mRNA Integrity by Agilent 5300 Fragment Analyzer

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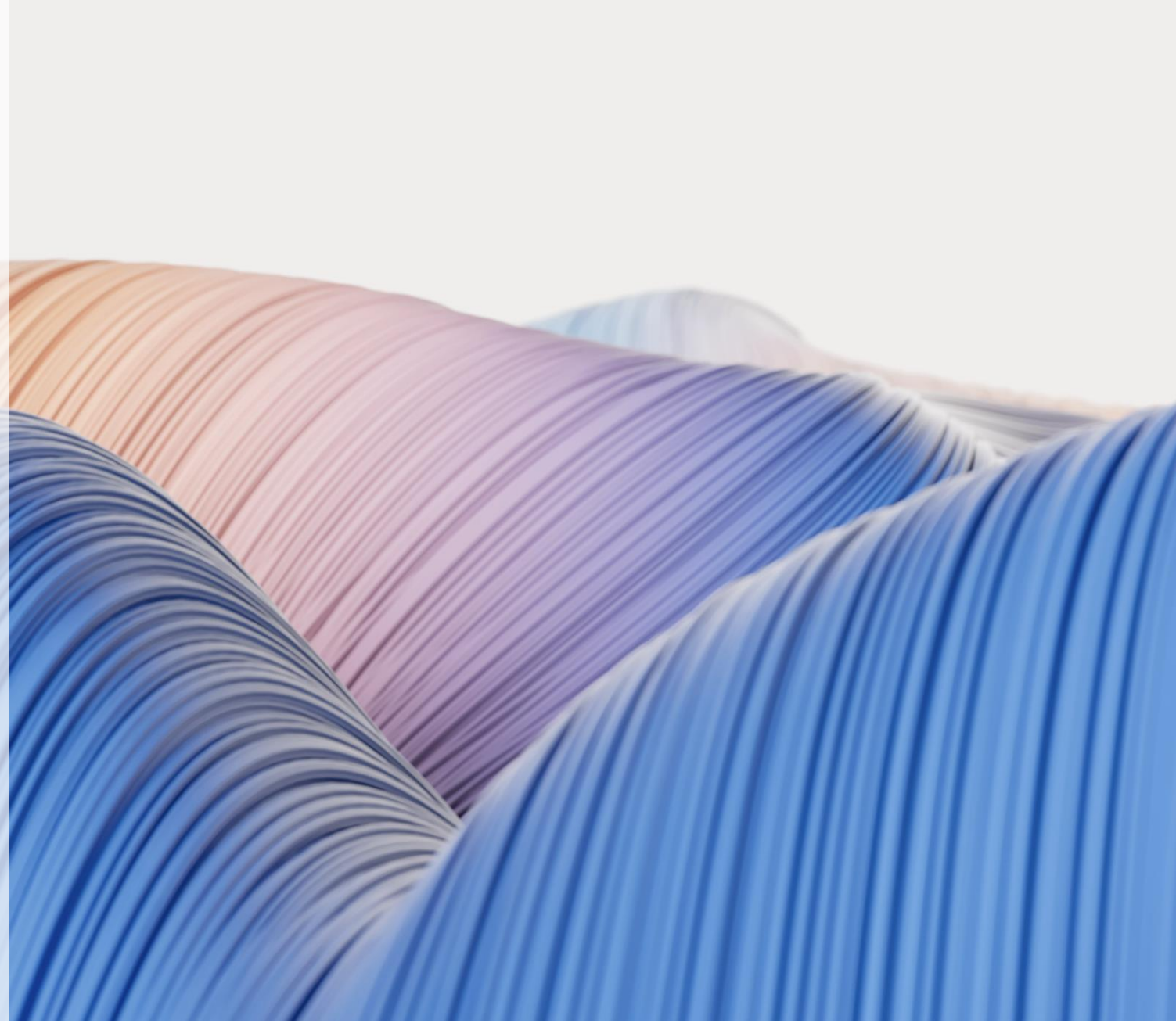
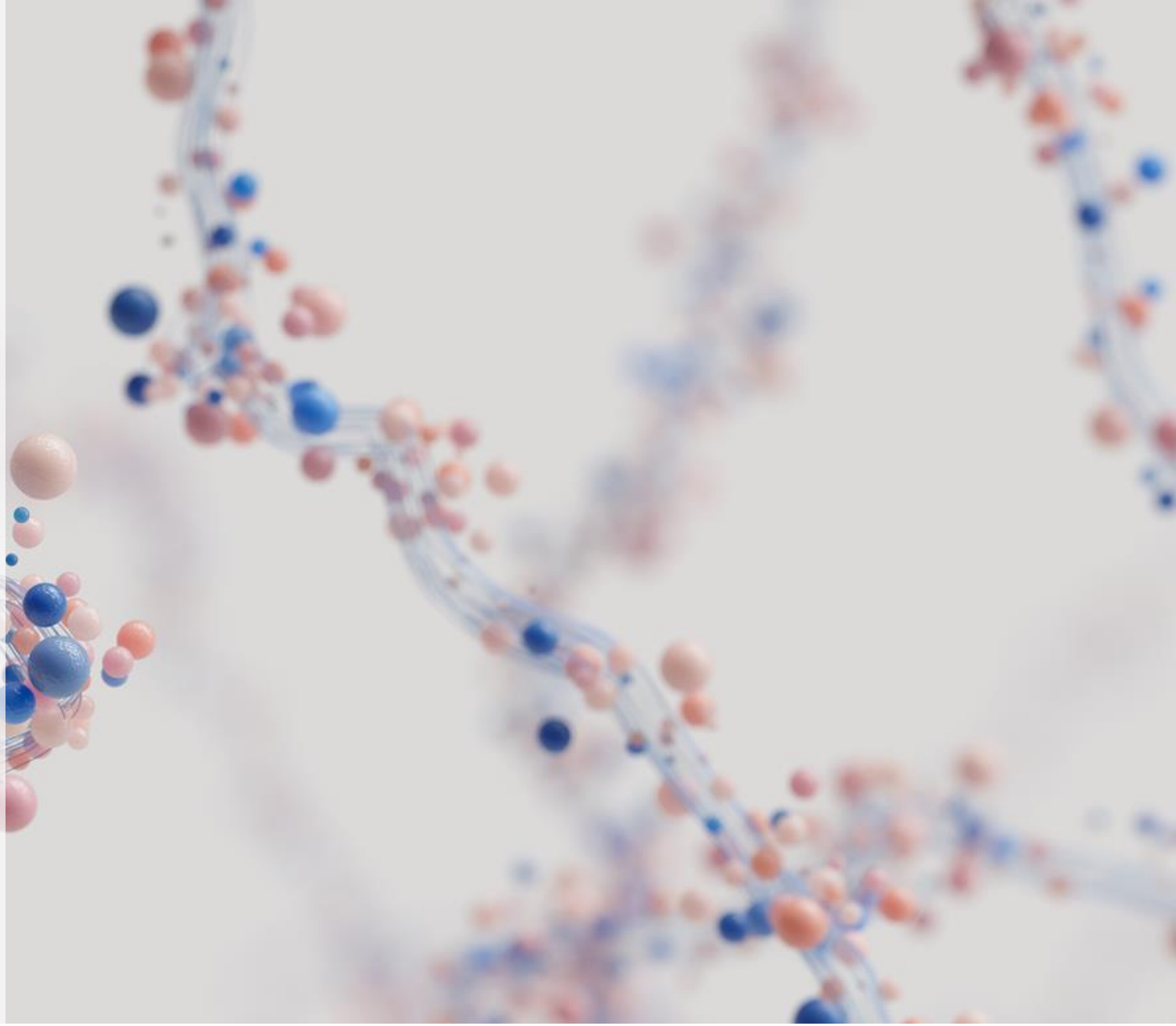


Outline

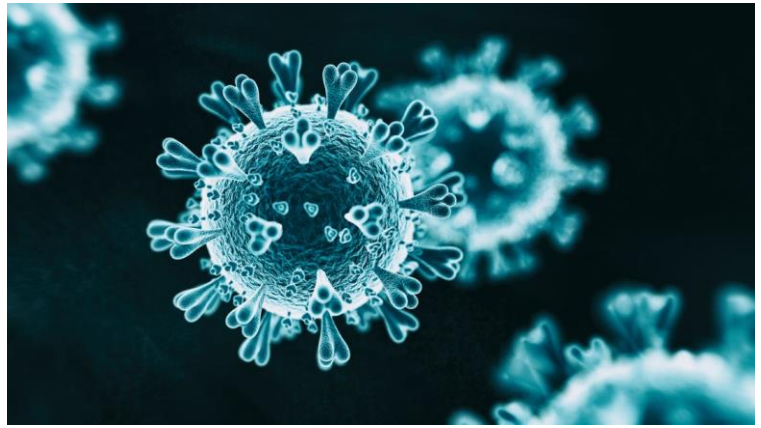
- mRNA Vaccines
- Agilent 5300 Fragment Analyzer Overview
- Case Studies
- Summary and Conclusions

mRNA Vaccines

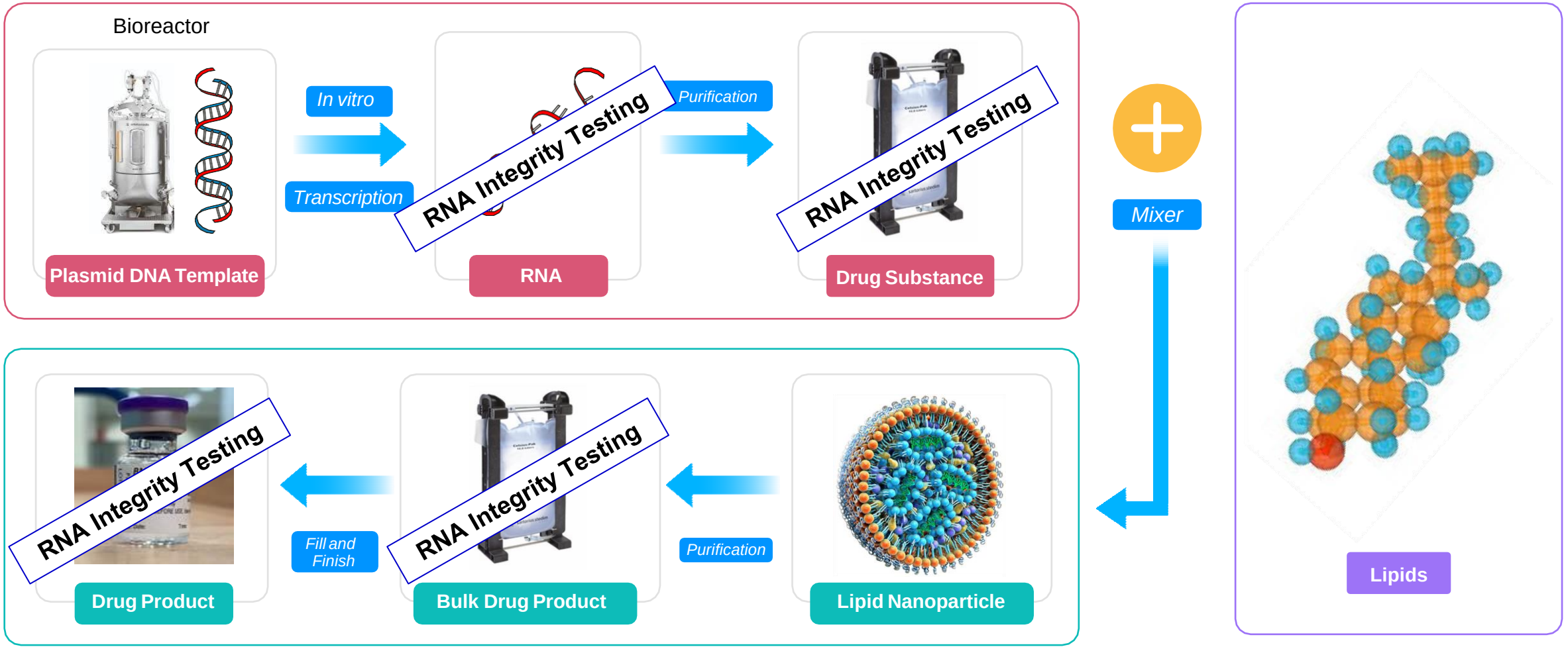


mRNA Vaccines and RNA Integrity

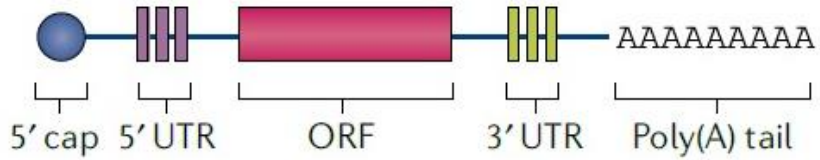
- Pfizer has several mRNA vaccine products with a wide variety of sizes and disease targets
- RNA integrity is a critical quality attribute
  - It directly impacts efficacy, product period of use, storage conditions, and shipping tolerances
- Pfizer's release purity method is capillary gel electrophoresis (CGE) using the Agilent 5300 Fragment Analyzer



Manufacture and Testing of mRNA



mRNA Drug Substance Quality Control Strategy



5' – Cap attributes by LC/MS & LC/UV

Poly(A) Tail attributes by PCR and LC/UV-MS

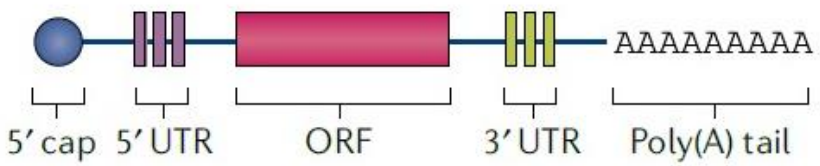
Platform QC Assays

- Compendial methods
- Purity by Capillary Gel Electrophoresis
- Concentration by UV spectroscopy
- Identity, Impurities by PCR-based methods
- Purity by Immunoblot

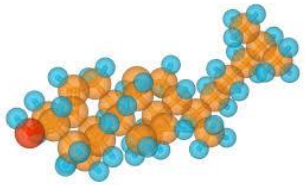
Characterization Assays

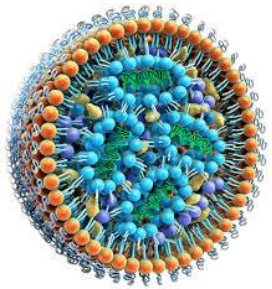
- NextGen Sequencing (NGS)
- Nucleoside/tide and Oligonucleotide mapping LC-MS/MS
- Higher Order Structure by Circular Dichroism (CD)
- Protein Expression Western Analysis

mRNA Drug Product Quality Control Strategy



Four Functional & Structural Lipids





Lipid Nanoparticle

Lipid ID and Content by LC-CAD
LNP Size and Polydispersity by DLS
In Vitro Expression by Cell-based FACS

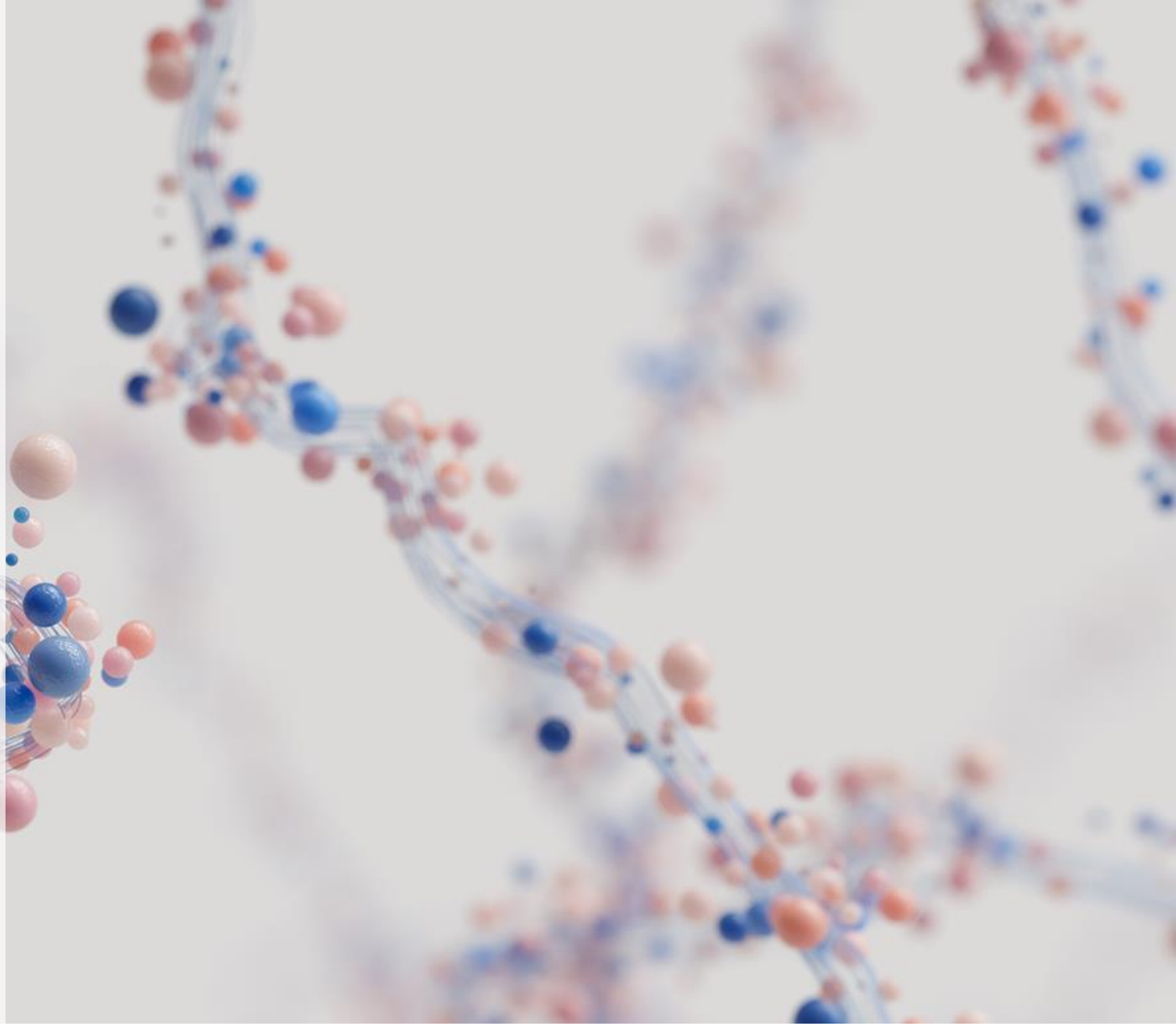
Platform QC Assays

- Compendial & Safety methods
- Purity by Capillary Gel Electrophoresis
- Content, RNA Encapsulation by Fluorescence Assay
- Identity by PCR-based method

Characterization Assays

- Lipid ID and content by LC-MS
- LNP surface properties by high-field NMR
- LNP surface charge by Zeta potential
- Orthogonal size measurements

Agilent 5300 Fragment Analyzer Overview



Fragment Analyzer Principles

- Multiplexed capillary electrophoresis system
  - 12, 48, or 96 capillary array
- Multiplexed - All samples are run simultaneously
- Analyze RNA
  - Kits are available to purchase
- Assay output
  - Relative purity of intact RNA vs. fragmented RNA
  - Approximate size (nucleotides)
- Separation is achieved by applying an electric field through the capillary array filled with gel, separating based on size
  - Smaller species migrating before larger species
- Detection of the separated RNA is achieved by fluorescence
- Run time is ~1.5 hours, FAST!

Sample Preparation

- Highly recommend preparing samples in a PCR hood or BSC with RNase free handling practices [Minimize lab induced fragmentation]
- Drug substance
  - Dilute to nominal concentration
- Drug product
  - Dilute to nominal concentration
  - Disrupt with detergent and alcohol solution
- In a 96 well plate, combine diluent marker (Agilent kit component that contains formamide to aid in denaturation) and sample
- Preform heat incubation for denaturation
- Sample preparation time is ~1.5 hours (varies based on # of samples)
- See Agilent kit literature for more details!

Drug Product Disruption

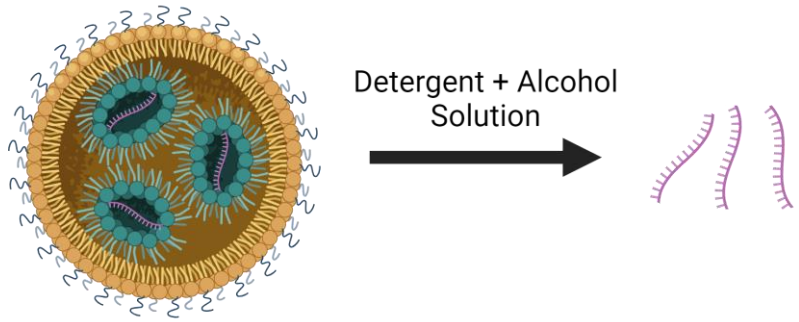
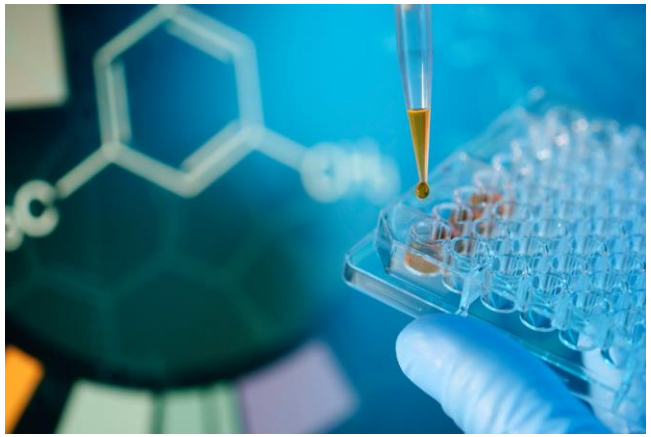


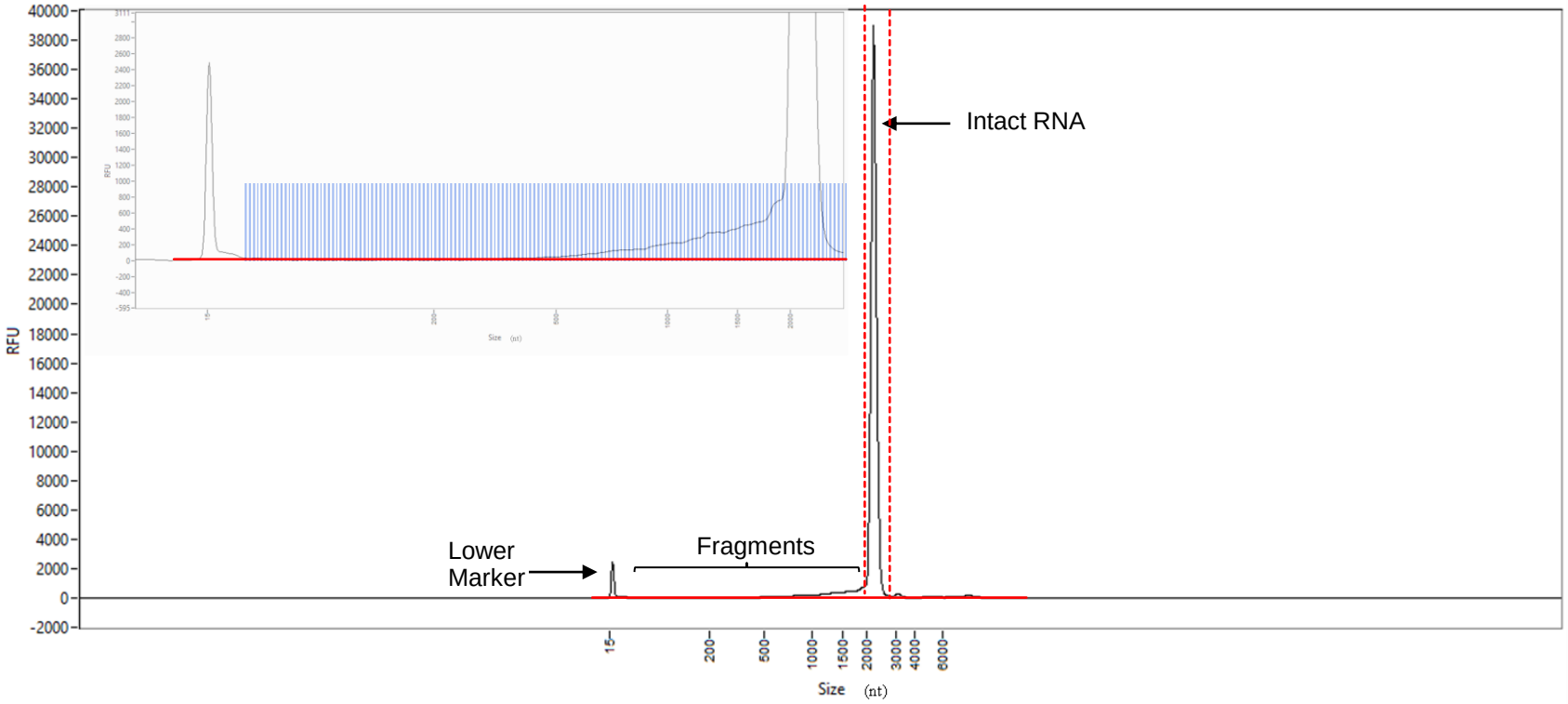
Figure generated using BioRender



Time Corrected Area (TCA) – A Brief Side Note

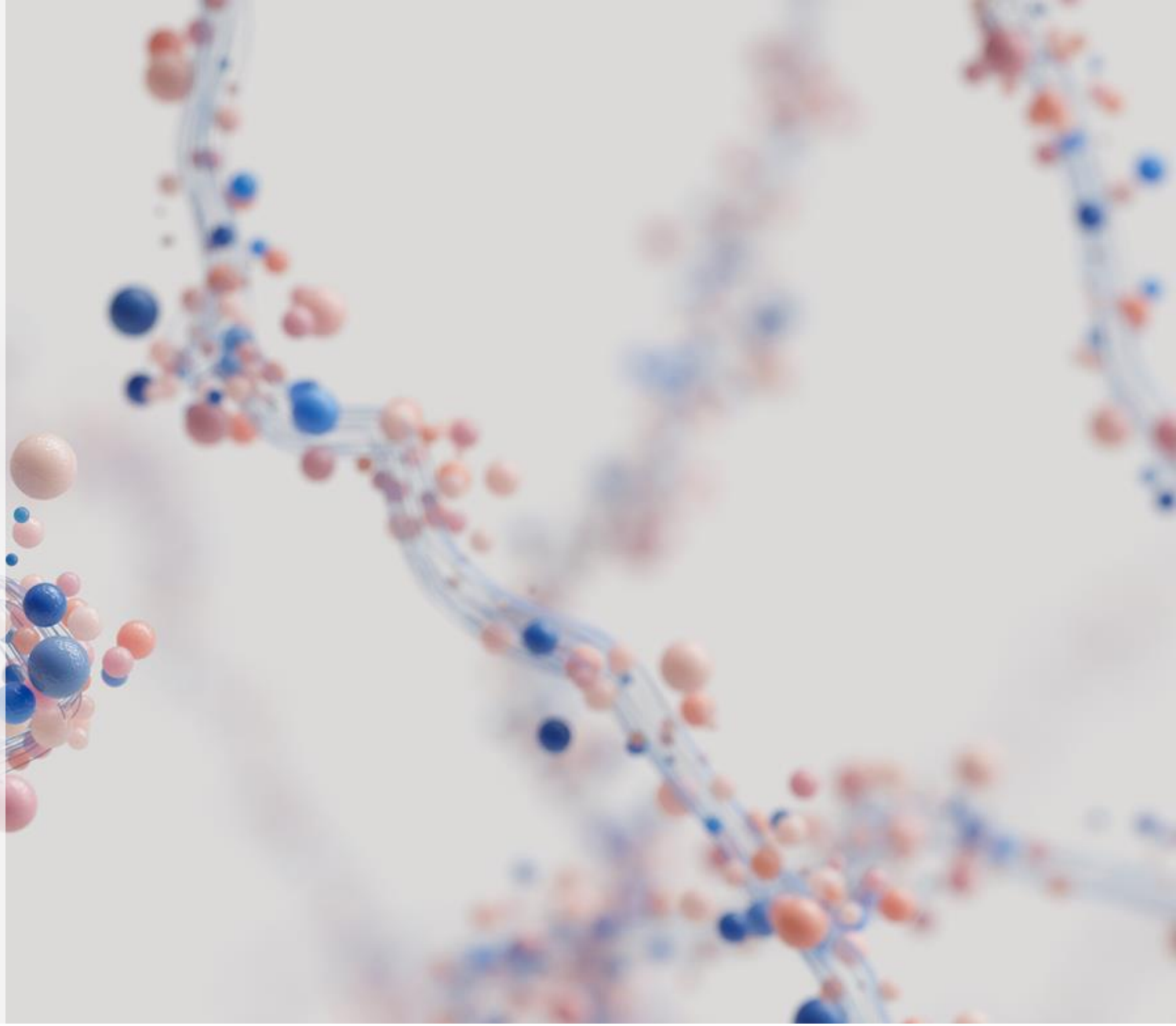
- CGE separation is based on size
- Smaller species migrate faster than larger species (migration velocity)
- Smaller species spend less time in front of the detector compared to larger species
- TCA accounts for the difference in migration velocity
- $TCA = \frac{Area}{Migration\ Time}$

Integration by Smear Analysis



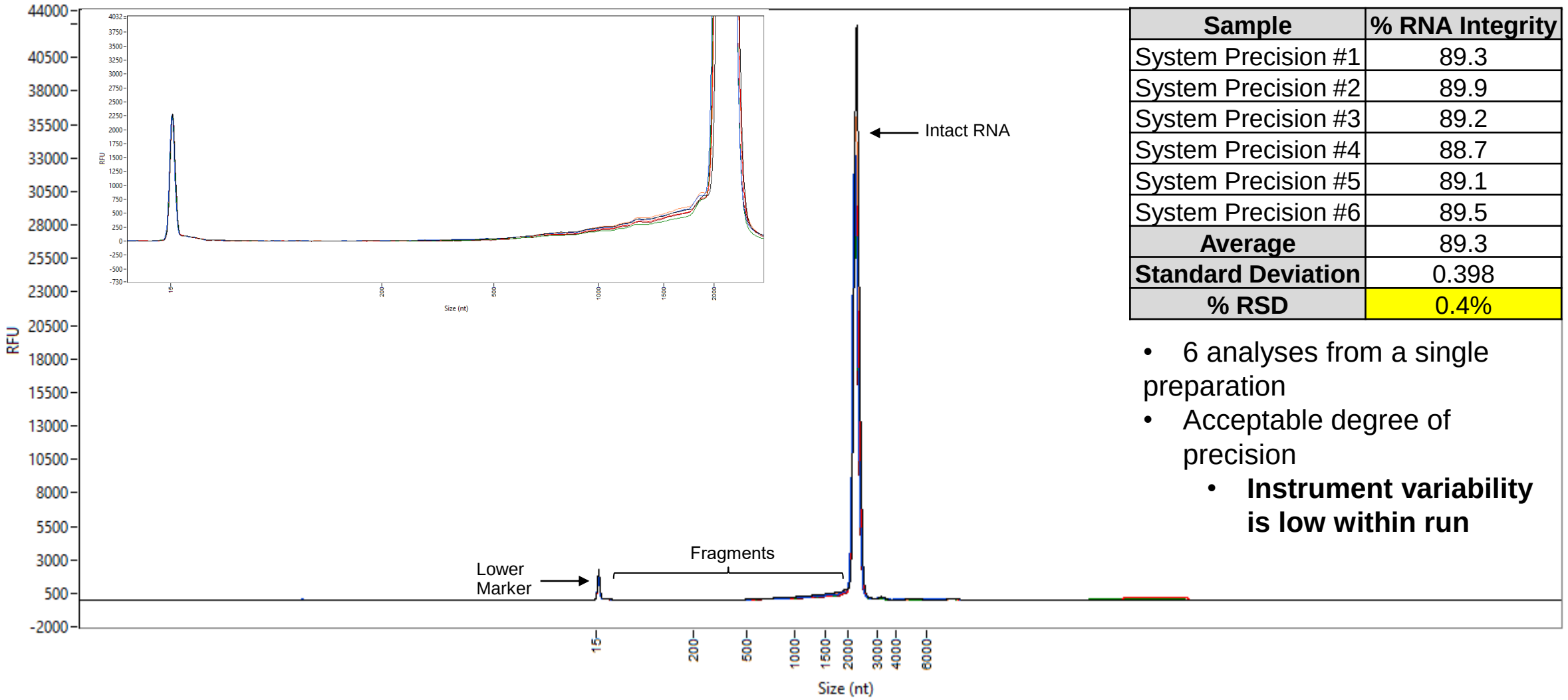
- The Agilent software ProSize is used to integrate results by smear analysis
- Smear ranges are set for a peak or region (i.e. intact RNA peak)
- % Species are calculated by total TCA above baseline, **instead of only integrated peaks**
- Area above baseline is auto sliced by the software (blue lines added for visualization, actual quantity of slices is near infinite)
- For each slice TCA is calculated in the background from smear algorithms using the time stamp of the slice, instead of using apex for entire region
- This is an efficient and appropriate way of calculating TCA for this type of data

Pre-Validation Case Study; Precision and Linearity



System Precision – mRNA DS

A measure of the degree of repeatability of the instrument for multiple aliquots from a single sample

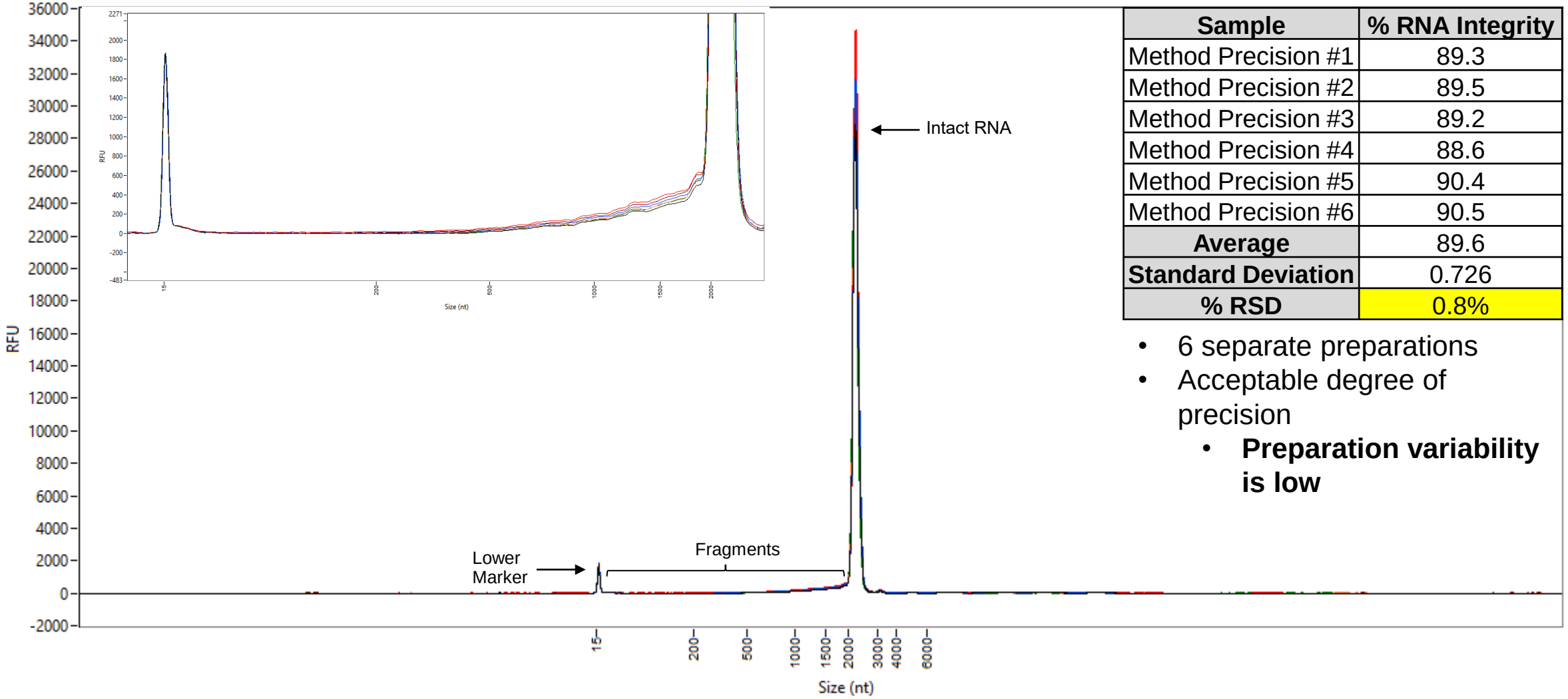


Sample	% RNA Integrity
System Precision #1	89.3
System Precision #2	89.9
System Precision #3	89.2
System Precision #4	88.7
System Precision #5	89.1
System Precision #6	89.5
Average	89.3
Standard Deviation	0.398
% RSD	0.4%

- 6 analyses from a single preparation
- Acceptable degree of precision
  - **Instrument variability is low within run**

Method Precision – mRNA DS

A measure of the degree of repeatability of the method for single aliquots from multiple sample preparations



Sample	% RNA Integrity
Method Precision #1	89.3
Method Precision #2	89.5
Method Precision #3	89.2
Method Precision #4	88.6
Method Precision #5	90.4
Method Precision #6	90.5
Average	89.6
Standard Deviation	0.726
% RSD	0.8%

- 6 separate preparations
- Acceptable degree of precision
  - **Preparation variability is low**

Intermediate Precision – mRNA DS

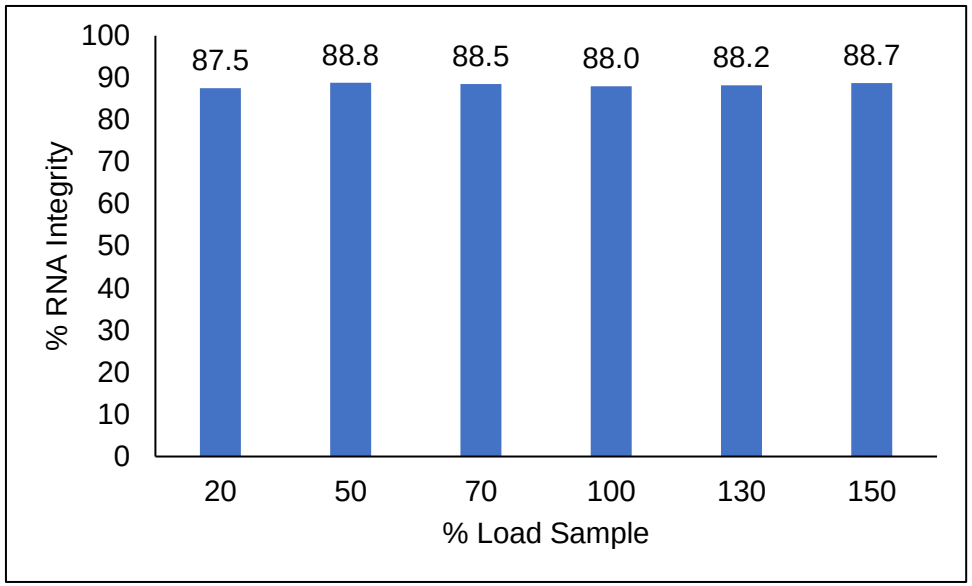
A measure of a variety of inter/intra-laboratory test conditions, multiple analysts/instruments/(may expand to labs)

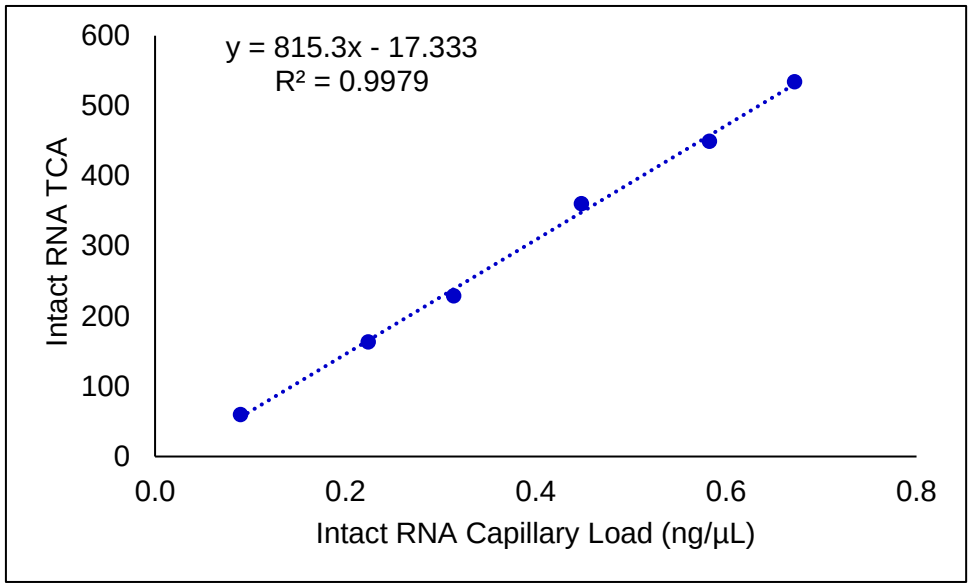
Lab	Instance	Analyst	Instrument	% RNA Integrity
1	1	1	1	89.3
	2	1	2	90.0
	3	1	3	89.1
	4	2	2	91.2
	5	2	3	89.3
	6	2	1	89.7
			Average	89.8
			Standard Deviation	0.774
			% RSD	0.9%

- More realistic view – evaluate variability impact of analysts/instruments/labs
- Acceptable degree of precision
  - **Analyst and instrument variability is low**

Linearity – mRNA DS

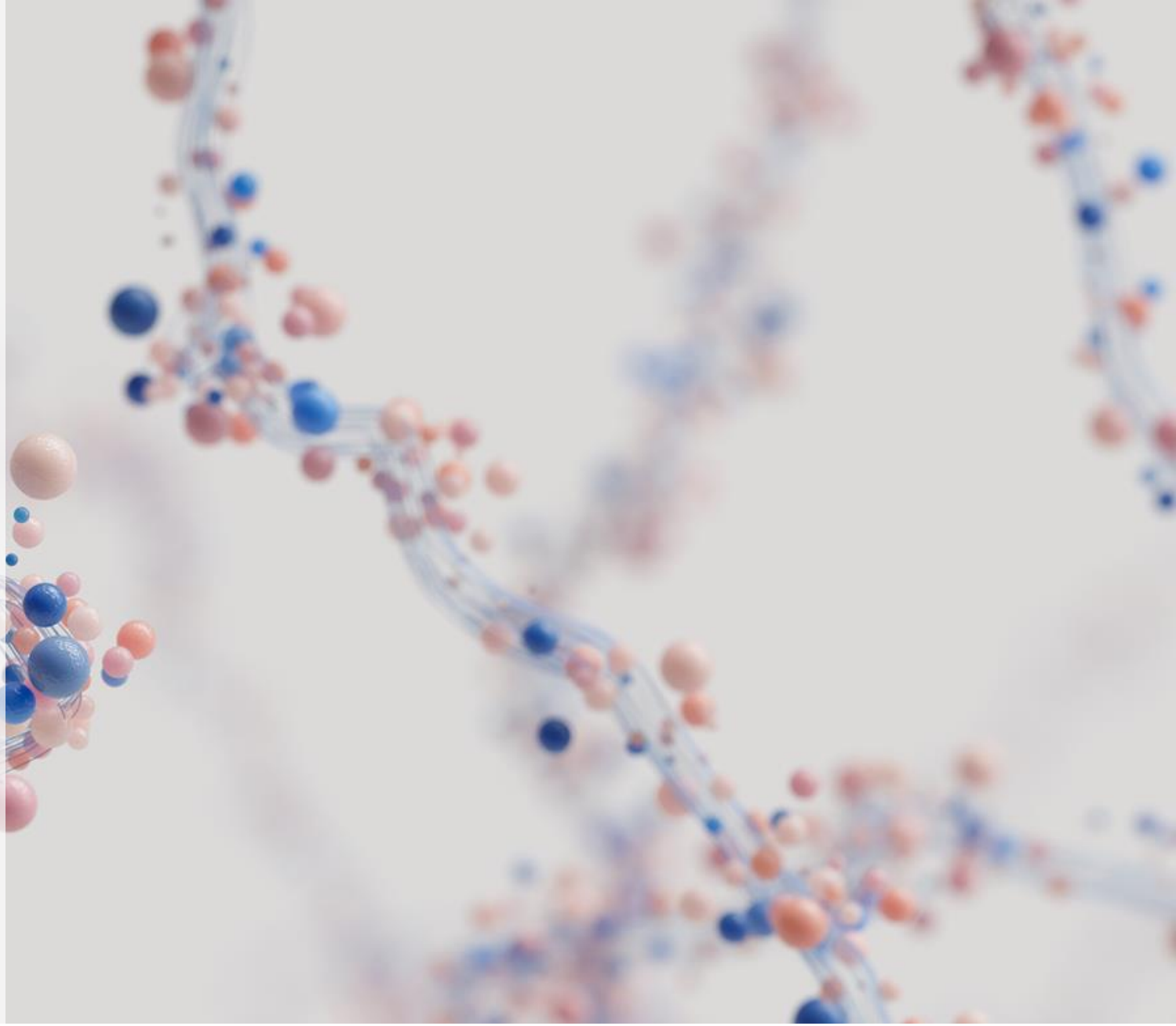
To determine the ability of the analytical method to elicit results that are directly proportional to the concentration of analyte in the sample over a given concentration range





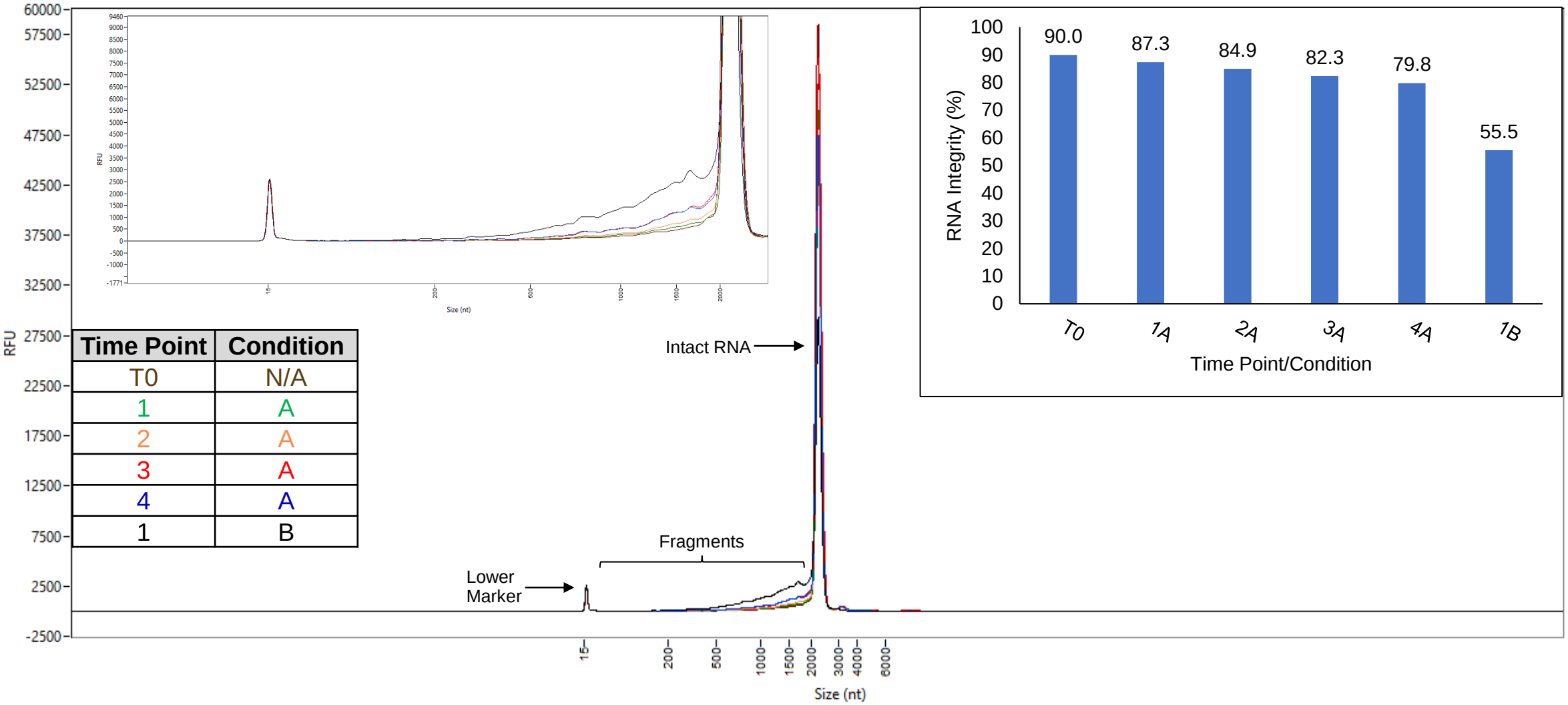
- Assay is linear from 20 to 150% of nominal load
- **Large linear range**

Forced Degradation Stability Case Study



Forced Degradation Stability Study - mRNA DS

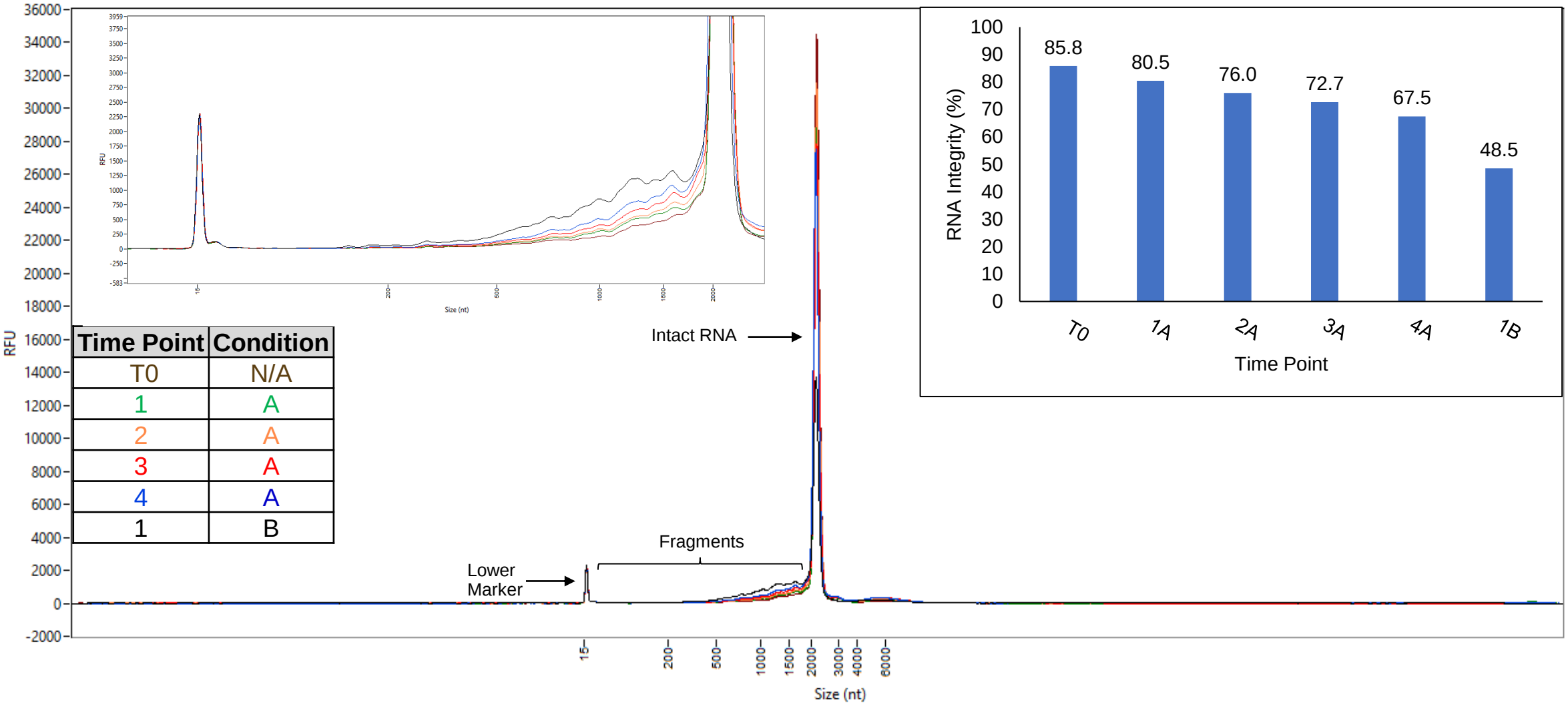
The assay is stability indicating



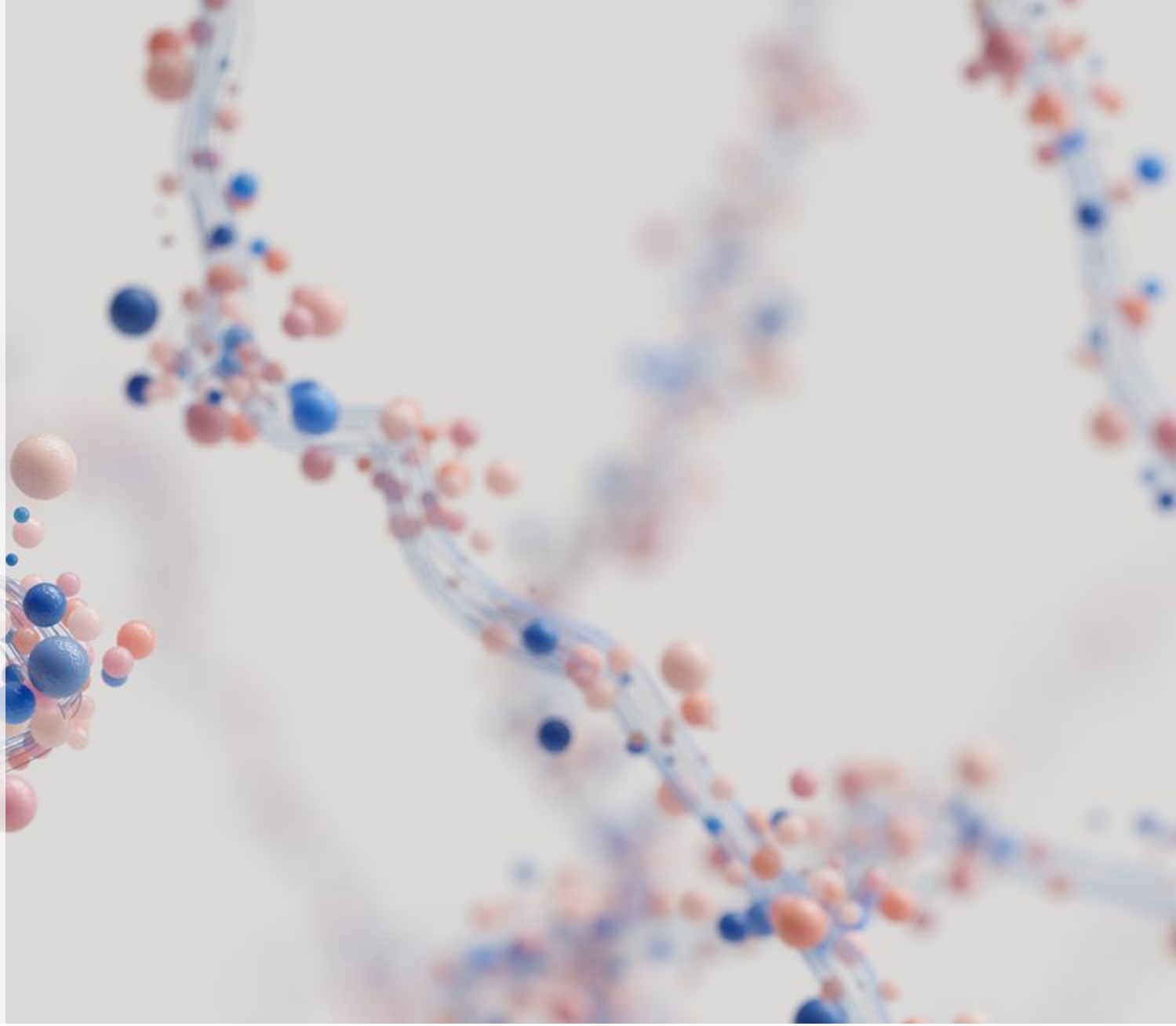
Time Point	Condition
T0	N/A
1	A
2	A
3	A
4	A
1	B

Forced Degradation Stability Study - mRNA DP

The assay is stability indicating



Standard Sensitivity Kit vs. High Sensitivity Kit Case Study



Standard Sensitivity (SS) Kit vs. High Sensitivity (HS) Kit

- SS and HS kits have similar separation and detection parameters
- The prepared sample concentration in the SS kit is higher and the injection duration is shorter (relative to the HS kit)
- The HS kit optimizes the intensity of the lower RNA marker peak and the RNA ladder to allow for low concentration samples to be injected for significantly longer injection durations
- Both kits achieve the same relative on-capillary load
- An established RFU range for main peak apex enables control of capillary load for both kit methods

Standard Sensitivity Kit

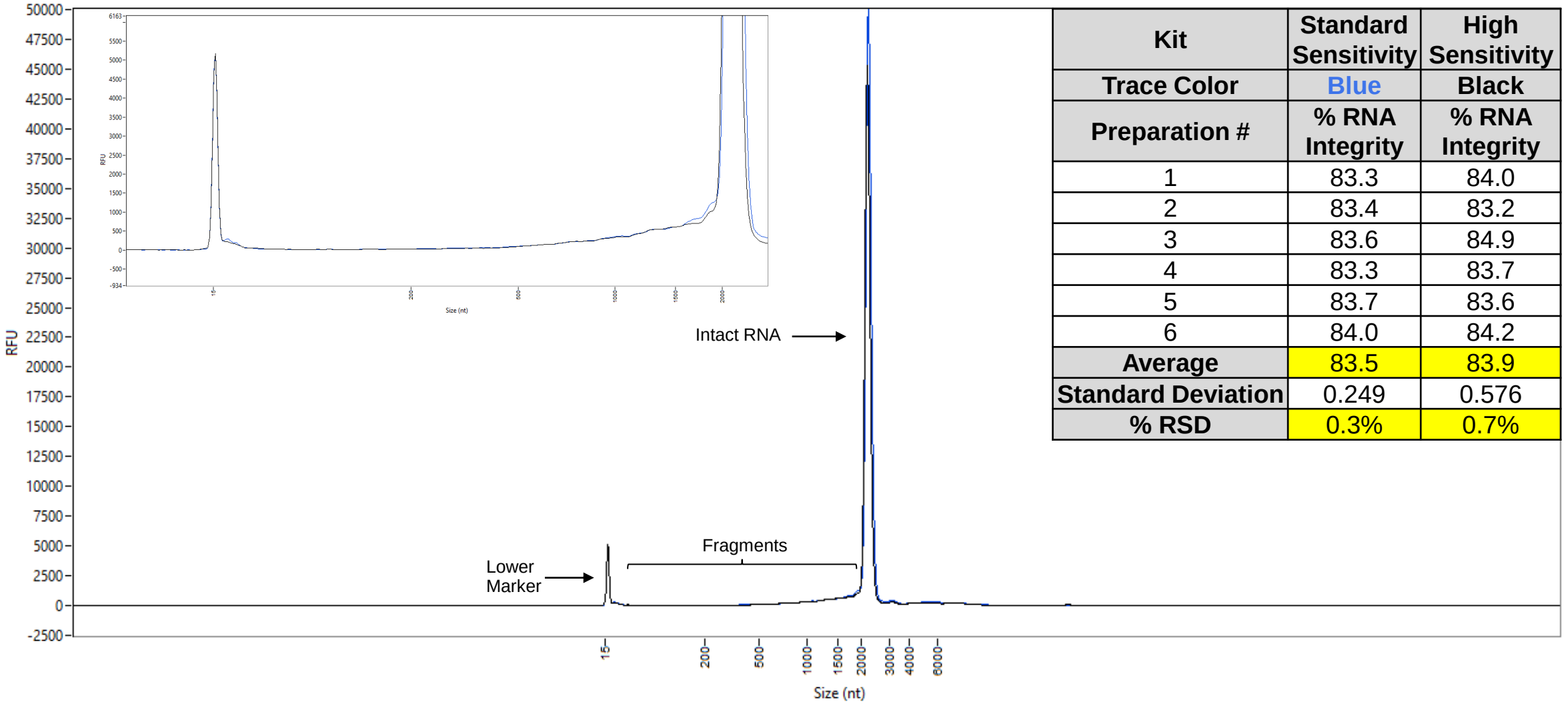
- Part # DNF-471-1000
- Higher concentration samples
- Low injection duration

High Sensitivity Kit

- Part # DNF-472-1000
- Low concentration samples
- High injection duration

Standard Sensitivity (SS) Kit vs. High Sensitivity (HS) Kit - mRNA DP

SS and HS kits provide comparable results



Kit	Standard Sensitivity	High Sensitivity
Trace Color	Blue	Black
Preparation #	% RNA Integrity	% RNA Integrity
1	83.3	84.0
2	83.4	83.2
3	83.6	84.9
4	83.3	83.7
5	83.7	83.6
6	84.0	84.2
Average	83.5	83.9
Standard Deviation	0.249	0.576
% RSD	0.3%	0.7%

Summary and Conclusions

- RNA integrity is a critical quality attribute which can be measured using the Agilent 5300 Fragment Analyzer
- Agilent 5300 Fragment Analyzer Pros:
  - High throughput
  - Fast sample preparation
  - Fast run time
  - Two kits available to purchase covering a wide range of sample concentrations
  - Robust method in routine use at Pfizer

Acknowledgments

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Thank You and Questions