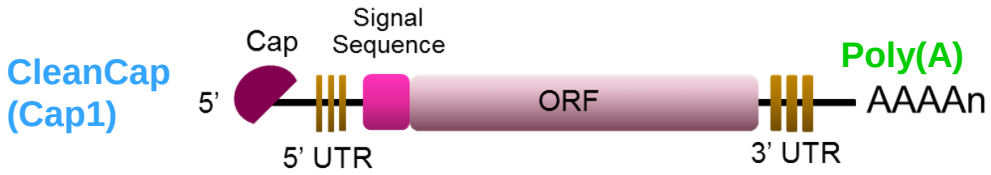
Drug Substance Manufacture

Double stranded Plasmid DNA coding for mAb light and heavy chains:





mRNA Drug Substance:



pDNA process

Transformed pDNA vial thaw

Production bioreactor

pDNA

Purification

Purified pDNA

Supercoiled

Intended storage of pDNA ≤ -65°C

mRNA process

Enzymatic linearization

Linearized pDNA

Linearized

IVT reaction

mRNA

2

CE for Plasmid DNA

Method Details

Analysis: eCap dsDNA 1000 kit by Sciex

System: Sciex PA800+

Sample Preparation:

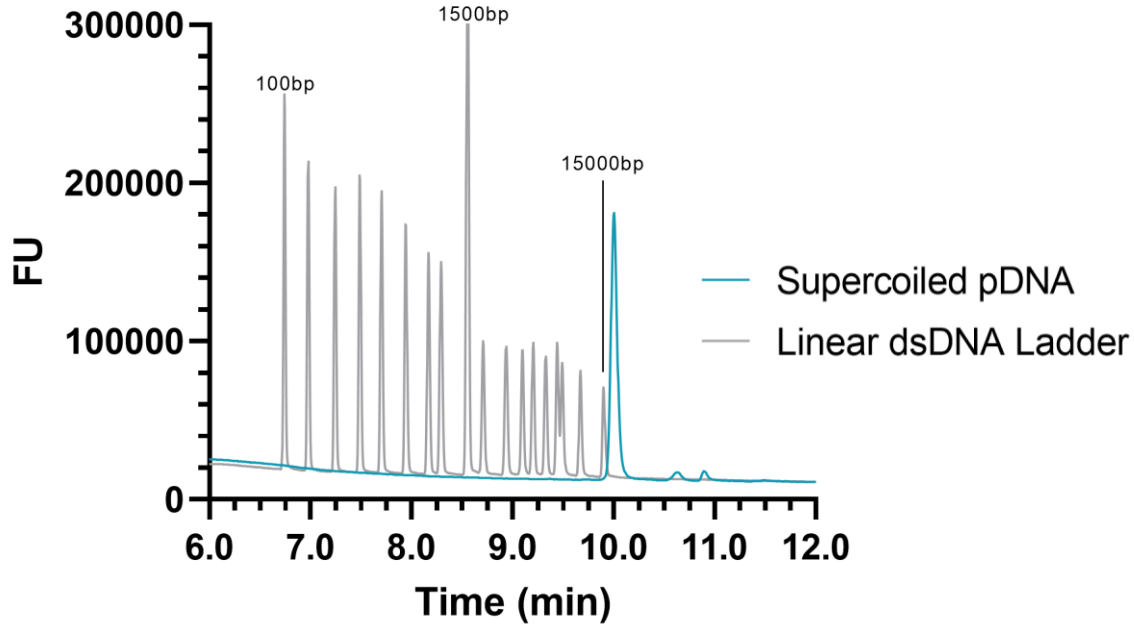
- Sample handling on wet ice
- 2 ng/µL in TE Buffer (10 mM Tris, 1 mM EDTA)

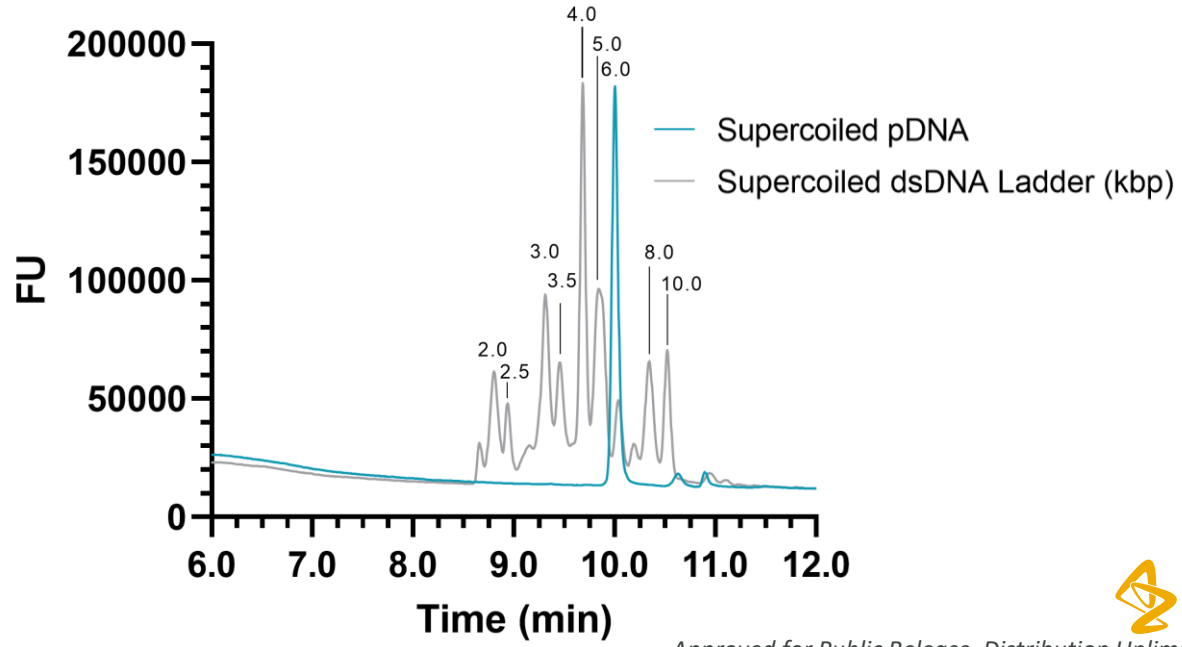
Separation:

- 100µm ID eCAP DNA capillary
- 40cm cartridge (30cm effective length)
- 20°C cartridge temp
- 5s pressure injection @ 0.2 psi
- 10 kV separation for 14mins

Detection:

- LIF
- 488 nm / 520 nm (excitation / emission)

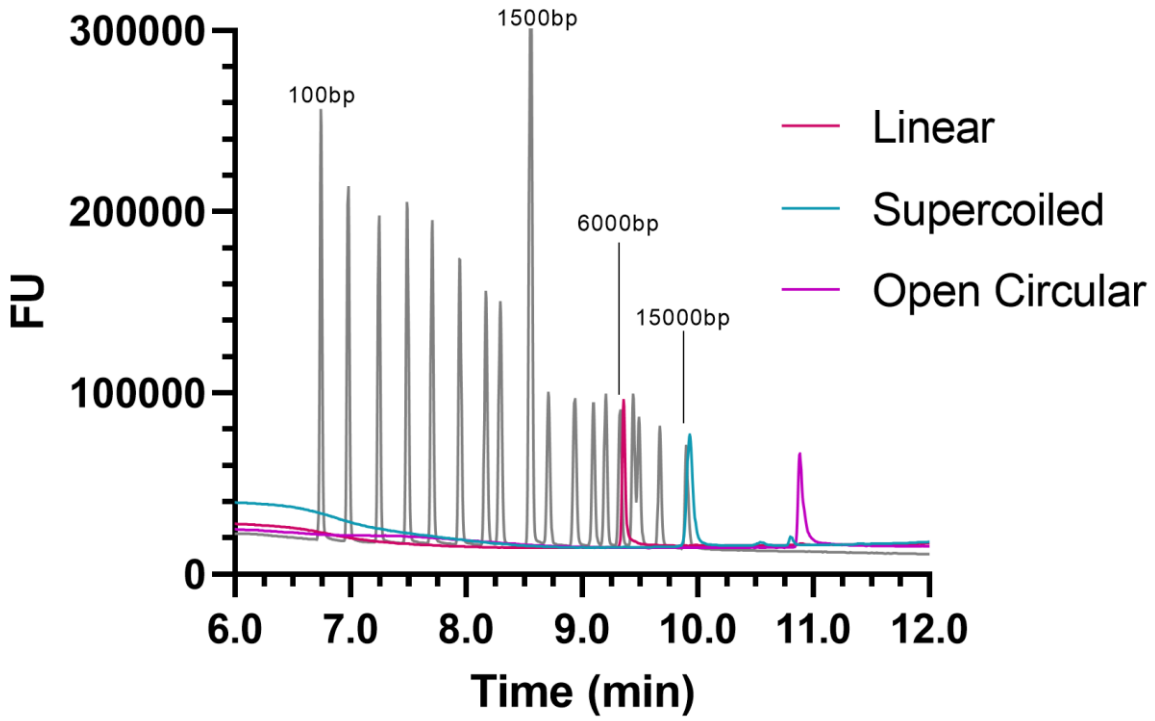




pDNA CE

- Ladder displays 18 peaks ranging from 100 - 15000 bp
- Variability in peak migration times observed
  - MT Δ up to ~0.4 mins
  - Δ 0.1 min > 1000 bp
- **Assay not suitable for accurate size determination**
- pDNA CE assay is sensitive to different pDNA isoforms
- Requires comparability to a suitable size ladder
- **Potential for complex profiles requiring peak ID**

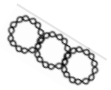
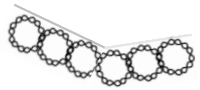
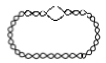
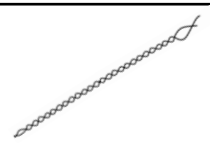
Overlay of pDNA Isoforms with 1kB Plus Ladder



Differences in Migration Time (MT Δ) of 1kB Plus Ladder Peaks

Ladder Peak Size	Run 1 MT (min)		Run 2 MT (min)		Run 3 MT (min)		Run 4 MT (min)		Max. Intra-assay MT Δ (min)	Inter-assay MT Δ (min)
100 bp	6.663	6.551	6.622	6.532	6.501	6.465	6.549	6.510	**0.112**	**0.198**
1500 bp	8.087	7.936	8.091	7.964	7.934	7.877	8.102	8.054	**0.151**	**0.225**
15000 bp	9.139	9.006	9.124	8.976	8.967	8.897	9.268	9.214	**0.148**	**0.371**

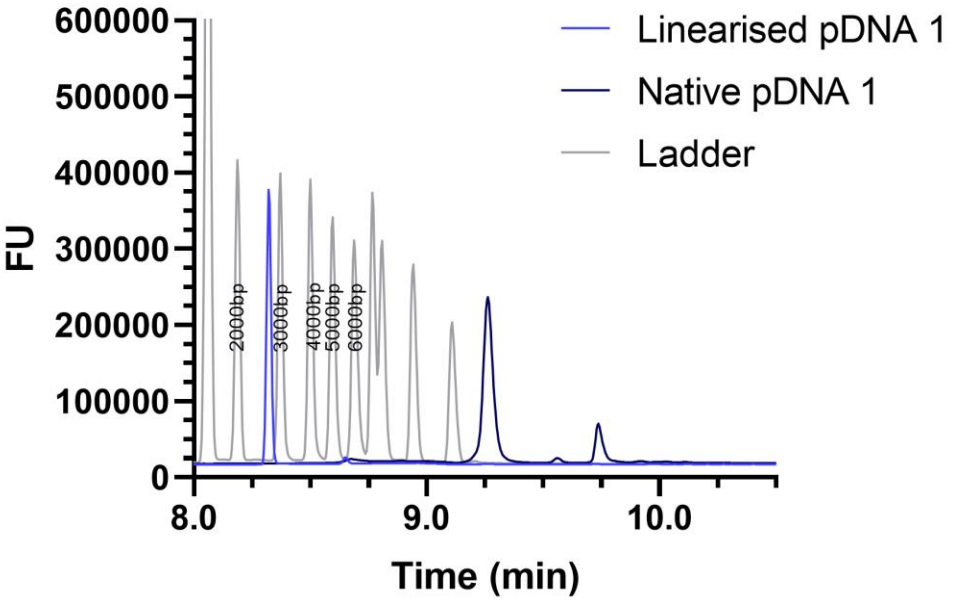
Plasmid DNA Isoforms

Supercoiled	
Open circular	
Linear	
Concatenated supercoiled	
Concatenated open circular	
Concatenated Linear	

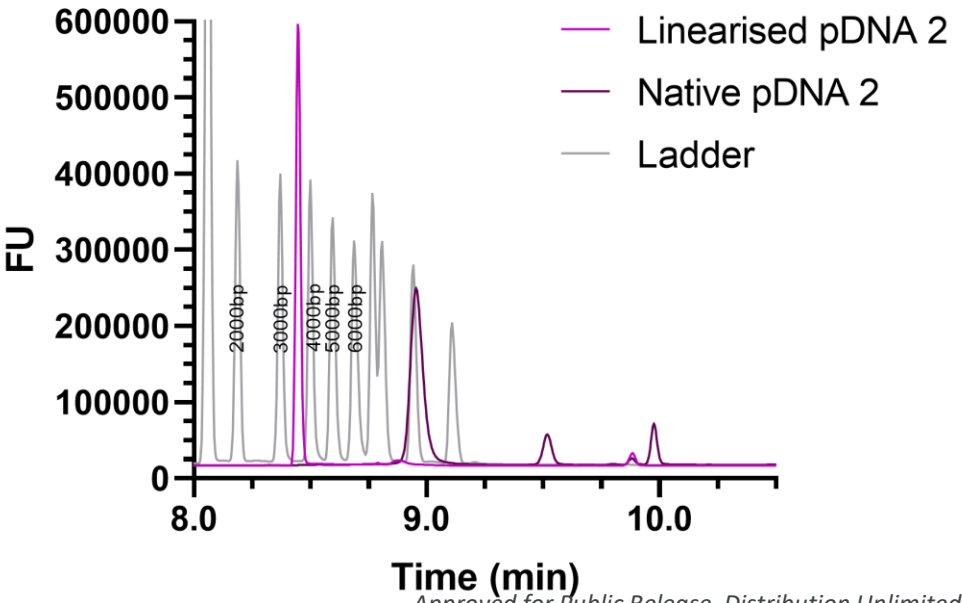
- Three major isoforms
  - Supercoiled (no breaks)
  - Open Circular (single strand break)
  - Linear (double strand break)
- Process can also generate concatemers
  - Multiple copies of the plasmid
  - Concatemers also display different isoforms when breaks occur
- Manufacture of the mRNA DS involves enzymatically linearising prior to IVT reaction

Need to determine an appropriate control strategy for the Plasmid DNA

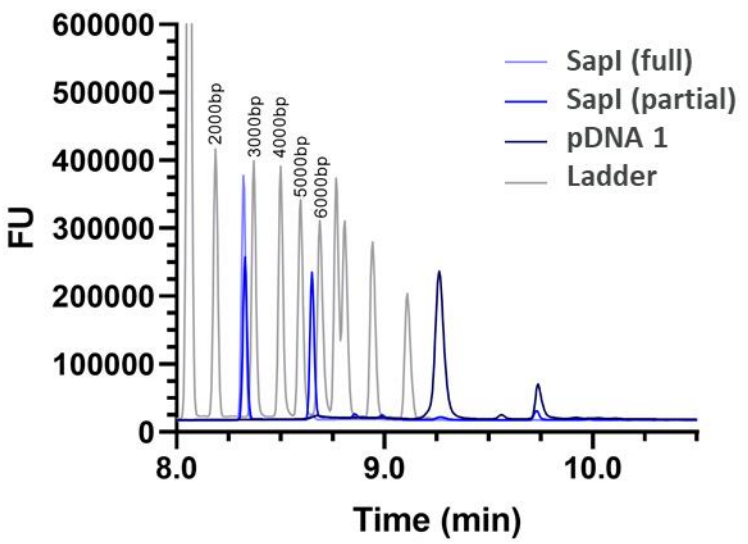
pDNA 1, mAb LC, ~3200 bp

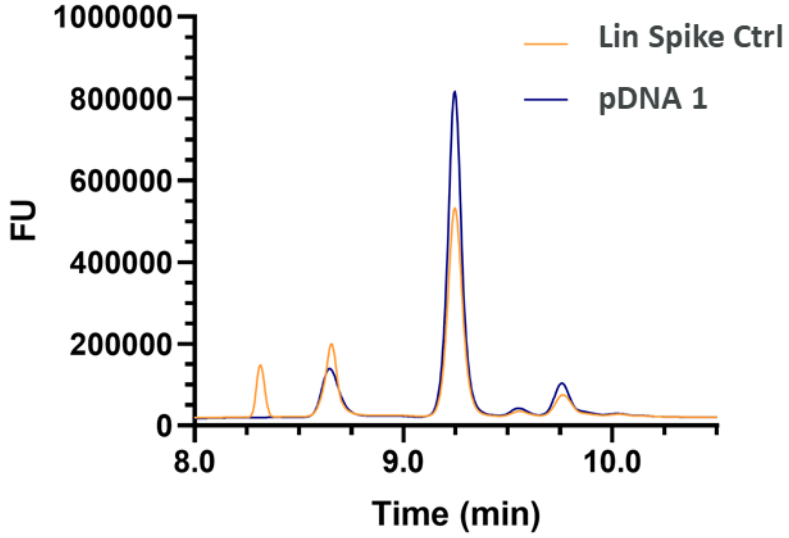


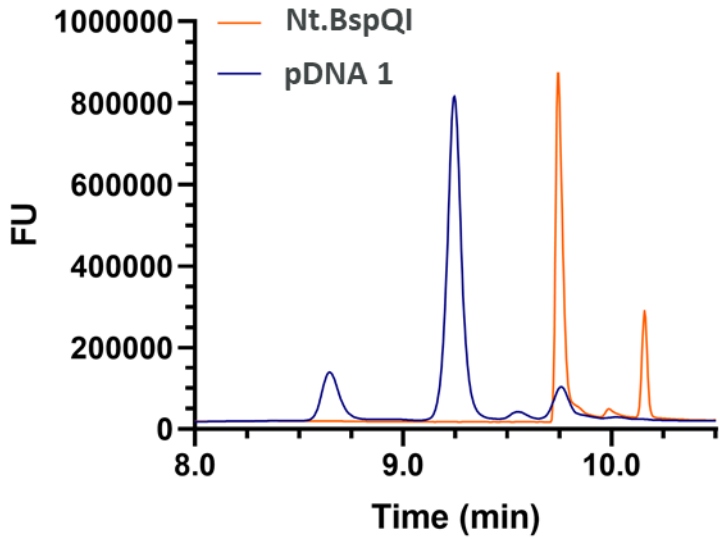
pDNA 2, mAb HC, ~4000 bp



pDNA 1 – Isoform Comigration





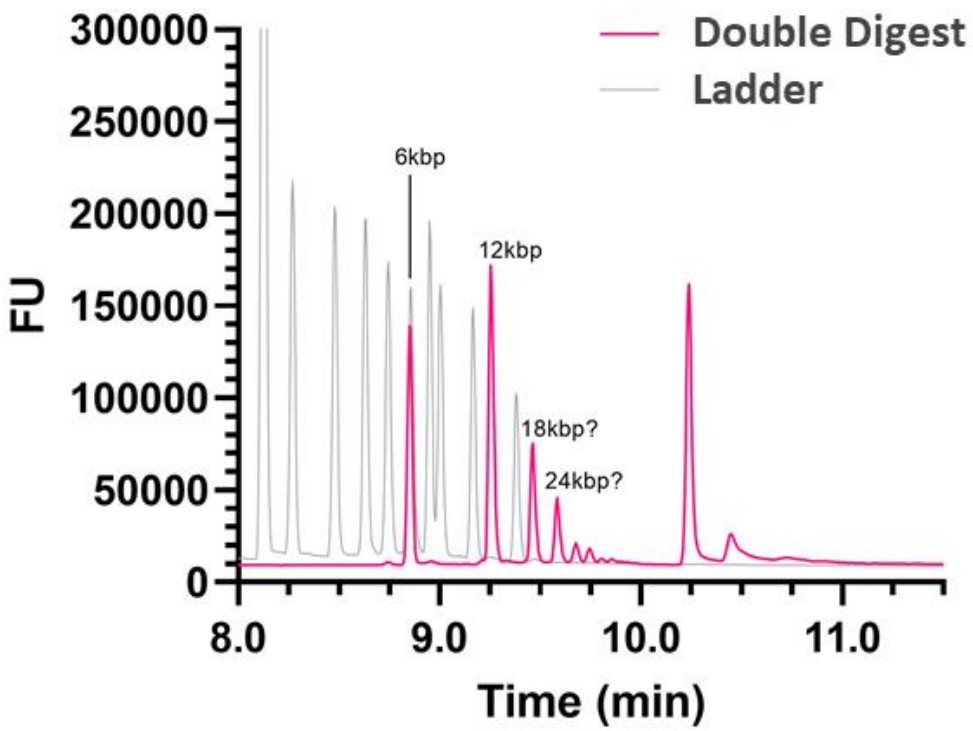


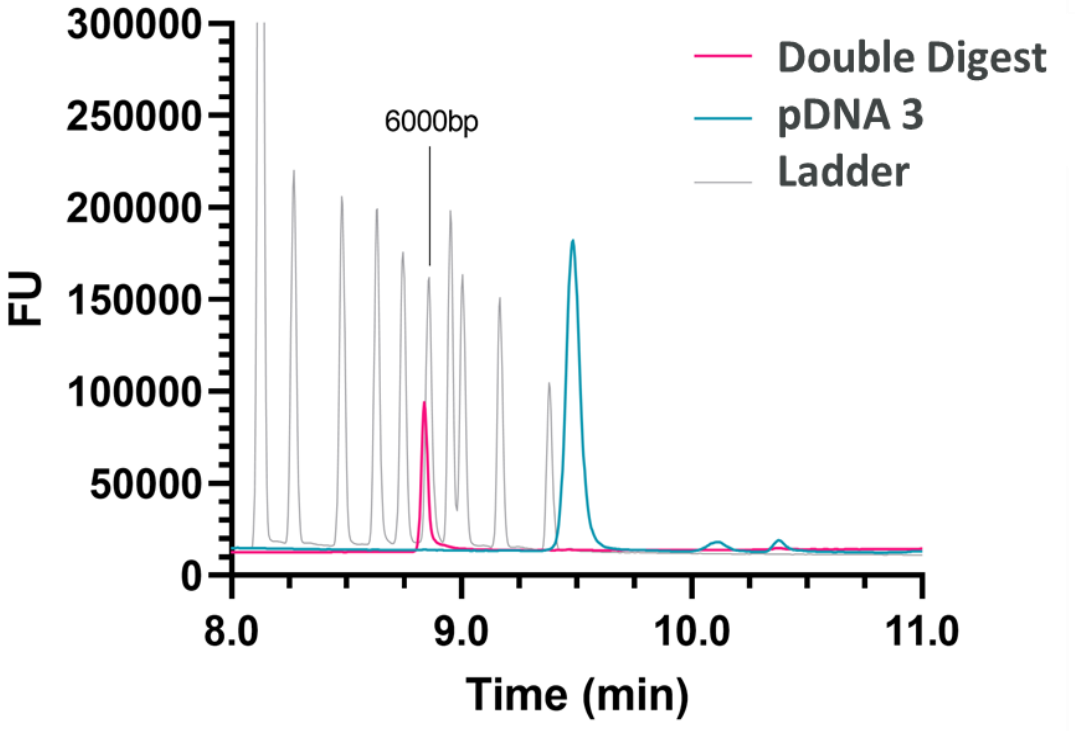
- Digest with restriction endonucleases aids peak ID
- Time restricted digests indicates presence of concatemers
- Concatemers not problematic for IVT

- New peak observed in additional lot of pDNA 1 (~15%)
- Spiking identifies peak as a concatenated linear isoform
- Uncontrolled linear isoforms considered unsuitable for IVT

- Nicking endonucleases results in new peak shift
- New peak reassigned as a supercoiled monomer
- Isoform co-migration problematic

pDNA 3 – Complex Peak ID

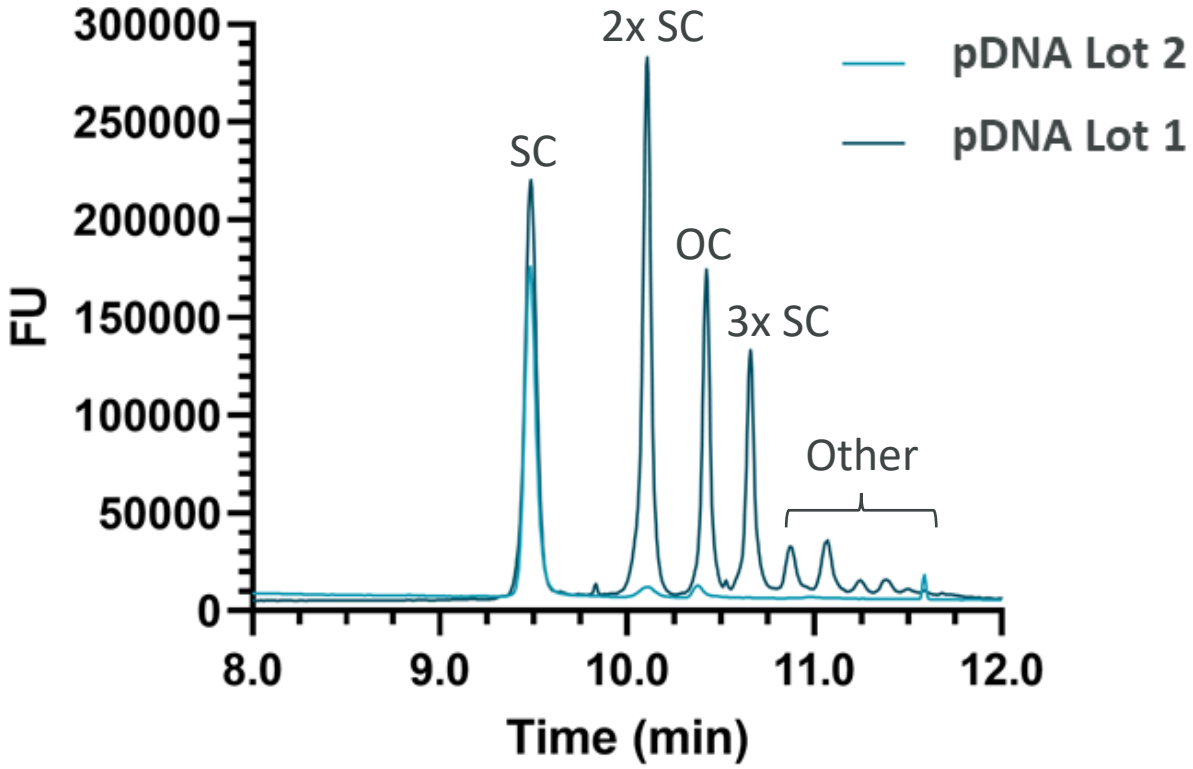




- Heterogeneous profile for pDNA 3
- Known co-migration of isoforms from experience
- Double digest aids peak ID (Nt.BbvCI [full] + SfiI [partial])
- Comparison vs ladder suggests concatemers of 2x, 3x, 4x …

- Different lots help considerably with peak ID
- Digest with Nt.BspQI identifies a clear open circular peak
- Double digest demonstrated main isoform is monomeric

pDNA 3 – Complex Peak ID



- Peak ID in heterogeneous batches is complex
- Clarity achieved though
  - Restriction enzyme digest
  - Double digests
  - Comparison of multiple lots

Plasmid DNA Strategy

- **Supercoiled is target pDNA isoform**
  - pDNA fully linearised prior to IVT for mRNA DS
    - Supercoiled concatemers also suitable
    - Uncontrolled linearised isoforms indistinguishable
  - Risk of premature termination of IVT reaction from open circular and uncontrolled linear isoforms
- **All critical lots require characterisation**
  - Restriction endonucleases aid peak identification
  - Comigration of different isoforms complicates accurate peak identification
  - Spike controls of digested material has limited value
  - Recommend characterisation of all critical lots
  - Recommend characterisation of any new peaks
- **pDNA released as a High Quality reagent for GMP**
  - CE method for % isoform only
  - Unsuitable for size determination or ID
  - Not a component of the Drug Substance
  - Control required to mitigate risk to mRNA Drug Substance yield & quality
  - Mini IVT prior to critical lots to check linearisation and expected mRNA quality
- **Ongoing work**
  - Formulation & stability studies impact on pDNA quality
  - Impact assessment of stressed pDNA material on mRNA quality
  - Impact assessment of different pDNA isoforms on mRNA quality

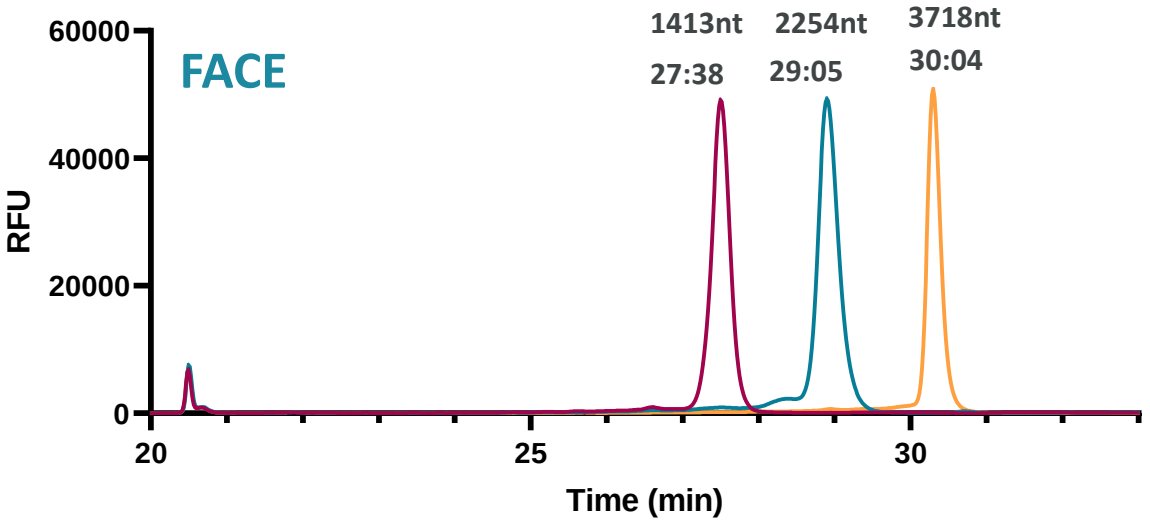
3

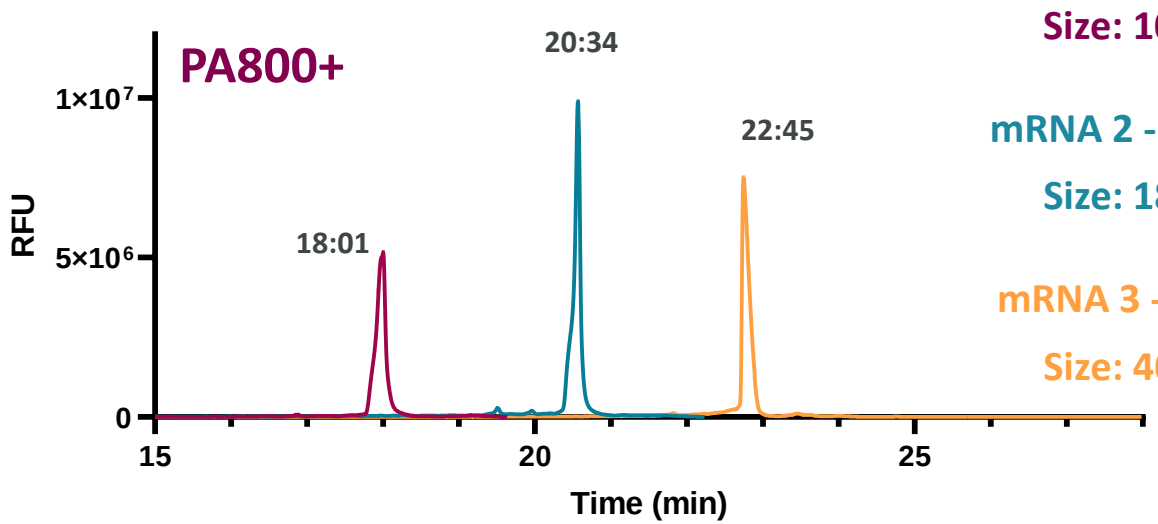
CE for Messenger RNA

FACE vs PA800+ CE Method Details

	FACE	PA800+
Components	High Resolution Fragment Analyser Kit for mRNA	Sciex eCAP dsDNA 1000 Kit
Sample concentration	↓	↑
Sample dilution buffer	TBE	Water
Injection	Voltage Injection	Pressure Injection
Capillary length (effective)	Equivalent	
Separation Voltage	Equivalent	
Run time	↑	↓
Temperature	↓	↑
Throughput	48 samples/1h	1 sample < 1hr

Comparison of mRNA Migration Times





mRNA 1 - mAb LC

Size: 1065nt

mRNA 2 - mAb HC

Size: 1809nt

mRNA 3 - protein

Size: 4000nt

ΔMT mRNA 1 to mRNA 2: + 1min 27s

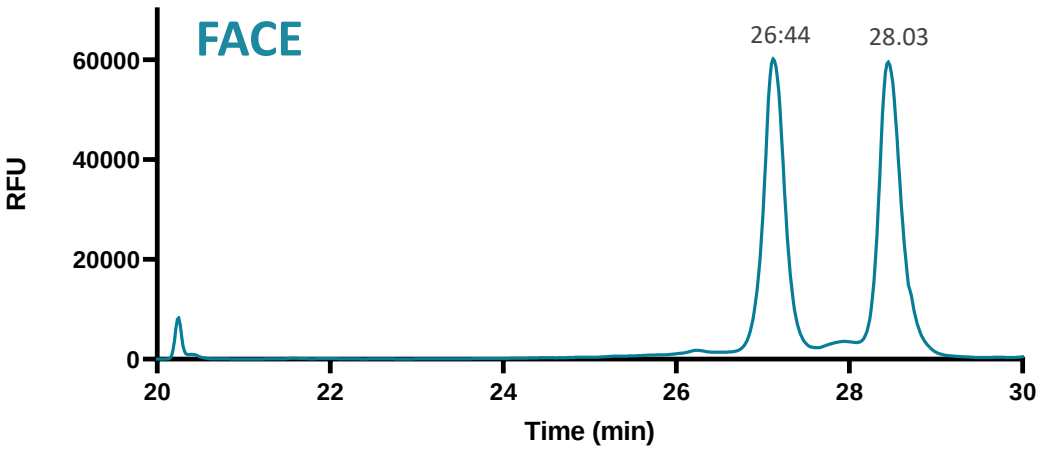
ΔMT mRNA 2 to mRNA 3: + 59s

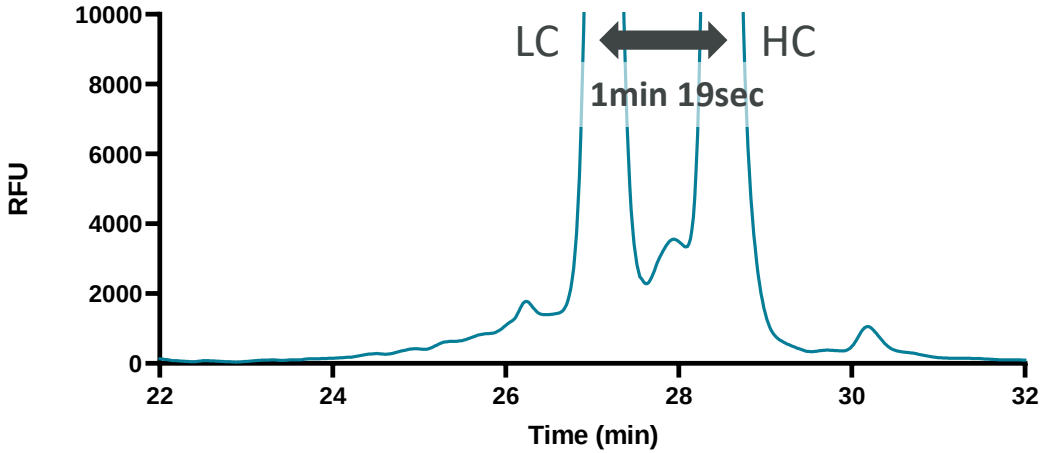
ΔMT mRNA 1 to mRNA 2: + 2min 33s

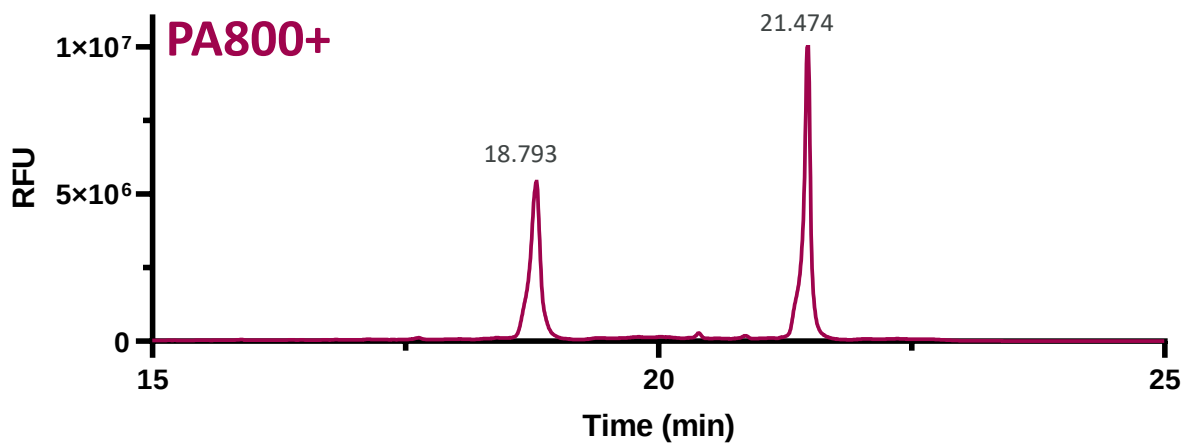
ΔMT mRNA 2 to mRNA 3: + 2min 11s

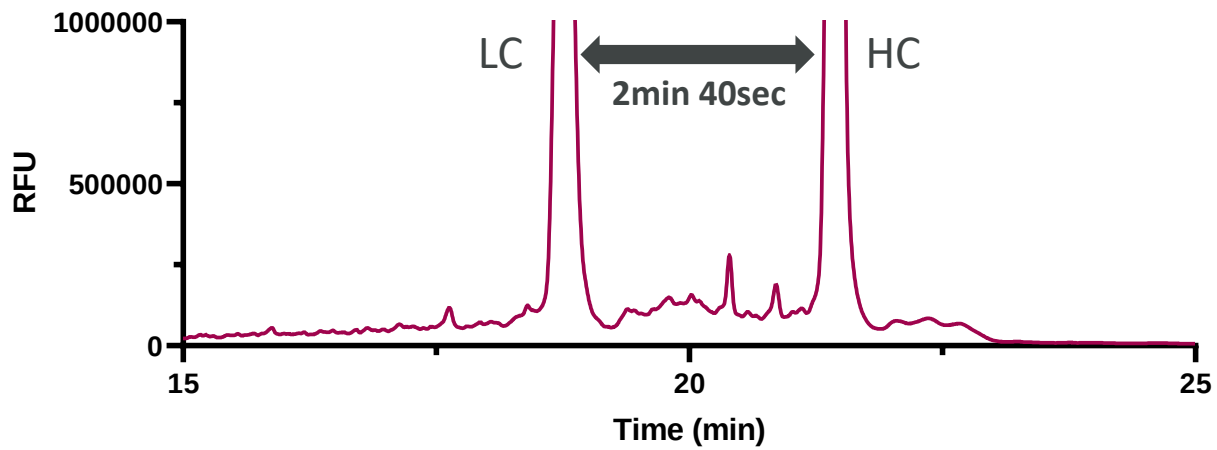
The CE method on the PA800+ provides better resolution. Both methods lose resolution with the increasing size of the mRNA molecules. Sizing not accurate but consistent from run-to-run.

Comparison of Profiles for LC and HC Mix





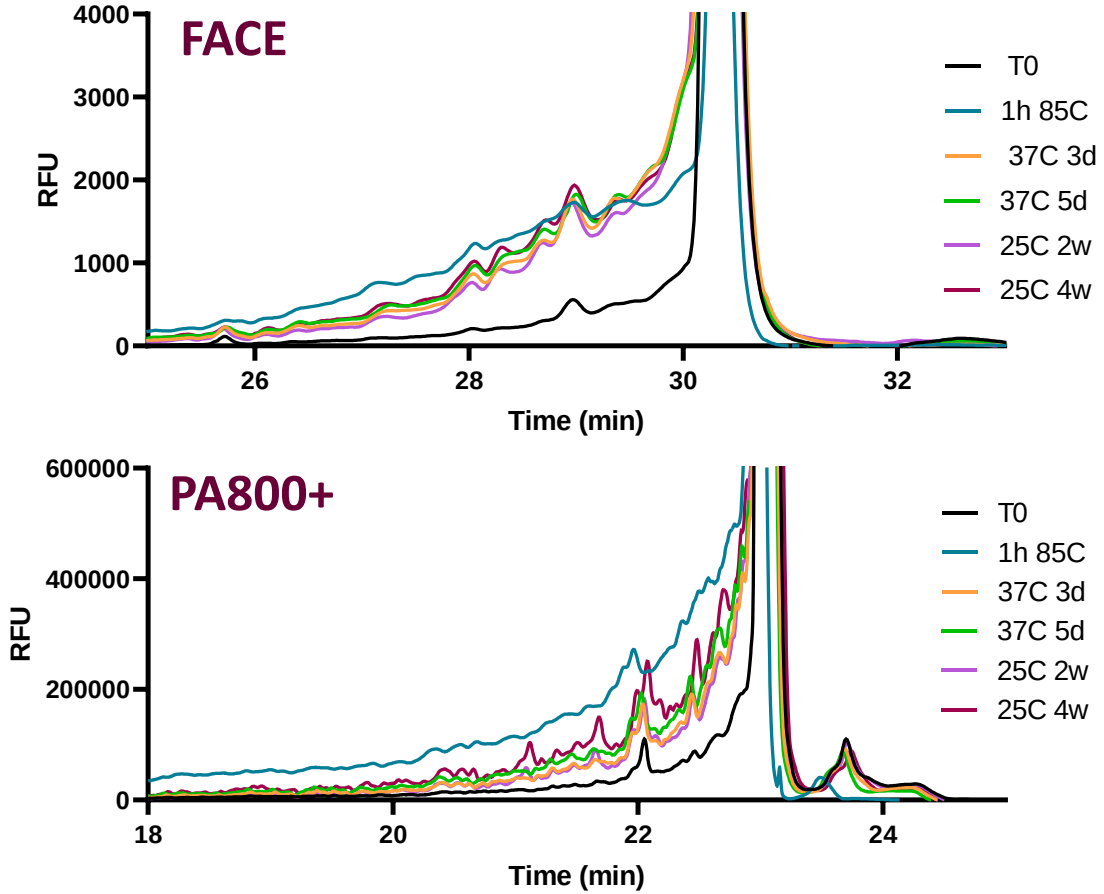




The improved resolution of the PA800+ method is even more clear when both mRNAs for LC and HC are mixed together for analysis (as they would be in the Drug Product)

Forced Degradation Assessment - % Main Peak

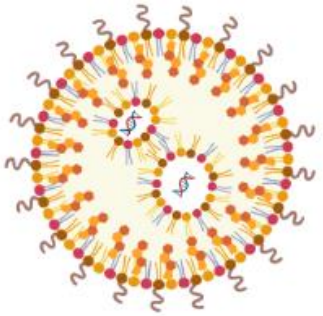
mRNA 3



	%MP FACE	%MP PA800+
mRNA 3 FD T0 2ng/uL	90.2	81.7
mRNA 3 FD 1h 85C 2ng/uL	32.5	26.0
mRNA 3 FD 37C 3d 2ng/uL	75.4	66.2
mRNA 3 FD 37C 5d 2ng/uL	67.2	58.2
mRNA 3 FD 25C 2w 2ng/uL	77.9	68.8
mRNA 3 FD 25C 4w 2ng/uL	62.4	53.4

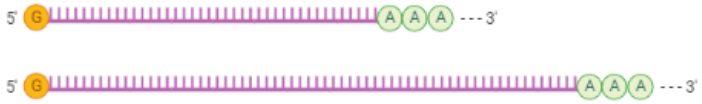
Both methods seem to be **stability indicating**.
Due to the higher resolution, the % of main peak is always lower for the PA800+ method.

Drug Product Analysis - mRNA after LNP Disruption

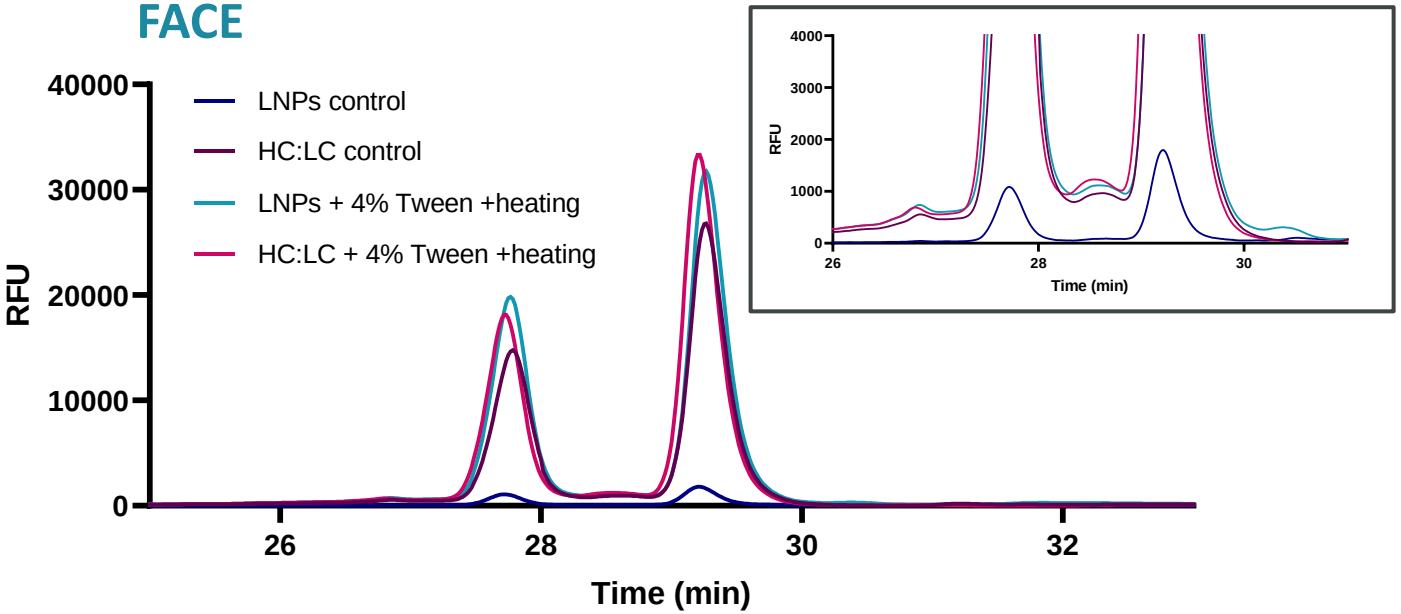


mRNA extraction from LNPs

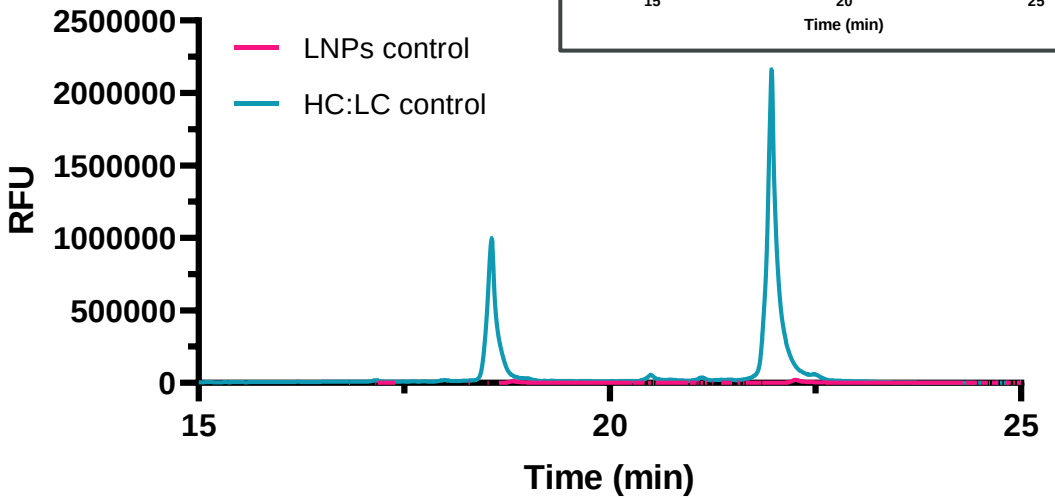
- **Detergent based methods**
- **Ethanol precipitation**

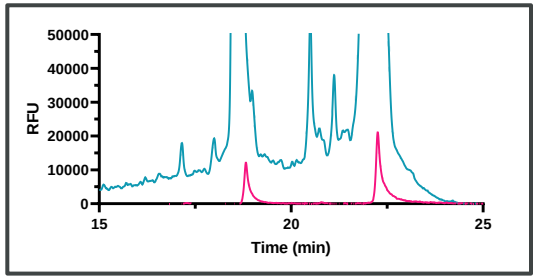


FACE



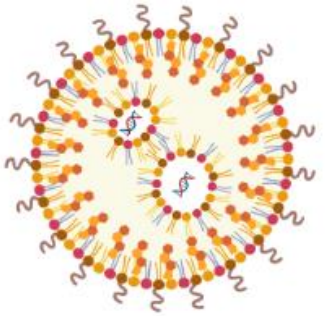
PA800+





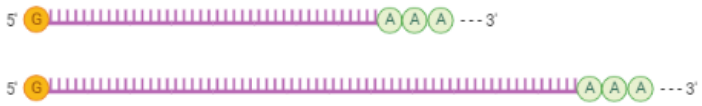
Disruption with detergents is an effective method to dissociate LNPs and recover the encapsulated RNA for analysis.

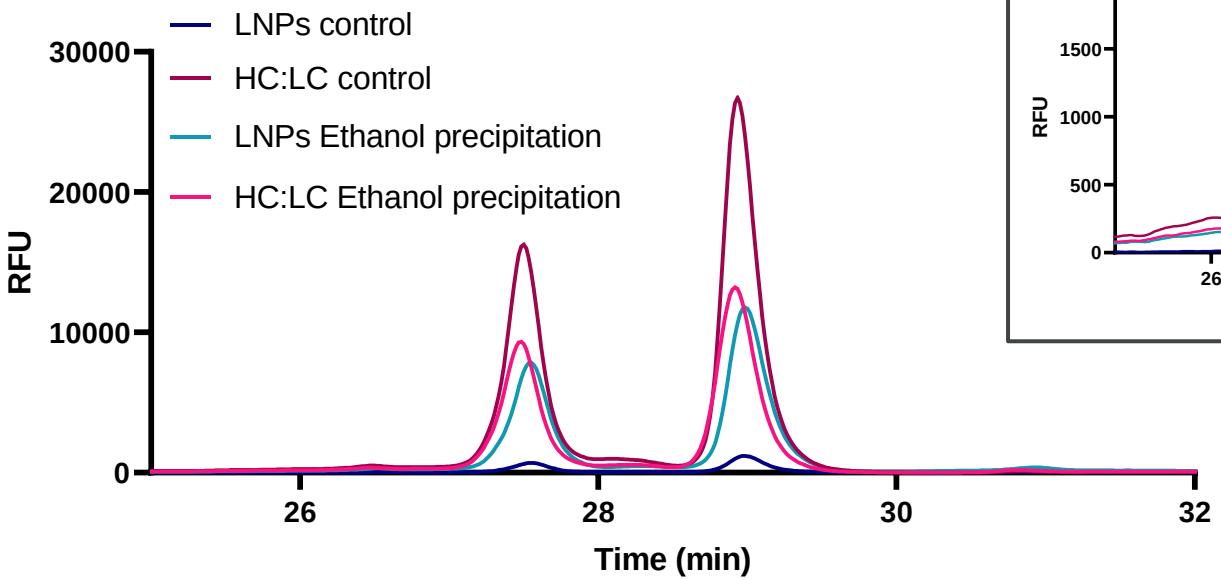
Drug Product analysis - mRNA analysis after LNP disruption

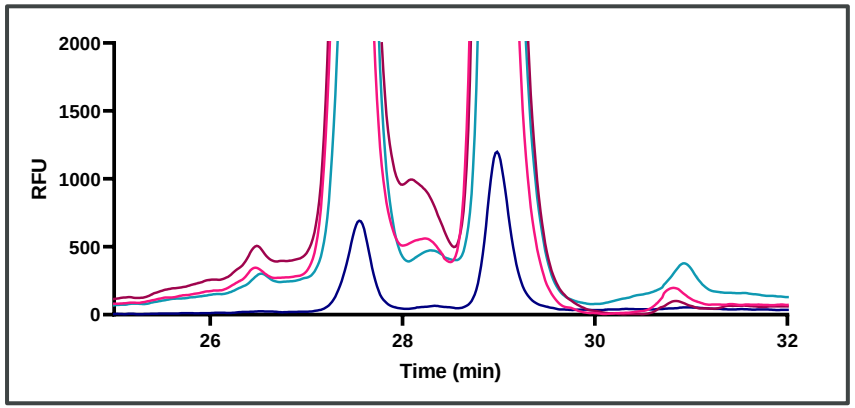


mRNA extraction from LNPs

- **Detergent based methods**
- **Ethanol precipitation**







Ethanol precipitation also provides a suitable means to recover encapsulated mRNA.

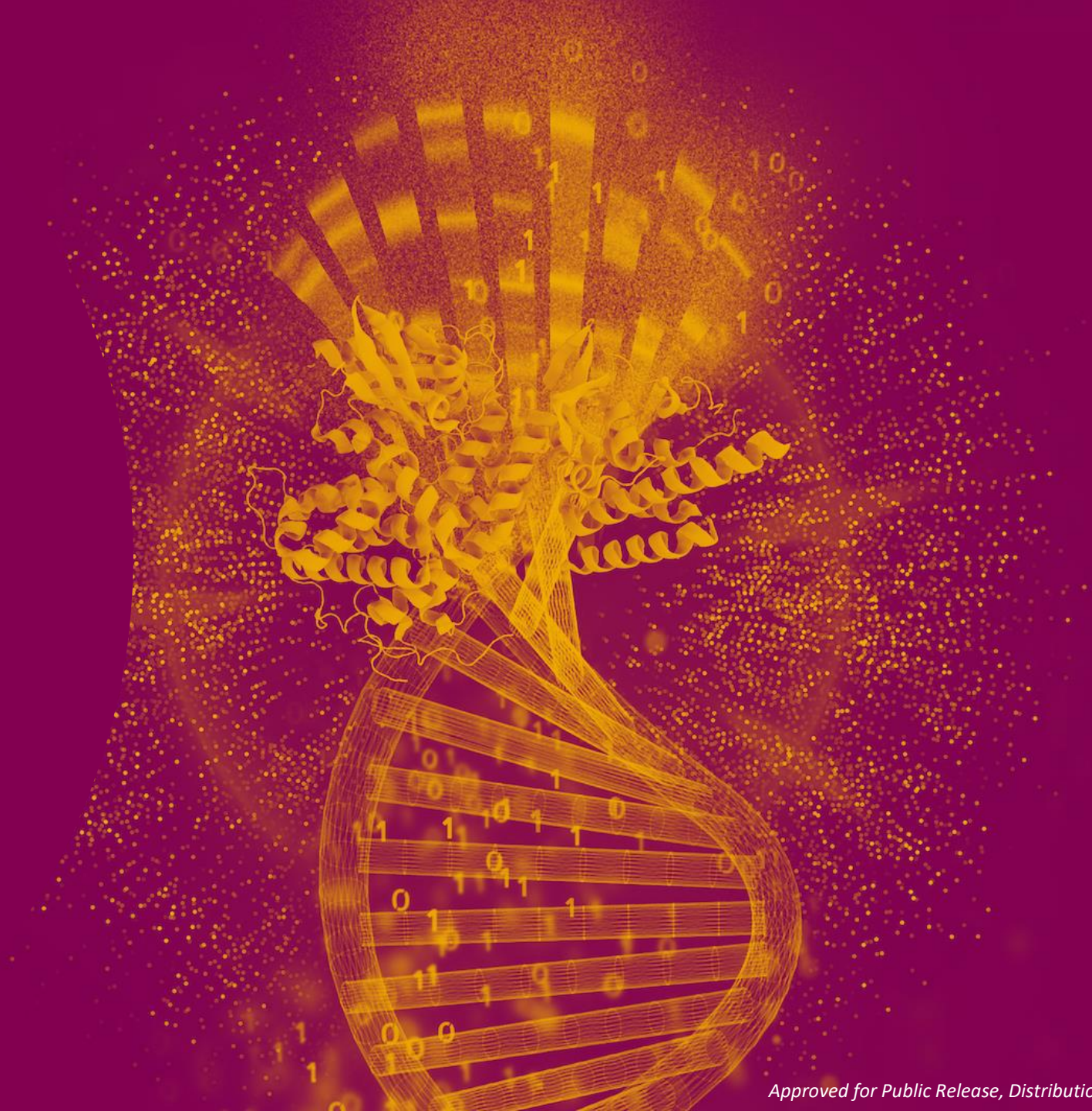
Both methods are suitable to use in sample preparation for CE, however avoiding detergents could be an advantage when preparing mRNA to use in other types of assays (e.g. HPLC/MS)

mRNA CE Method Summary

- **FACE CE Method Summary**
  - Suitable for % purity
  - Not suitable for size determination or ID
  - Lower sample conc, electrokinetic injection
  - Stability indicating
  - Comparability between free DS and DP released mRNA
  - Higher throughput
  - Data processing with ProSize
  - Recommended for HT process development support
- **PA800+ CE Method Summary**
  - Suitable for % purity
  - Not suitable for size determination or ID
  - High sample conc, pressure injection
  - Stability indicating
  - Comparability between free DS and DP released mRNA
  - Greater resolution
  - Data processing with Empower
  - More suitable for lot release / product characterisation
- **Ongoing work**
  - Further optimise mRNA recovery from LNPs
  - Method evaluation/optimisation for accurate mRNA LC / HC molar ratio determination

4

Summary & Control Strategy



pDNA

- **Plasmid DNA analysis using Sciex eCAP dsDNA 1000 kit**
  - Appropriate for pDNA isoform distribution
  - Not suitable for size analysis
  - Not suitable for ID
- **Release strategy dependant upon use**
  - GMP release not required if not component of DS/DP
  - Control mitigates business risk
  - Consider appropriate inclusion / exclusion of concatemers and certain isoforms
- **Thorough characterisation recommended**
  - Digests with restriction endonucleases
  - New / critical lots
  - Lots displaying new peaks

mRNA

- **Release strategy**
  - Full mRNA molecule required for biological activity – fragments result in incomplete or no translation
  - Method for stability monitoring important as hydrolysis is the most relevant degradation route
- **Evaluation of mRNA CE using the FACE and PA800+**
- Both methods suitable for:
  - % purity / fragments
  - mRNA from DS and LNP DP
- Both methods give only approximate size determination
- CE method could be used as identity method together with other assays / differentiate between molecules produced in the same facility

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