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CE PHARM
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Nucleic Acid Analysis by CGE: Developing and Implementing a Multivalent Analytical Assay to Characterize and Control In-Process Mixtures of Cell Therapy Products

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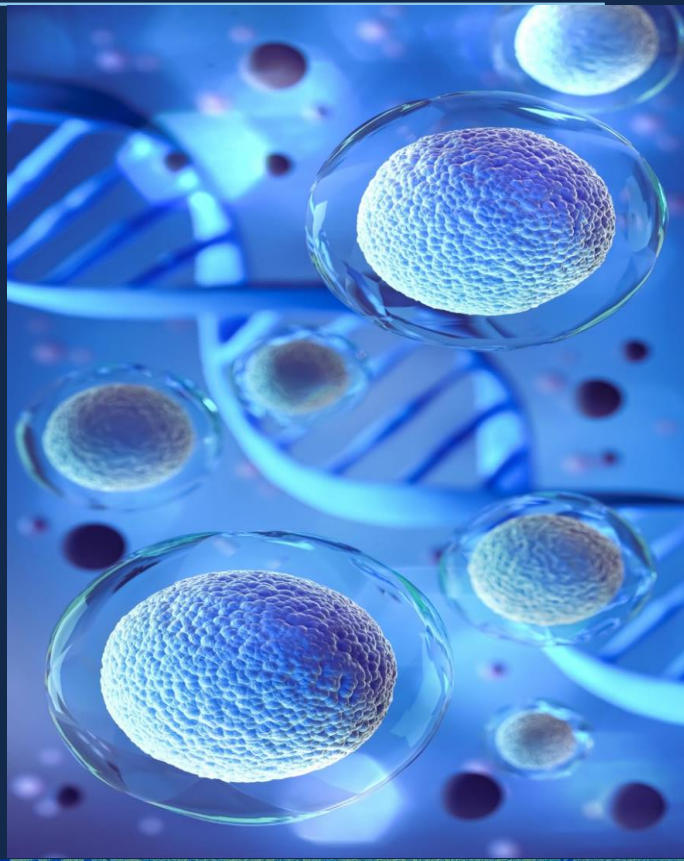
OUTLINE

1. Introduction
2. Method Development
3. Multivalent Analysis
4. Conclusions and Next Steps



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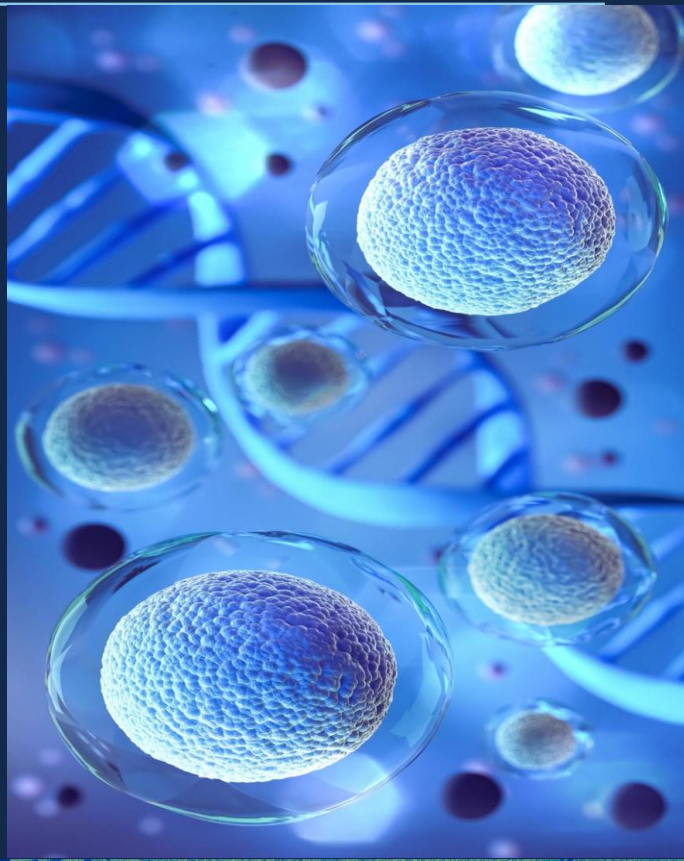
1. Introduction



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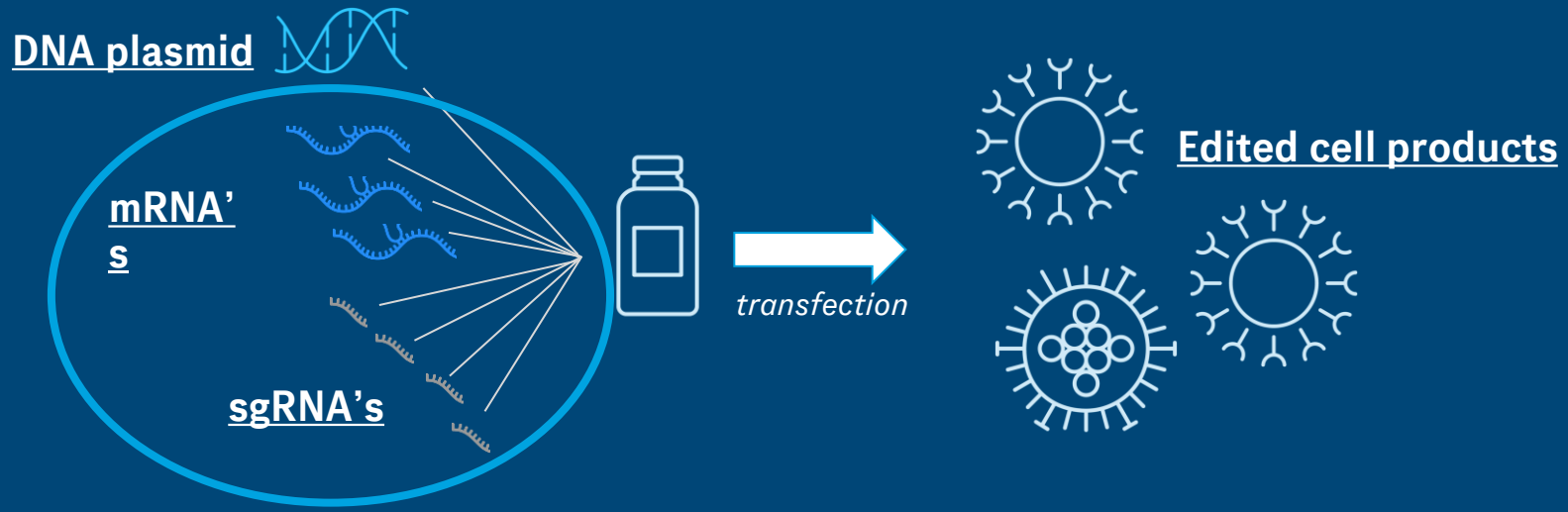
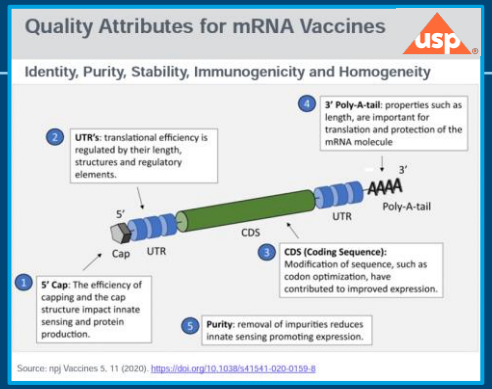
IN-PROCESS NUCLEIC ACIDS FOR CELL THERAPY PRODUCTS



In-process mixtures that transfect cell therapy products require **characterization and control** of each RNA and DNA plasmid (separately, and within a mixture)

- Comparability between different in-process runs
- Stability and shelf life considerations while on manufacturing floor
- Quality of these in-process materials impacts knock-in/knock-out efficiency of our cell therapy products

mRNA CHARACTERIZATION



Identity: must be correct

5' Cap: must be fully capped

PolyA tail: must have sufficient length (<80 nt)

Integrity/Purity: must be fully intact

mRNAs have several **critical quality attributes (CQA's)** that affect successful transfection and translation of cell therapy products (USP reference in right hand corner)

- In addition to the above: pH, concentration, and potency, as well as in-process impurities such as residual protein, genomic/template DNA, NTPs, and dsRNA
- One of the most important CQA: **purity**, can be assessed by IPRP-UV and/or **CGE-LIF**
- **CGE-LIF** will be the subject of this talk

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WHY CAPILLARY GEL ELECTROPHORESIS (CGE)?

Individual nucleic acids

- mRNA/sgRNA **integrity/purity** (degraded, intact, aggregates of total RNA)
- mRNA **polyA tail** distribution (tail-less, longmer/shortmer variants)
- **dsRNA** presence (3'-extended transcripts)

Complex mixture for transfection

- **Identity** of each mRNA/sgRNA can be inferred with sizing (via retention time)*
- **Integrity/purity** possible if the RNAs has enough baseline separation (i.e. 100 nt vs 3000 nt RNA)

CGE-LIF sizes RNAs with considerable resolution and with sensitive detection (laser-induced fluorescence LIF) with the potential to be used as both an IPC and QC method; while primarily used to assess RNA **integrity/purity**, other size-based attributes, such as **polyA tail** and **dsRNA**, can be inferred with the support of other assays.

*this does not confirm the coding sequence

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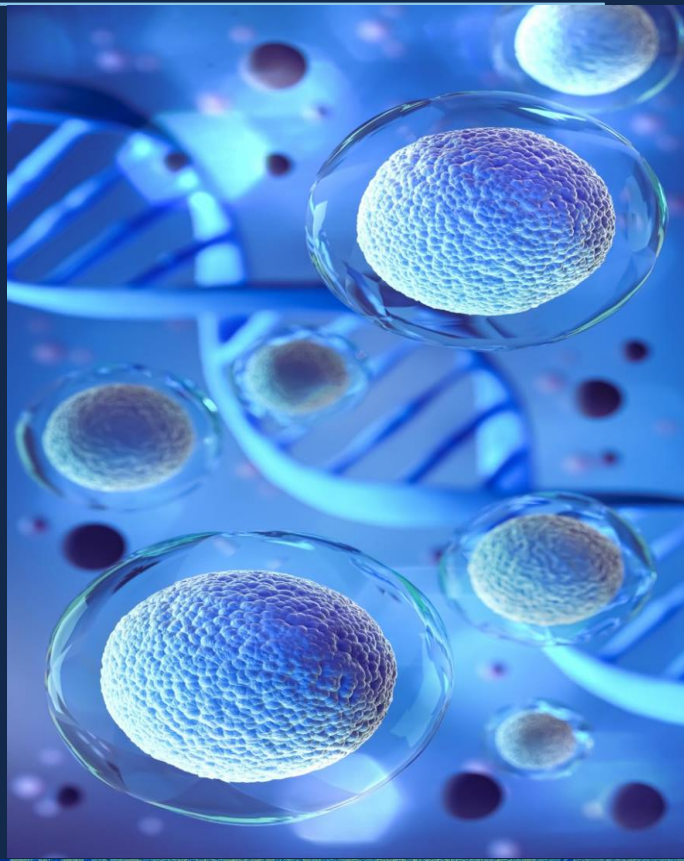
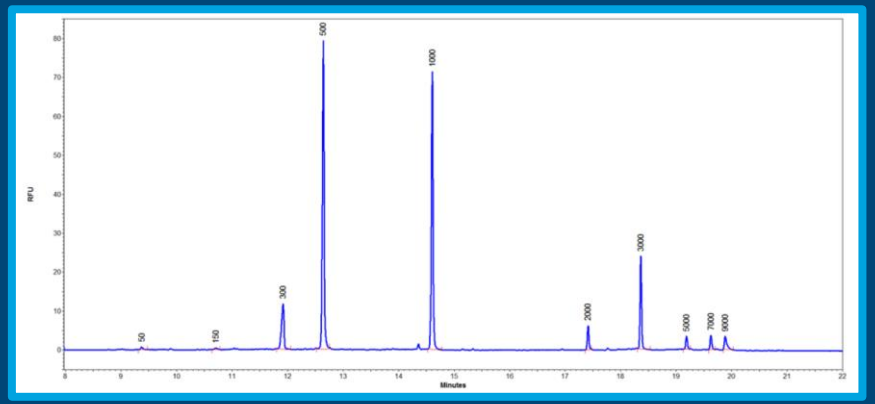


RNA 9000 KIT ON THE PA800+ (SCIEX)

PA800+

Bare-fused capillary
520 nm filter
CE vials and caps

ssRNA ladder (50 - 9000 nt)



RNA 9000 kit

Nucleic acid gel
SYBR Green II RNA dye
Acid wash and water

https://sciex.com/content/dam/SCIEX/pdf/flyers/ruo-mkt-07-14191-a-rna-kit-flyer.pdf

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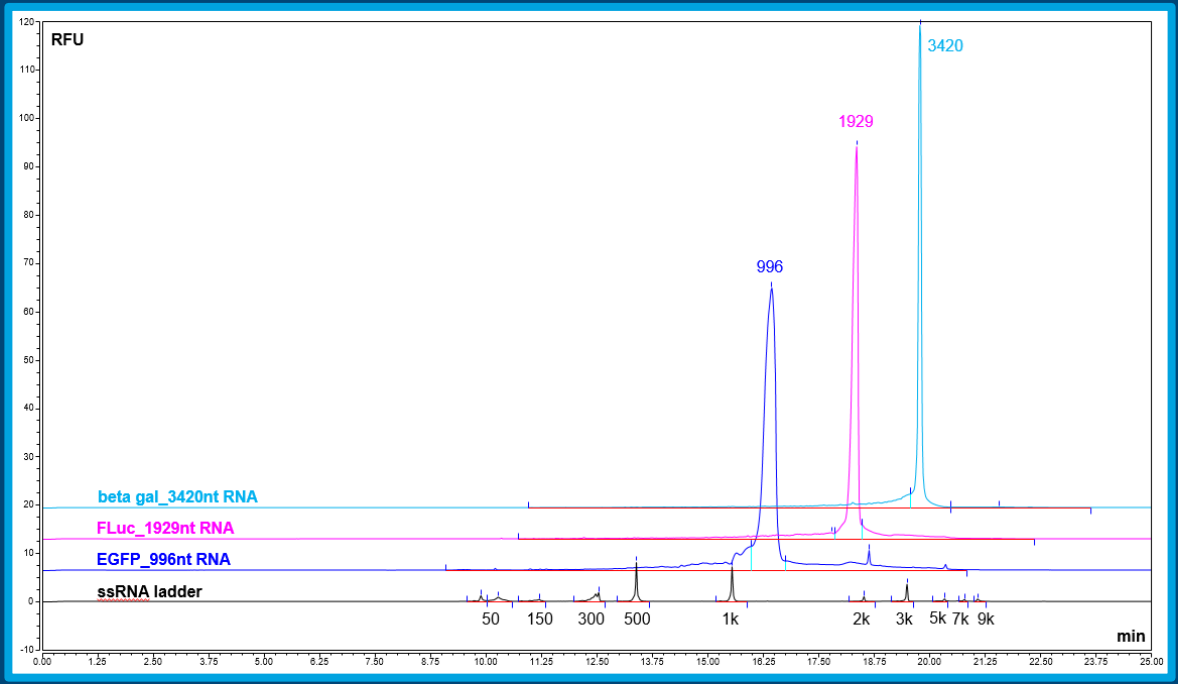
FIRST ASSESSMENT



SciEx kit conditions:

- 30 cm capillary (20cm to detector)
- 30°C capillary temperature
- 1kV 3s injection
- 6kV, 22 minute separation

Assessment:

- n=3 injection per RNA sample
- total peak area: **16% to 26% RSD**
- main peak % (integrity/purity): **2% to 5% RSD**
- shallow shoulders between main peak and pre/post-peaks

Development needed to improve resolution and injection repeatability

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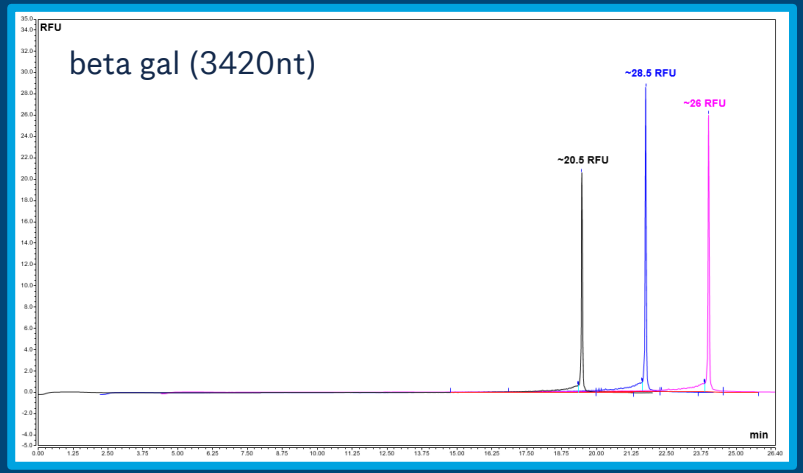
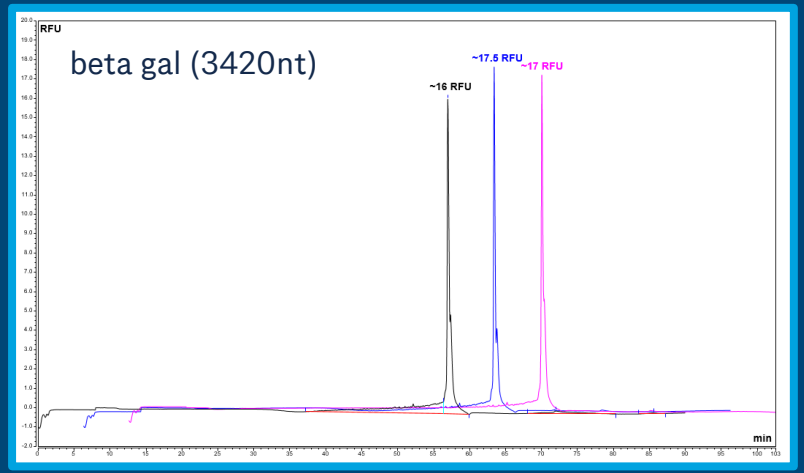
INJECTION MODE

Electrokinetic (1 kV, 3s)



16% RSD (total peak area, n=3)

Pressure (2 psi, 10s)



10% RSD (total peak area, n=3)

Overall, **improved peak area repeatability** with pressure injections, which vary from 5-10% RSD overall for several mRNAs/sgRNAs (100nt to 3500nt)

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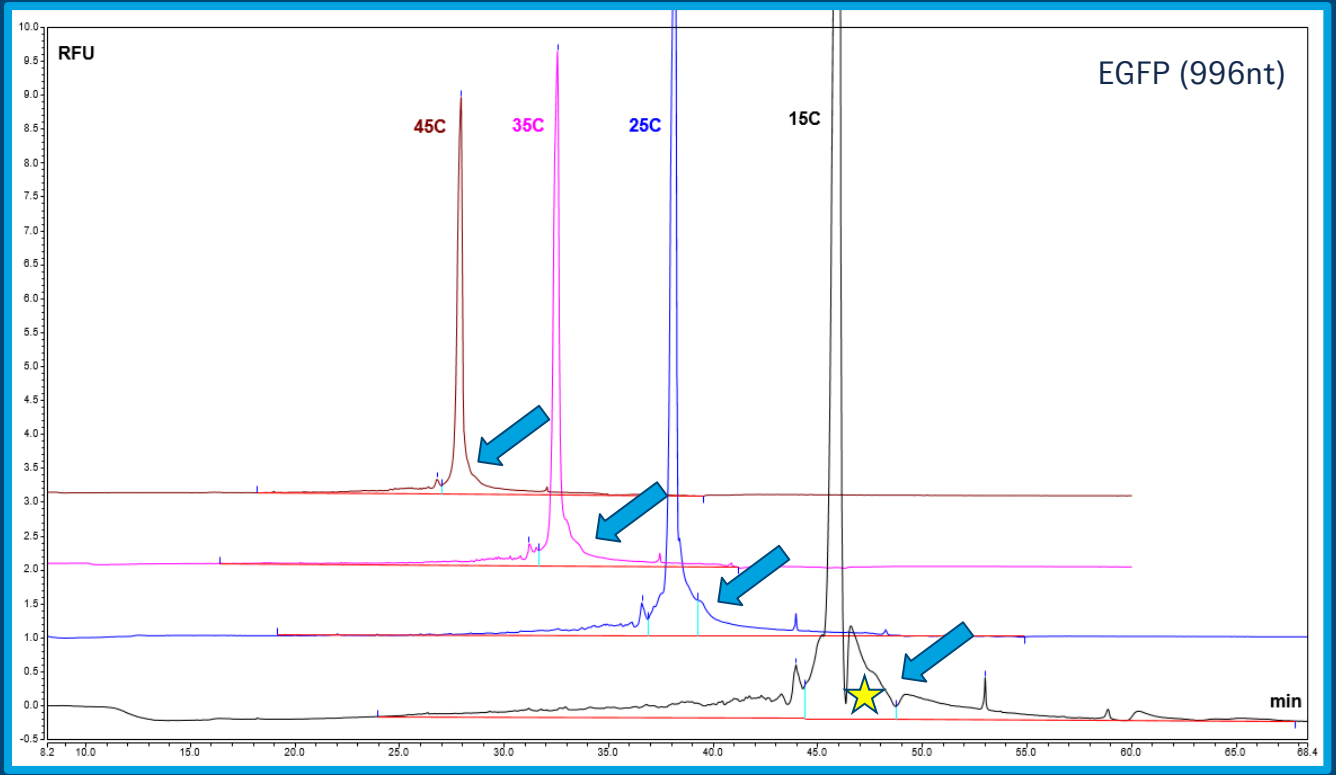
IMPROVING RESOLUTION



Extended capillary length from 30 cm to 60 cm (keeping same V/cm during separation)

Lower capillary temperatures slow RNA mobility, improving resolution of larger species

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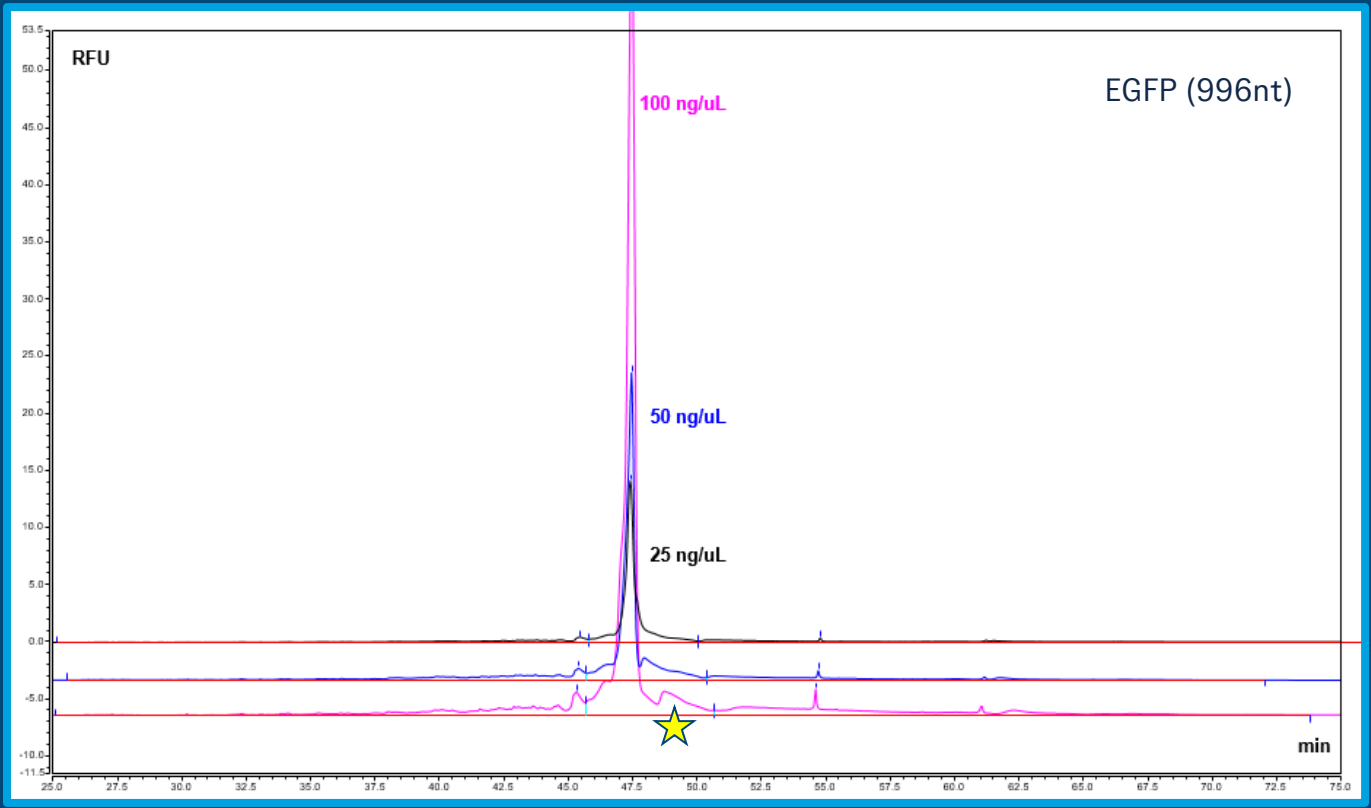
IMPROVING RESOLUTION - SPLIT PEAKS



Linearity experiment: 72% - 77% intact RNA variability, r^2 = 1.00 for total peak area vs [RNA]

Split peak observed in 15°C condition disappears with lower concentrations of RNA (50% of target, 25 ng/uL)

Split peak could be polyA tail variant or other intact RNA variant that loses resolution when dye:RNA ratio changes

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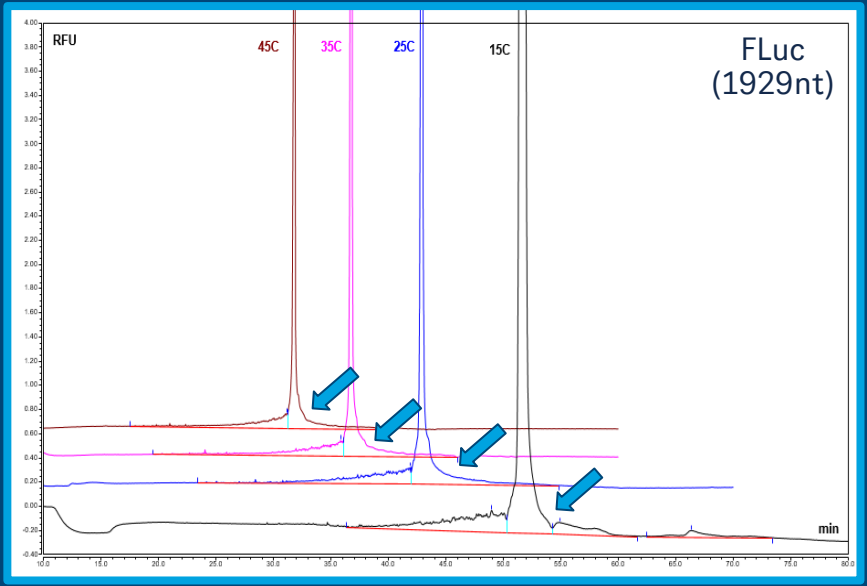
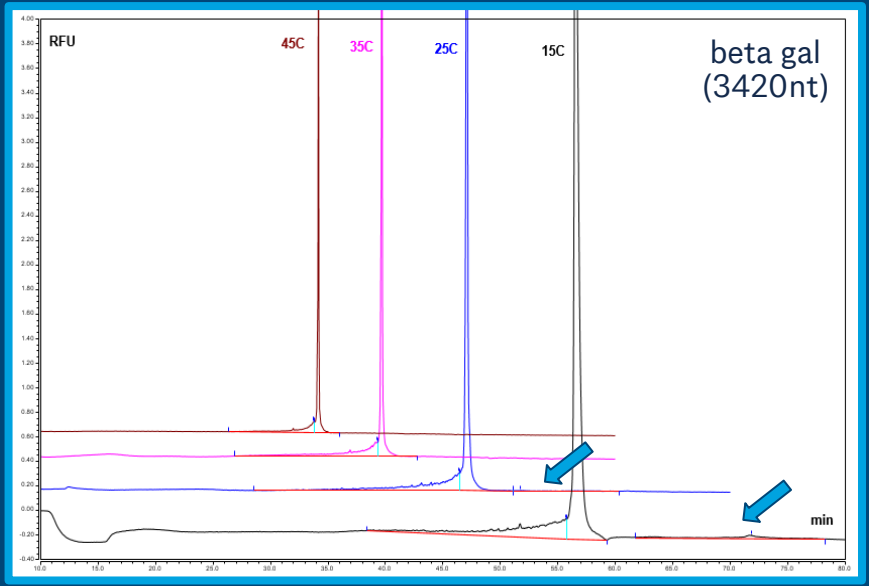
IMPROVING RESOLUTION - LARGER mRNAs

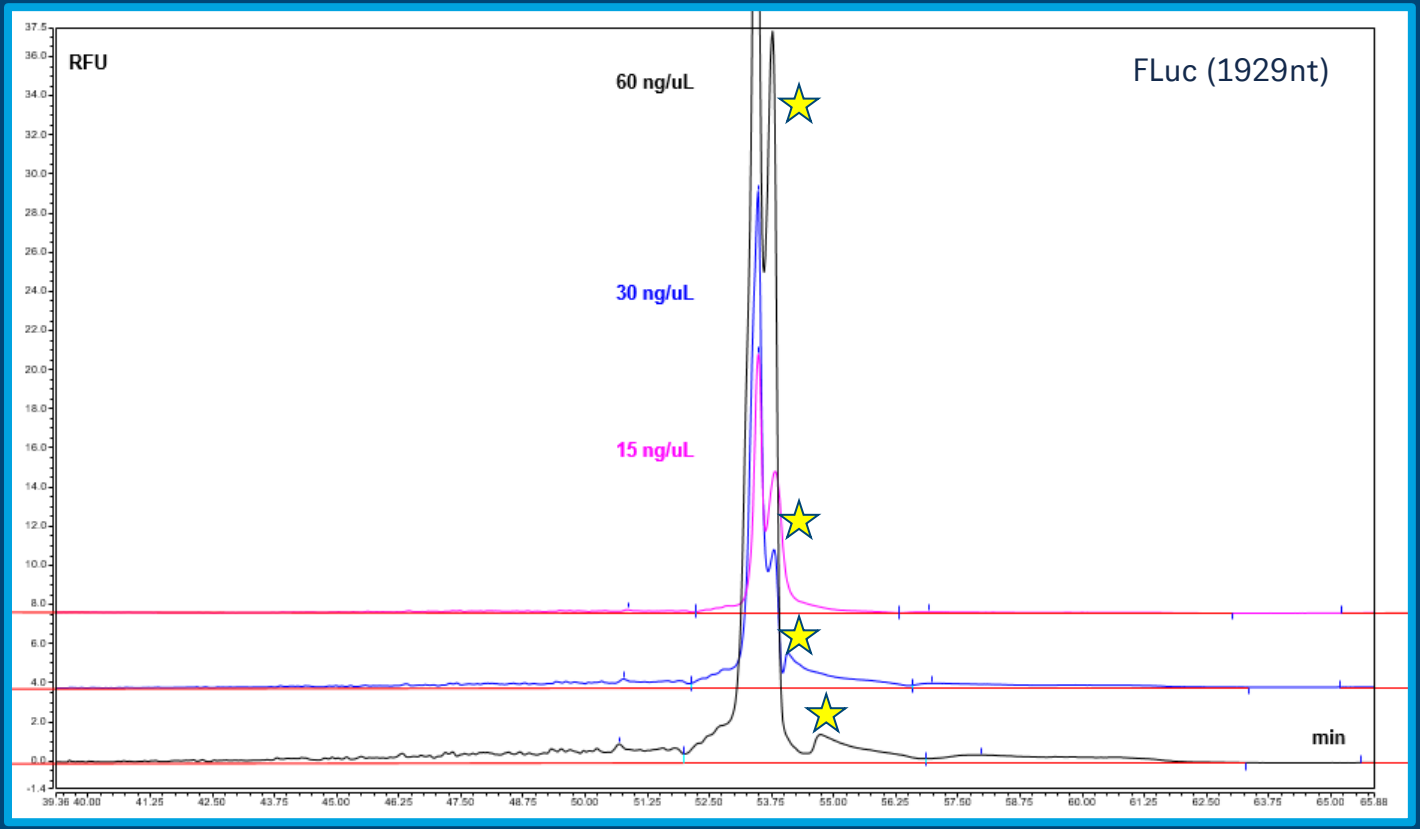




Similar resolution gains of post-peak region with larger RNA constructs (1929, 3420 nt)

Split peak of intact RNA observed in 15°C condition as well for both constructs (next slide)

IMPROVING RESOLUTION - SPLIT PEAKS



3 split peaks at target (30 ng/uL) and 200% of target (60 ng/uL); 2 observed at 50% of target (15 ng/uL)

Again, dye:RNA ratio may be playing a role in resolving these variants

Linearity (FLuc): 79% - 80% intact RNA variability, r^2 = 0.99

Linearity (beta gal): 64% - 72% intact RNA variability, r^2 = 0.99

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FINAL METHOD CONDITIONS

Pressure injections

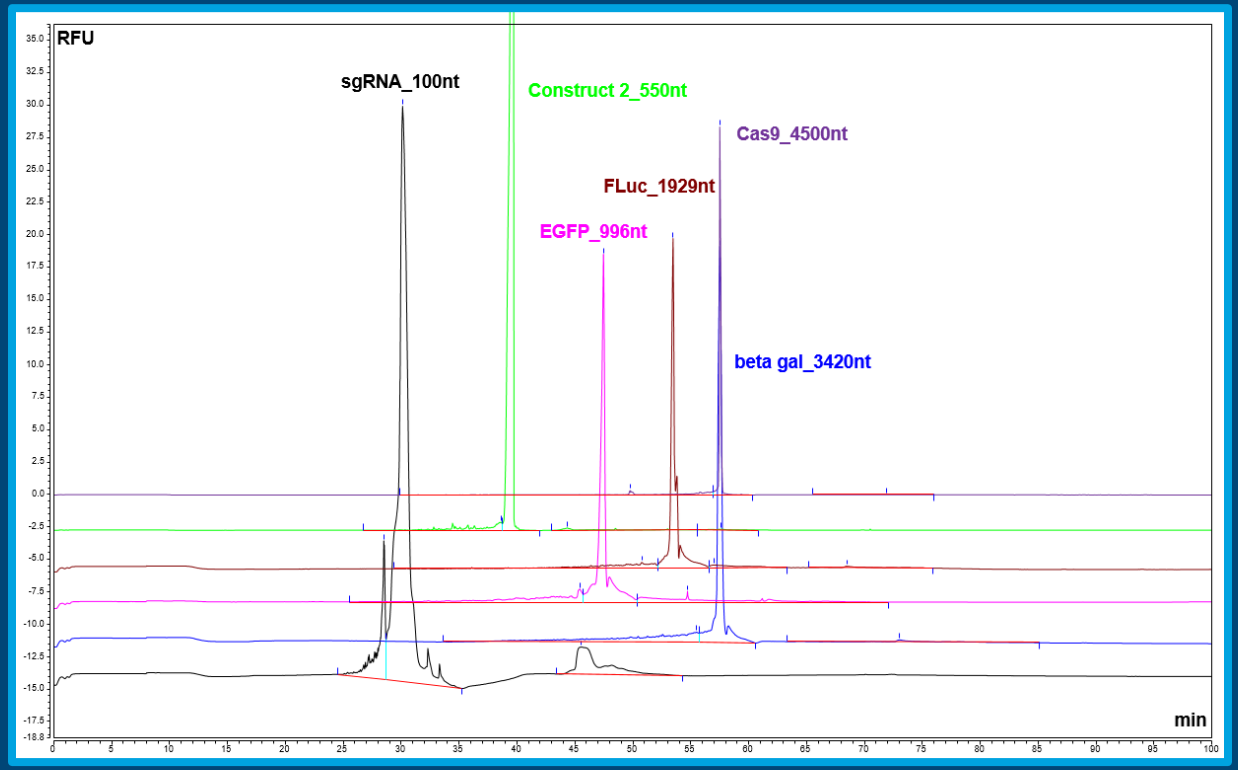
15°C capillary temperature

60 cm capillary length

Conditions per reagent kit

Capable of RNA **identity** within a mixture per retention time shift (up to ~2000 to 3000 nucleotides)

sgRNA ladder resolves up to 9000 nt, but RNA constructs may have more complex hydrodynamic radii



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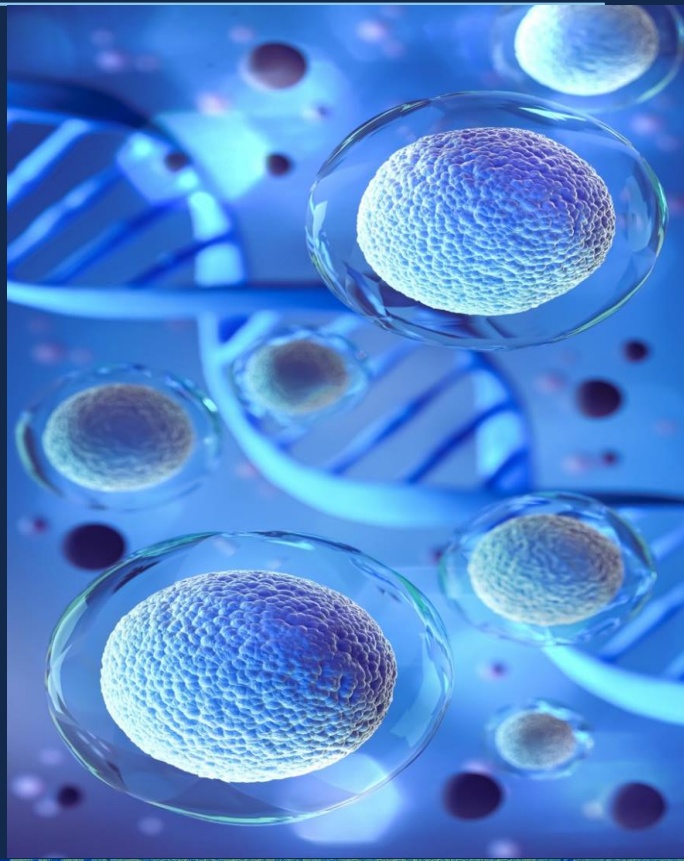

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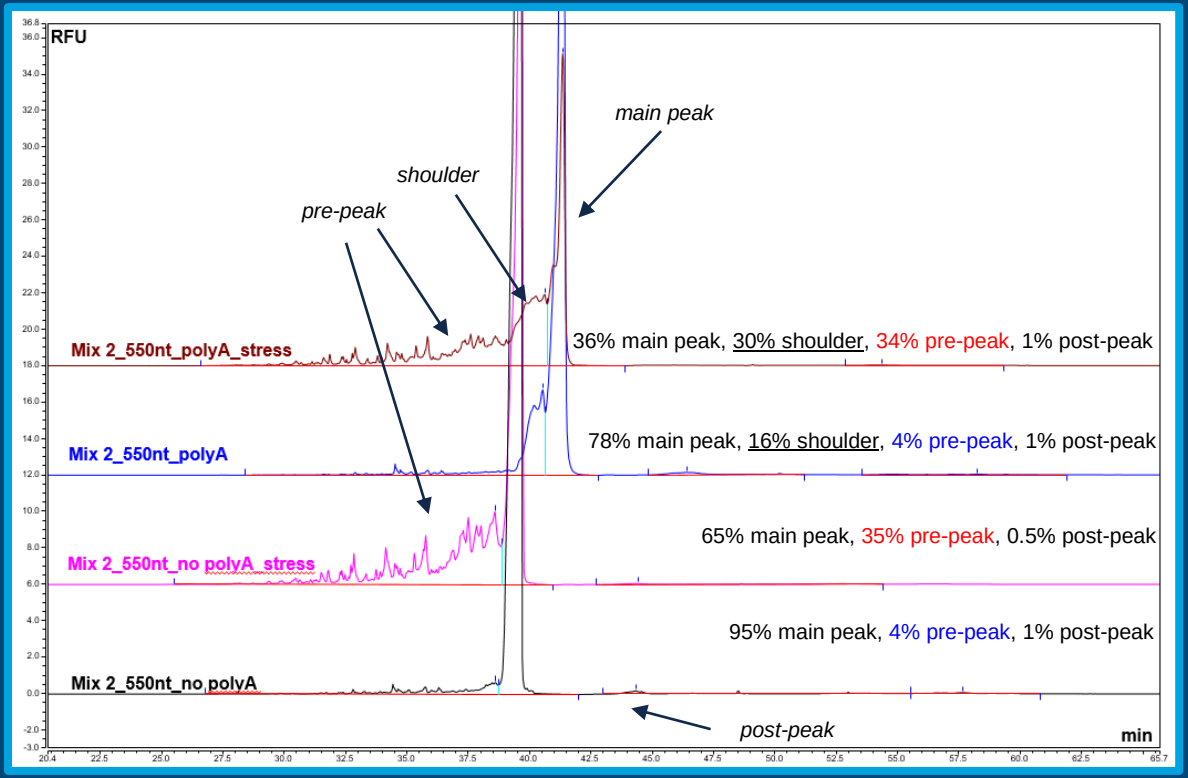
CGE IS A STABILITY-INDICATING METHOD (POLY-A TAIL EXPERIMENT)



Retention time shift observed when polyA tail present

40C 5d stress change similar for both samples (with and without poly A), confirming shoulder peak is a polyA tail variant

Less pronounced retention time shift as RNA gets bigger (550 nt small enough for sufficient resolution between tail-less and tailed constructs)

Purities confirmed with orthogonal IPRP-UV assay

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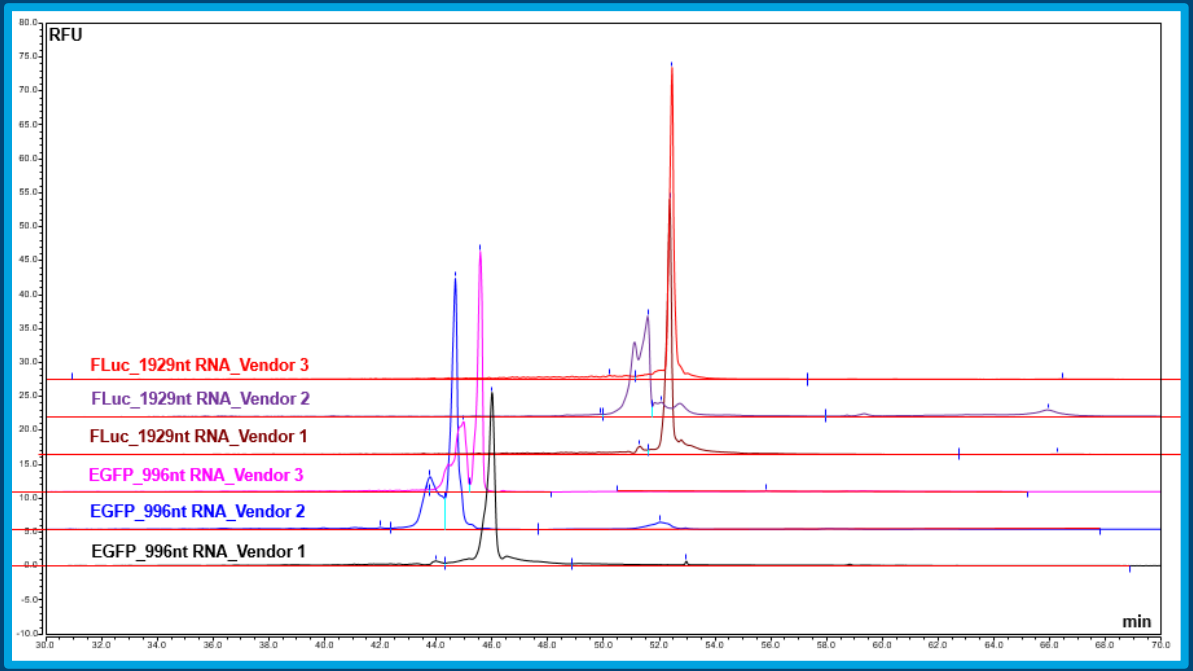
mRNA VENDOR ASSESSMENT (POLY-A TAIL LENGTH DIFFERENCES)

Different vendors of the same construct show retention time shifts, likely due to differences in 5' and 3' UTRs + polyA tail length distribution (differences in vendor manufacturing process)

PolyA tail assay: EGFP_Vendor 1 has most longmer tails (20%), EGFP_ Vendor 2 has most shortmer tails (15%), which may suggest retention shifts

In addition, Vendor 1 has 150 nt polyA, while Vendor 2 has 120 nt



Overall, **resolution decreases** within the gel as RNA's get bigger: therefore, less retention time differences in FLuc construct (but same order of elution by vendor)

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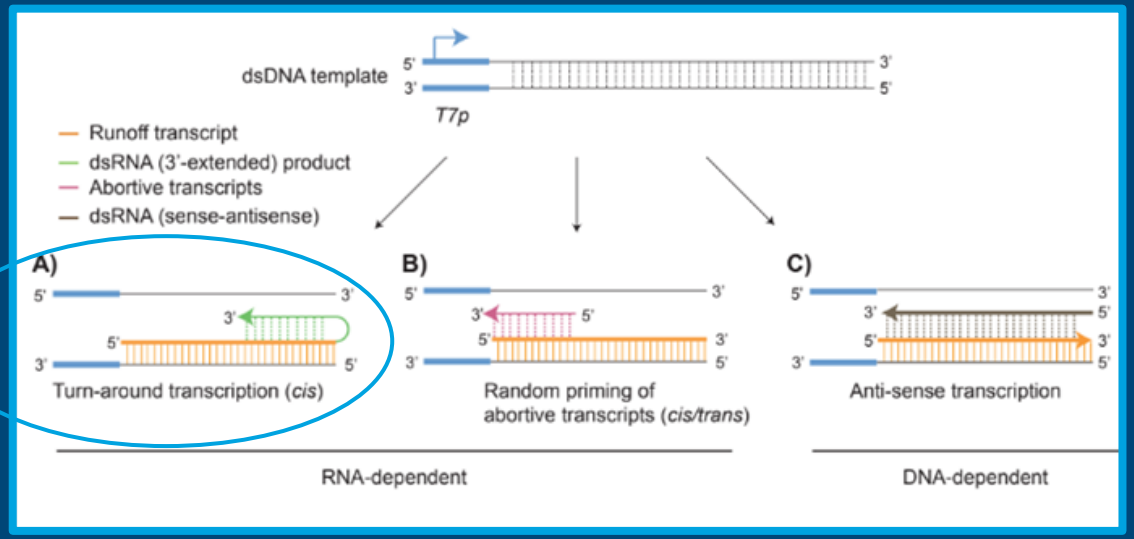
dsRNA AS LONGMER IMPURITIES

A CQA that may have negative impact on cell electroporation, and therefore expression & efficacy



Turn-around transcript (**3'-extended dsRNA**) retains structure during denaturing conditions of a CGE separation, and therefore should elute in the post-peak (longmer variant) region

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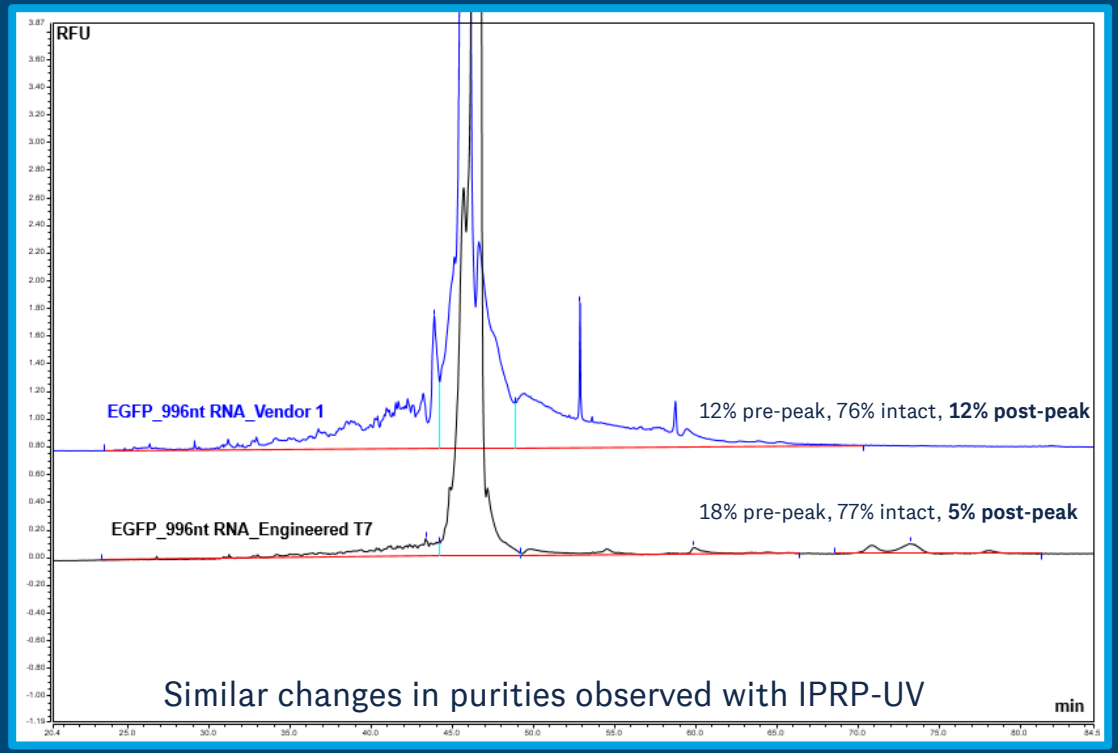
Figure reference: "Understanding and Overcoming the Immune Response from Synthetic mRNAs," B. Roy and M. Wu, 3 Dec 2019, https://www.liebertpub.com/doi/10.1089/gen.39.12.15

dsRNA AS LONGMER IMPURITIES

Engineered T7 polymerase used in IVT reaction of EGFP mRNA construct versus control (vendor 1)

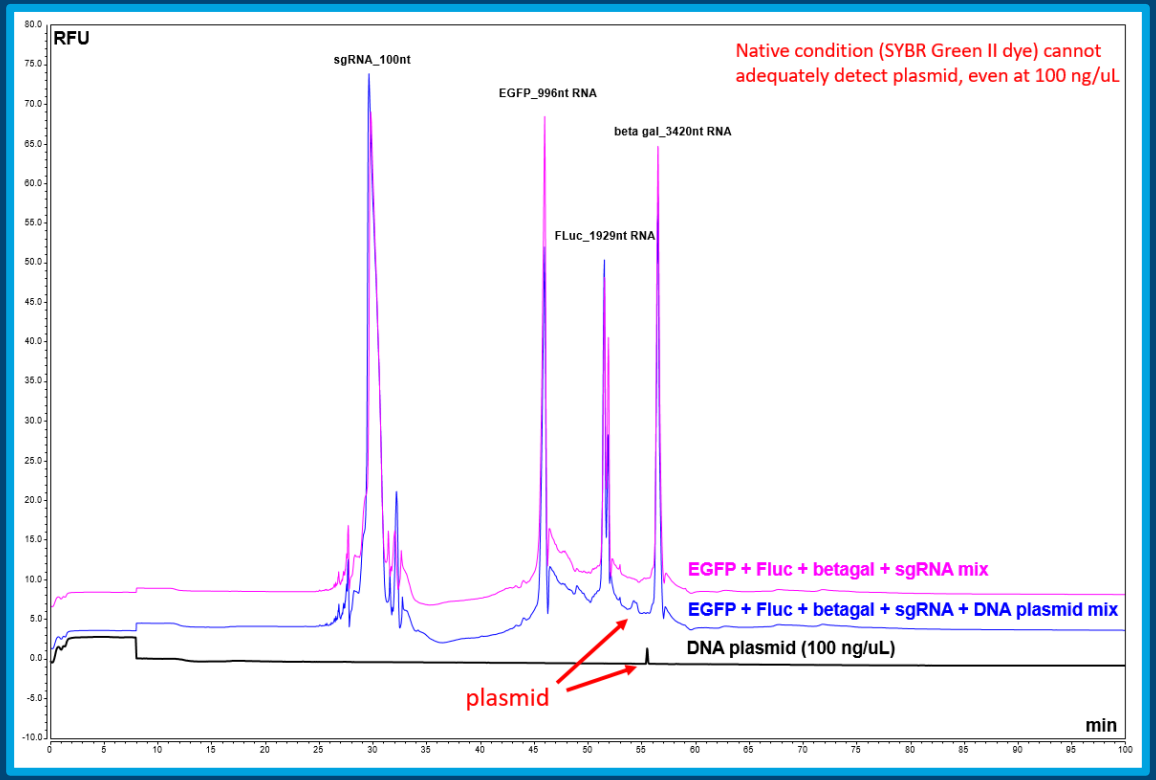
Engineered T7 polymerase shown to reduce dsRNA based on literature (reference below)

Since only 3'-extended dsRNA remains intact in **denaturing conditions**, presence within post-peak (longmer) region is likely



Publication: "An engineered T7 polymerase that produces mRNA free of immunostimulatory byproducts," A. Dousis, K. Ravichandran, E. Hobert, M. Moore, and A. Rabideu, https://www.nature.com/articles/s41587-022-01525-6

WHAT ABOUT DNA PLASMID?



A complex mixture detects main peaks of RNA but cannot quantitate purity if co-elution occurs (**identity** assay instead)

DNA plasmid binds and detects poorly with SYBR Green II dye (even at 100 ng/uL, which is higher than all other RNAs in the mixture)

Likely competing with the other RNAs within the mixture, which the dye has greater affinity with chemically

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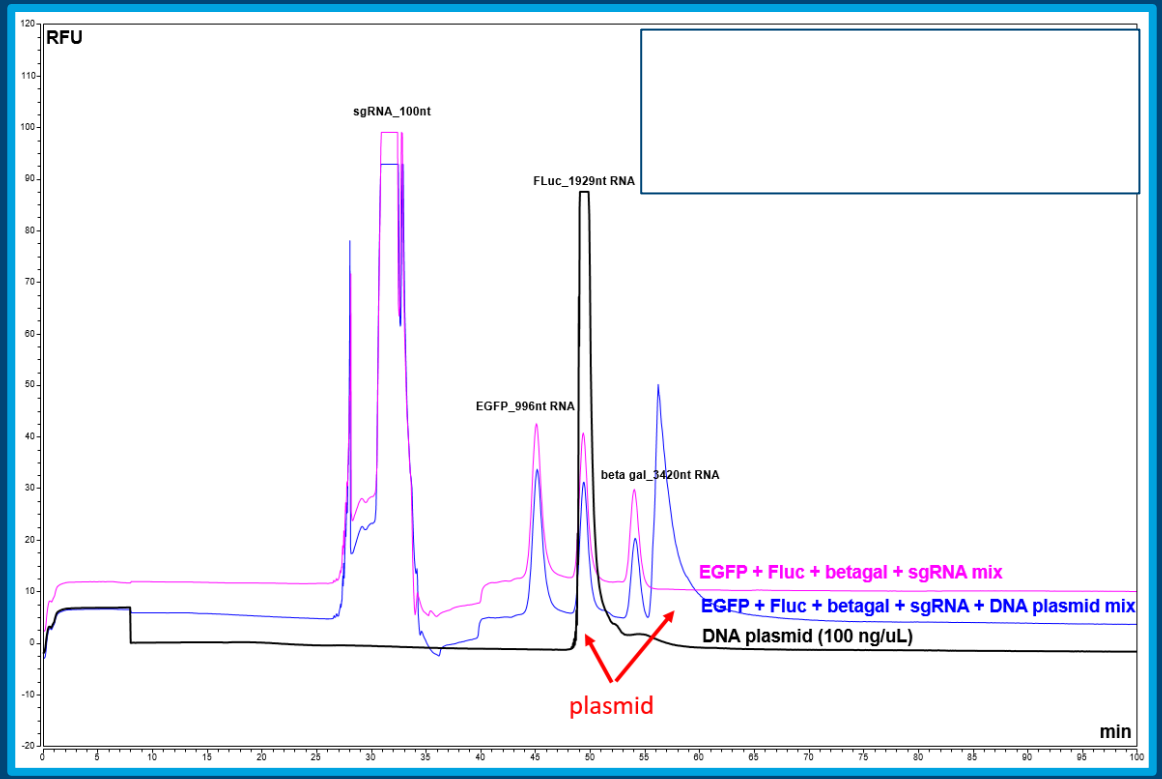
WHAT ABOUT DNA PLASMID?

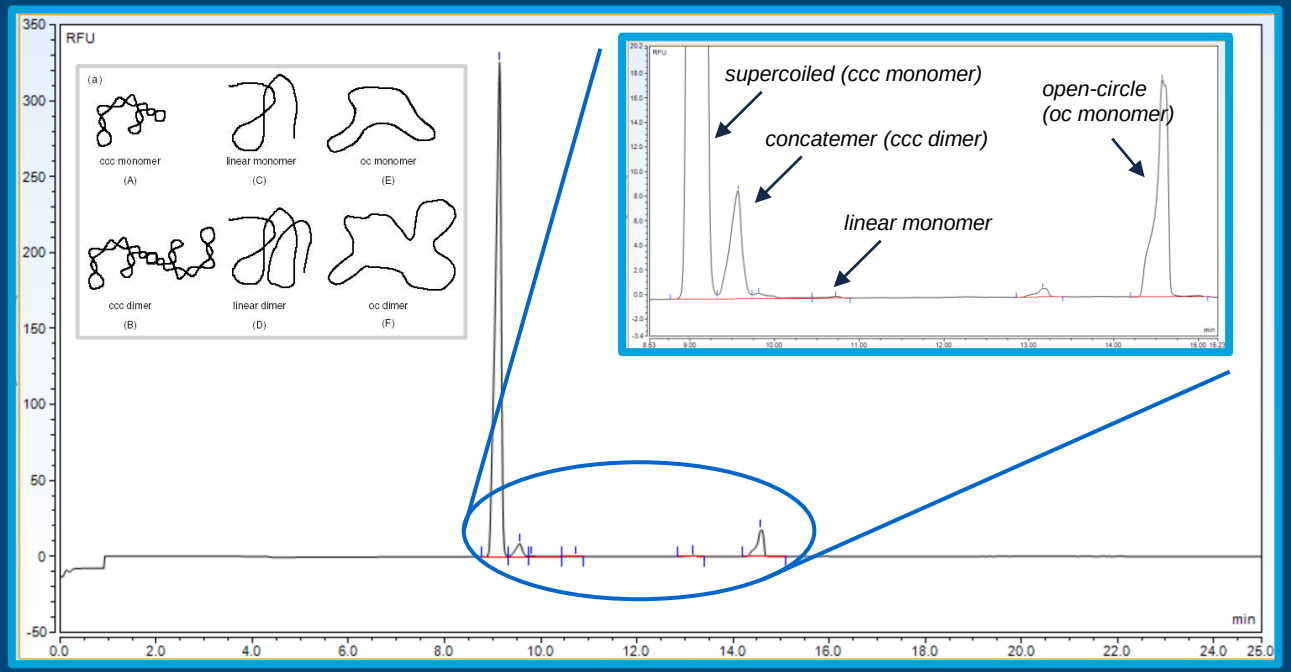


Adding SYBR Gold dye to the native condition of the assay allows identity of all RNAs + DNA plasmid

Notably, resolution of peaks are reduced when adding SYBR Gold and Green to the same gel

An alternative assay condition to confirm an in-process mixture has necessary components for transfection

WHAT ABOUT DNA PLASMID? (A DIFFERENT CGE-LIF ASSAY)



Sciex's DNA 1000 kit with SYBR Gold dye can measure purity of DNA plasmid (**DNA topology**)

Different gel and dye chemistry but similar CGE separation mechanism (sieving gel, dye-induced LIF)

Peaks confirmed with enzyme digests and fraction collection

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Figure reference: "Production of non-viral DNA vectors," M. Schleef, M. Blaesen, M. Schmeer, R. Bair, C. Marie, G. Dickson, D. Schermen, https://pubmed.ncbi.nlm.nih.gov/21054244/

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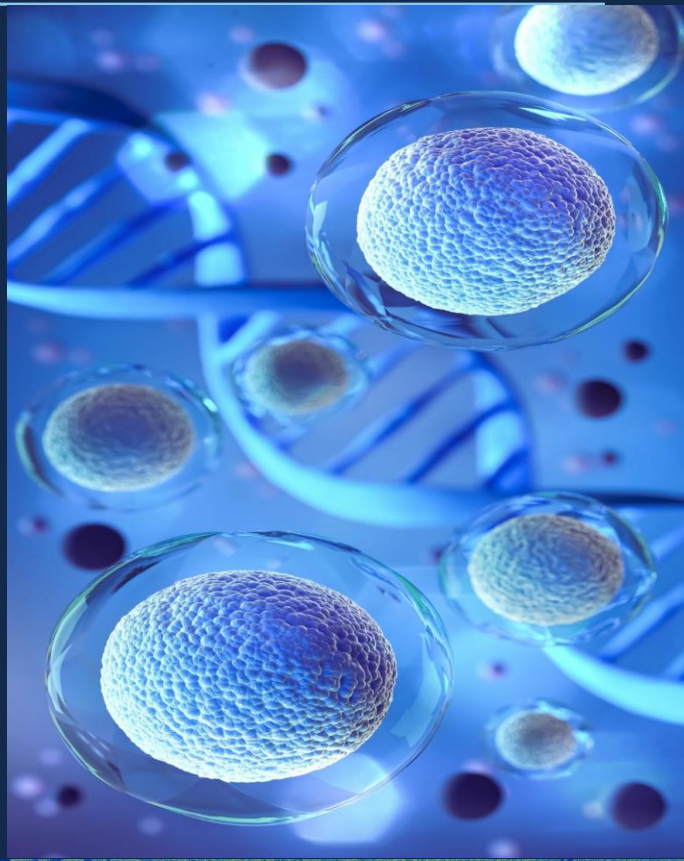

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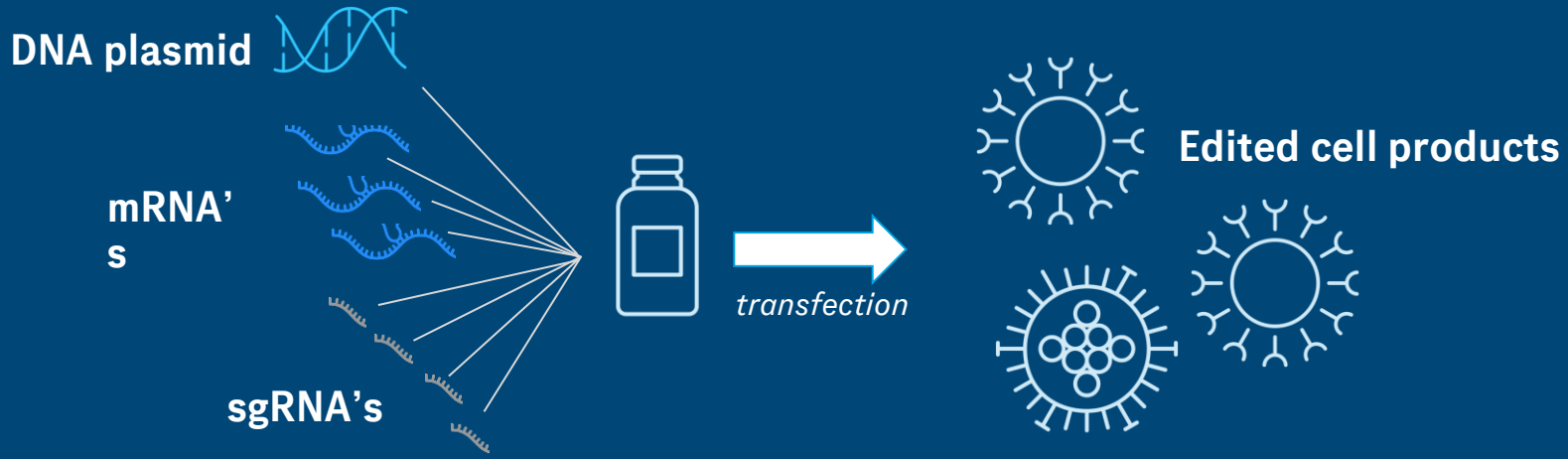
CONCLUSIONS: WHAT CAN CGE-LIF DO?



In-process mixtures: **identity** of sgRNA, mRNA, and DNA plasmid based on migration time; **integrity/purity** possible with sufficient baselines resolution

Individual nucleic acids: **integrity** of sgRNA and mRNA; some **polyA tail** distribution (tail-less, longmer/shortmer variants); some **dsRNA variants (3'-extended** variant)

- **Integrity** is the primary attribute measured; orthogonal assays such as RNase T1 HPLC (polyA tail distribution) and dsRNA ELISA (most dsRNA variants) help infer CE peaks

NEXT STEPS

- Running by UV detection may help discern any labelling biases of SYBR Green II dye
- Alignment on integration strategies of both CGE and HPLC, since both profiles have heterogeneous peak regions with somewhat poor resolution
- Further exploration of improving injection repeatability (to 5% RSD or less), which is is not necessary for in-process sampling, but also inadequate for a release test (should the need arrive for a different project)
- Further exploration of improving resolution to achieve baseline resolution between peaks (especially for larger RNAs)

THANK YOU

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