REPLICATE

Analysis of srRNA poly(A) tails with single nucleotide resolution by capillary gel electrophoresis using ultraviolet (CGE-UV) and laser-induced fluorescence (CGE-LIF) detection

Jacqueline Posy
Senior Scientist, CMC
Amanda McDonnell
Principal Associate Scientist, CMC
Replicate Bioscience



Replicate Bioscience company overview

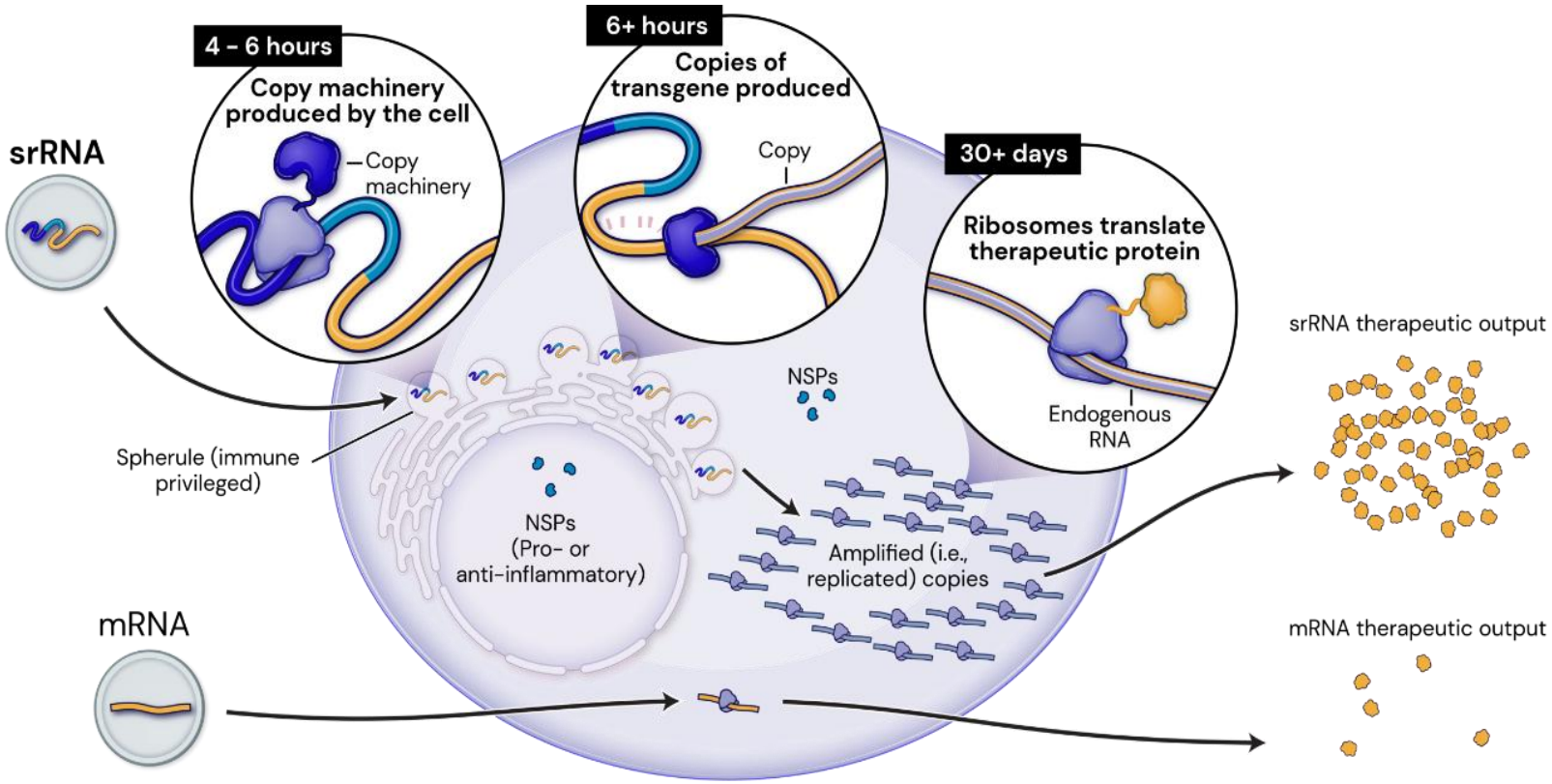
- Founded in early 2020 just prior to the start of the SARS-CoV-2 pandemic
- Based in San Diego, California
- Currently ~20 employees
- Focused on developing proprietary 2nd generation self-replicating mRNA vectors
- Clinical stage company with established CDMO manufacturing for 9-16 kb srRNA drug substance and LNP formulated drug product
- Phase 1 clinical data from Rabies vaccine de-risks platform





srRNA: an instruction manual for your body's own cells to create fully natural mRNA

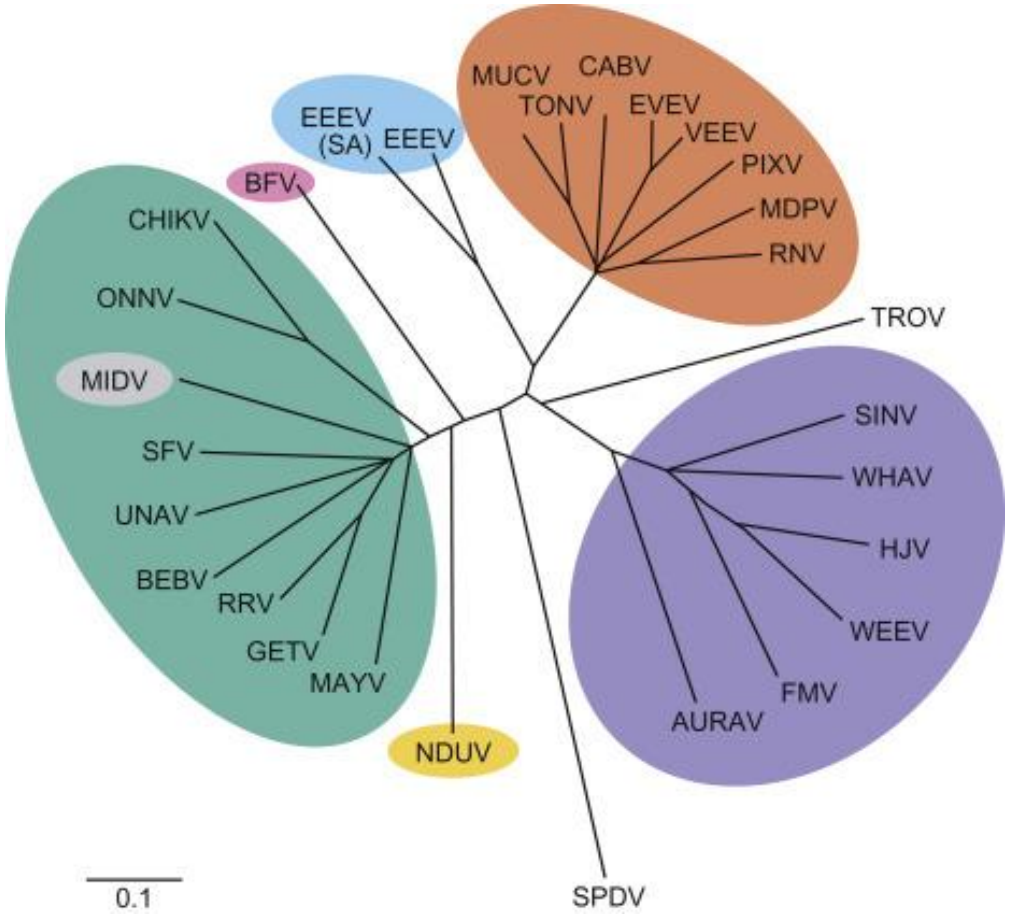
Mechanism for protein expression



Replication machinery is only created the first few hours; mRNA production is a self-limiting process

Blakney A., et al., Vaccines 2021

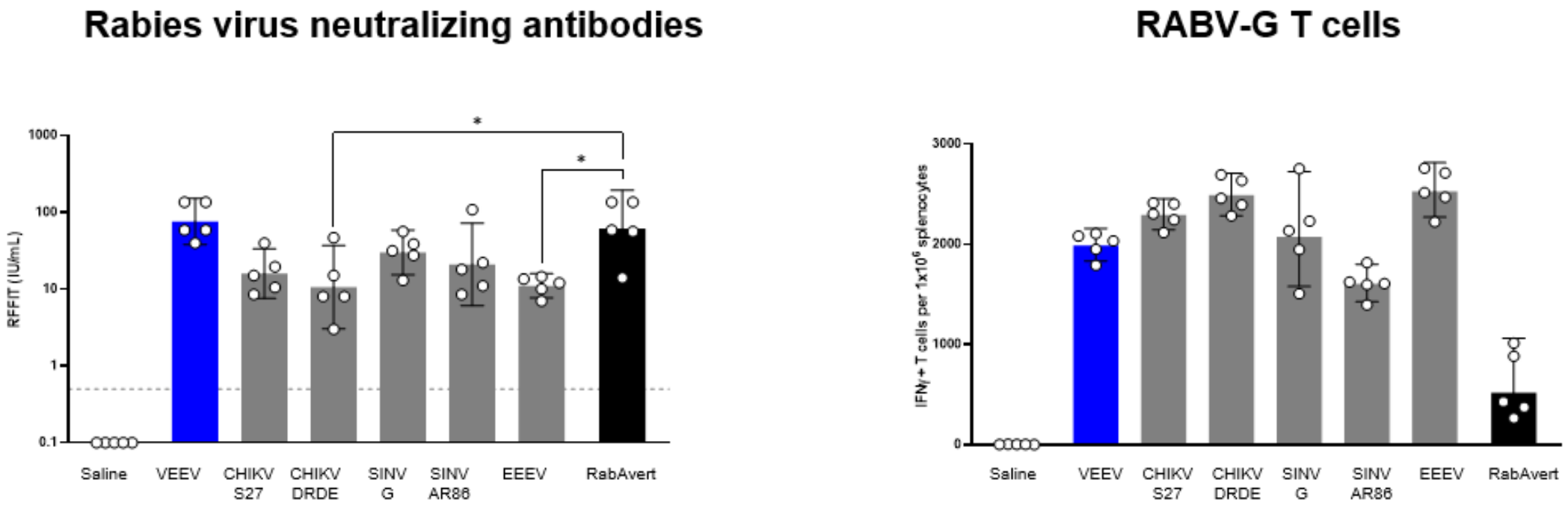
Replicate has vectorized new members of the alphavirus family (2nd generation srRNA)



- Most 1st generation srRNA strategies use VEEV-based vectors
- VEEV was the 1st to be characterized and published by the US Army in late 1990's
- Over 45+ species and 1800+ sequences (including subspecies) of alphaviruses have been documented
- Replicate are the first to unlock the potential of the alphavirus species and now have 15+ novel vectors
- We are the first to effectively mine the diversity of alphaviruses and optimize non-coding regions

Blakney A., et al., Vaccines 2021

Novel srRNA vector screening for customizable vaccine development



Serum titers of rabies virus neutralizing antibodies were measured by RFFIT assay after two administrations of srRNA/LNP vaccine (0.15 mcg) and RabAvert at 1/10 human dose. Dashed line refers to the established WHO indirect immune measure of protection of 0.5 IU/mL. Splenic RABV-G-specific T cells were measured after two administrations of srRNA vaccine and RabAvert. Statistical significance between VEEV srRNA vaccine compared to non-VEEV srRNA vaccines and RabAvert was performed by a Kruskal-Wallis analysis, where *$p<0.05$.

Spasova et al. Novel srRNA vectors for customizable vaccine development. Vaccine Insights 2025; 4(2), 55–62

srRNA vaccine encodes RNA replicase and protein(s) of interest making them very large ranging from 9kb to over 16kb

Conventional mRNA



Self-replicating RNA (srRNA)



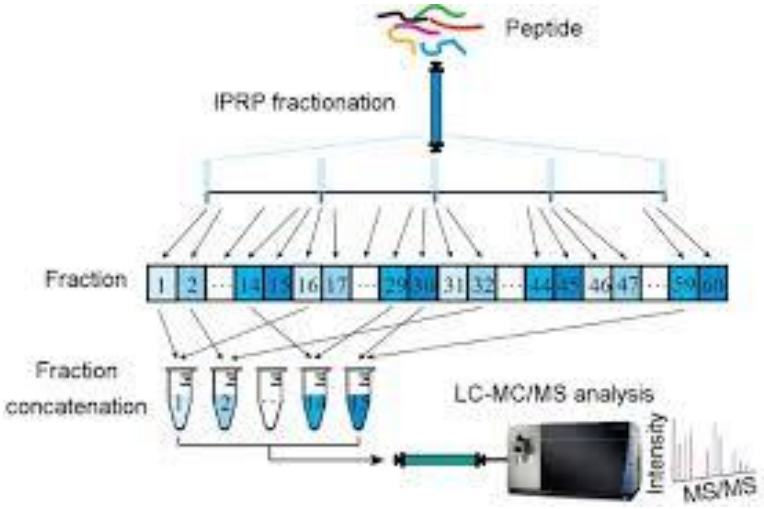
- Benefits of srRNA vaccine allows delivery of much lower doses than conventional vaccines to achieve similar or better levels of immune response
- Length of poly(A) tail is critical in dictating stability and translation efficiency of srRNA
- CGE-LIF optimized methods have been developed specifically for purity analysis of a 16kb srRNA using the PA 800 Plus system and 32 Karat software v10.2

https://www.mdpi.com/2076-0817/12/1/138

Poly(A) tail length analysis is difficult to sequence and accurately measure

- Two major analytical method have been used but both pose problems:
  - Ion-pair reversed-phase liquid chromatography (IPRP-LC) quantifies percentage of mRNA containing poly(A) tail, but lack ability to determine tail length
  - Next-generation sequencing (NGS) can achieve single-nucleotide resolution in separating poly(A) tail length, but it's technically challenging and very expensive requiring advanced bioinformatics software tools





https://link.springer.com/article/10.1007/s40242-022-2166-y

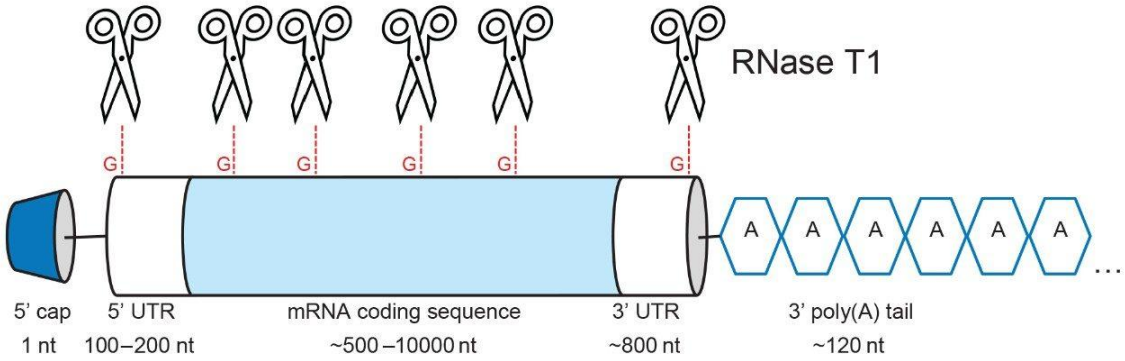
CGE-UV and CGE-LIF methods offer determination of poly(A) tail length and quantitation of peaks

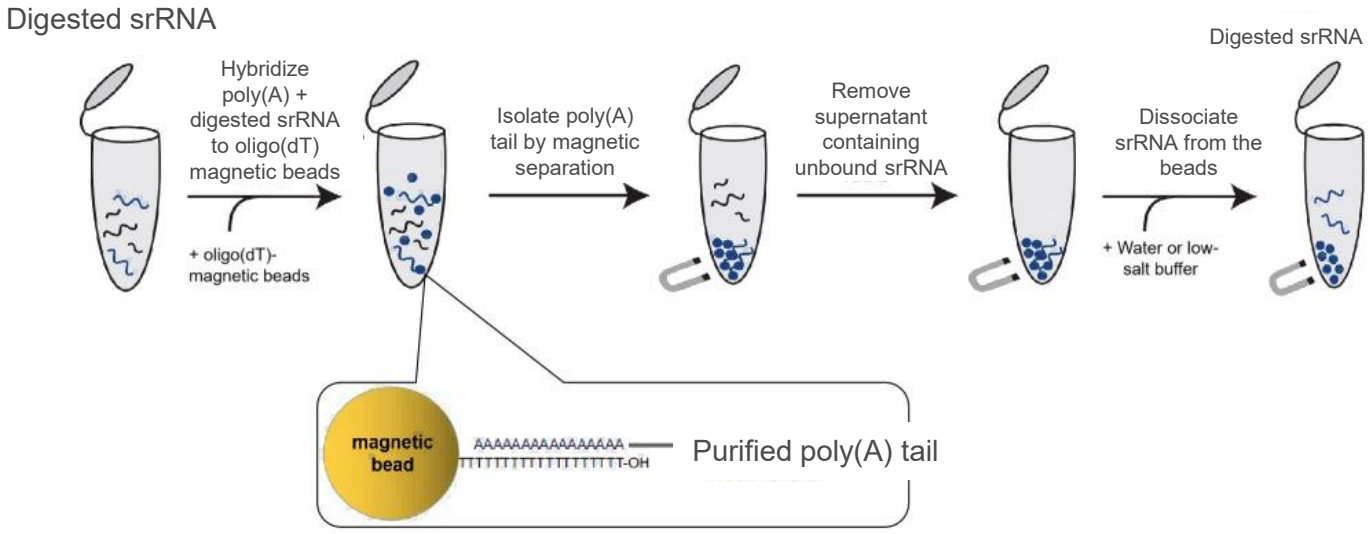
- SCIEX has previously demonstrated workflow for mRNA poly(A) tail analysis by CGE-UV using the ssDNA 100-R Kit on the PA 800 Plus system
- Recently, SCIEX has developed a new kit, applicable to large srRNA poly(A) tail CGE analysis
  - Ready-to-use denaturing gel and reagents allows for separation of single-stranded oligonucleotides
  - CE methods are compatible for use with bare-fused silica (BFS) capillary
  - Capillary conditioning methods, sample injection and separation methods are applicable to both UV and LIF detector

Luo et al., n.d. Retrieved September 8, 2026, from https://sciex.com/content/dam/SCIEX/pdf/tech-notes/biopharma/MKT-30825-APolyA_tail_20240314.pdf

Sample preparation for srRNA poly(A) tail purification

- 50 pmol of 9kb and 16kb srRNA were pre-heated at 70℃ for 5 minutes then placed on ice to denature secondary and tertiary structures
- Sample was digested with RNase T1 and RNase H reaction buffer for 37℃ for 1 hour to release poly(A) tail
- Poly(A) tail was purified with magnetic Dynabeads Oligo (dT)25, washed 3x, and eluted with nuclease free water
- OPTIONAL: Sample was desalted with a spin column and poly(A) tail was collected and prepped for CE analysis (data not included)





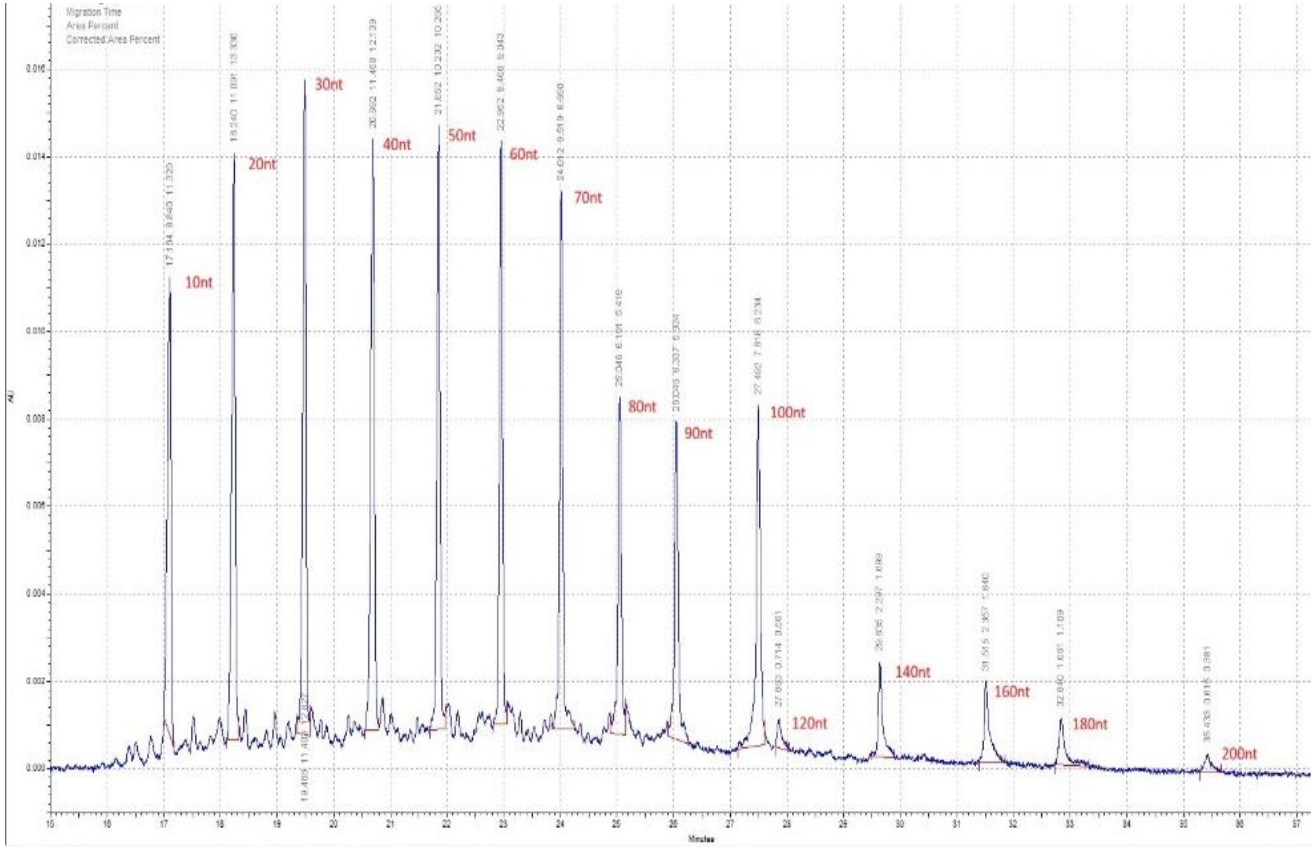
Yeasen Bio. 2023. Various types of magnetic beads in NGS: DNA\RNA\mRNA magnetic beads. www.yeasenbio.com
Gilar and Gaye. 2023. on-Pair Reversed-Phase Liquid Chromatography Method for Analysis of mRNA Poly(A) Tail Heterogeneity. www.waters.com

Assay Method (PA 800 Plus)

- Oligo 200 nt Purity and Integrity kit*
- Capillary: Pre-assembled bare-fused silica capillary
- Detector:
  - UV detector at 254nm
  - LIF detector at 520nm with SYBR Gold added at 25000x
- Separation condition: Electrokinetic
  - Sample injection – 8kv, 5 seconds
  - Sample separation – 8.5kV, reverse polarity
- Samples (Sanger sequence of DNA template):
  - 9.5kb srRNA with 64 Poly(A) tail
  - 15.6kb srRNA with 68 Poly(A) tail
  - 9.7kb srRNA with 154 Poly(A) tail
- Sample Prep:
  - Poly(A) tail purification
  - Dilute to a final concentration of 3µg/mL
    - Add Single-stranded Oligo standard (15 nt)* at 2000x dilution
  - Denature for 70°C for 5 minutes on thermal cycler
    - Immediately place on ice for 5 minutes

*Prototypes were used in the evaluation of this kit

Clear resolution obtained with Oligo 200 nt ladder*

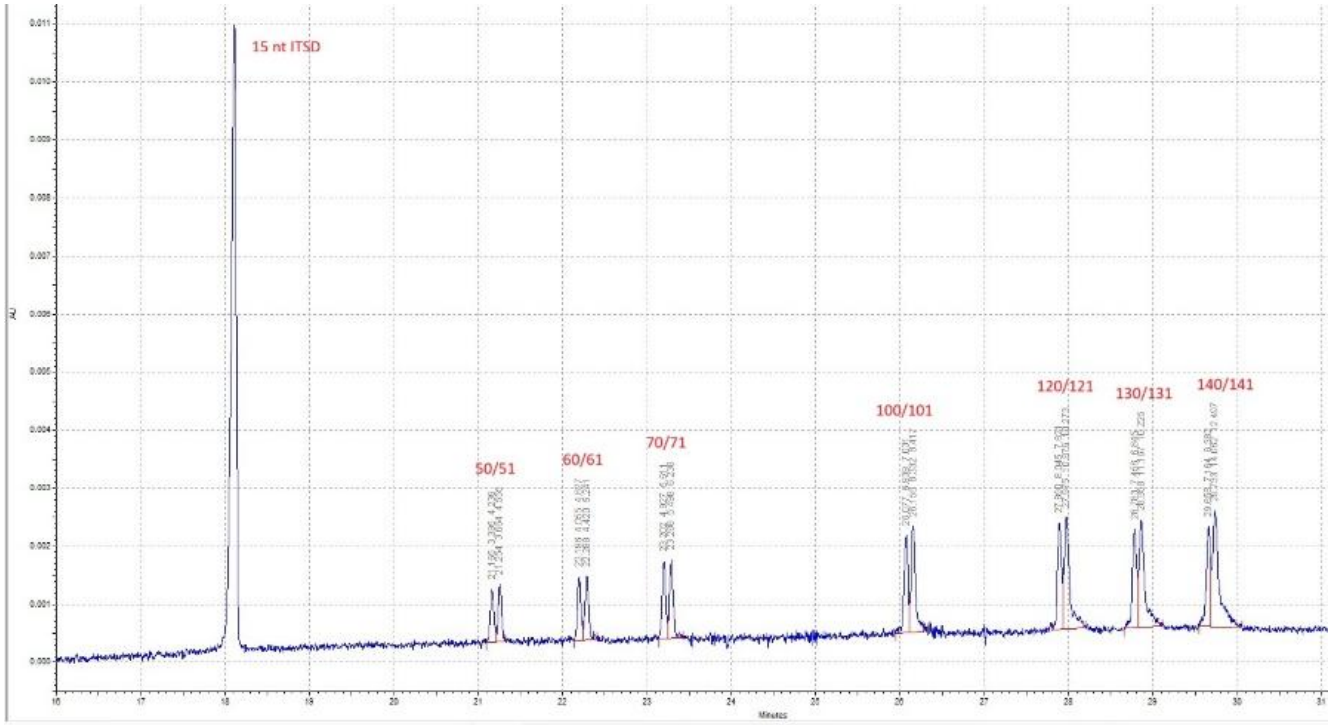


Peak (nt)	Migration Time (min)	Migration Time STDEV (min)	Corrected Peak Area (%)	Corrected Area STDEV (%)
10	17.2	0.1	11.4	0.1
20	18.3	0.1	13.3	0.1
30	19.6	0.1	13.2	0.5
40	20.8	0.2	12.2	0.02
50	22.0	0.2	10.3	0.1
60	23.1	0.2	9.3	0.3
70	24.1	0.2	8.6	0.1
80	25.2	0.2	5.4	0.1
90	26.2	0.2	5.2	0.2
100	27.6	0.2	6.3	0.1
120	28.0	0.2	0.5	0.1
140	29.8	0.2	1.6	0.1
160	31.7	0.2	1.5	0.1
180	33.0	0.2	0.9	0.3
200	35.6	0.2	0.4	0.04

- Results are reported as an average of 2 sample separation reps
- Standard deviation for migration time and corrected area is <0.5 for all peaks

*Prototypes were used in the evaluation of this kit

Single-nucleotide separation achieved with Oligo 200 nt test mix*



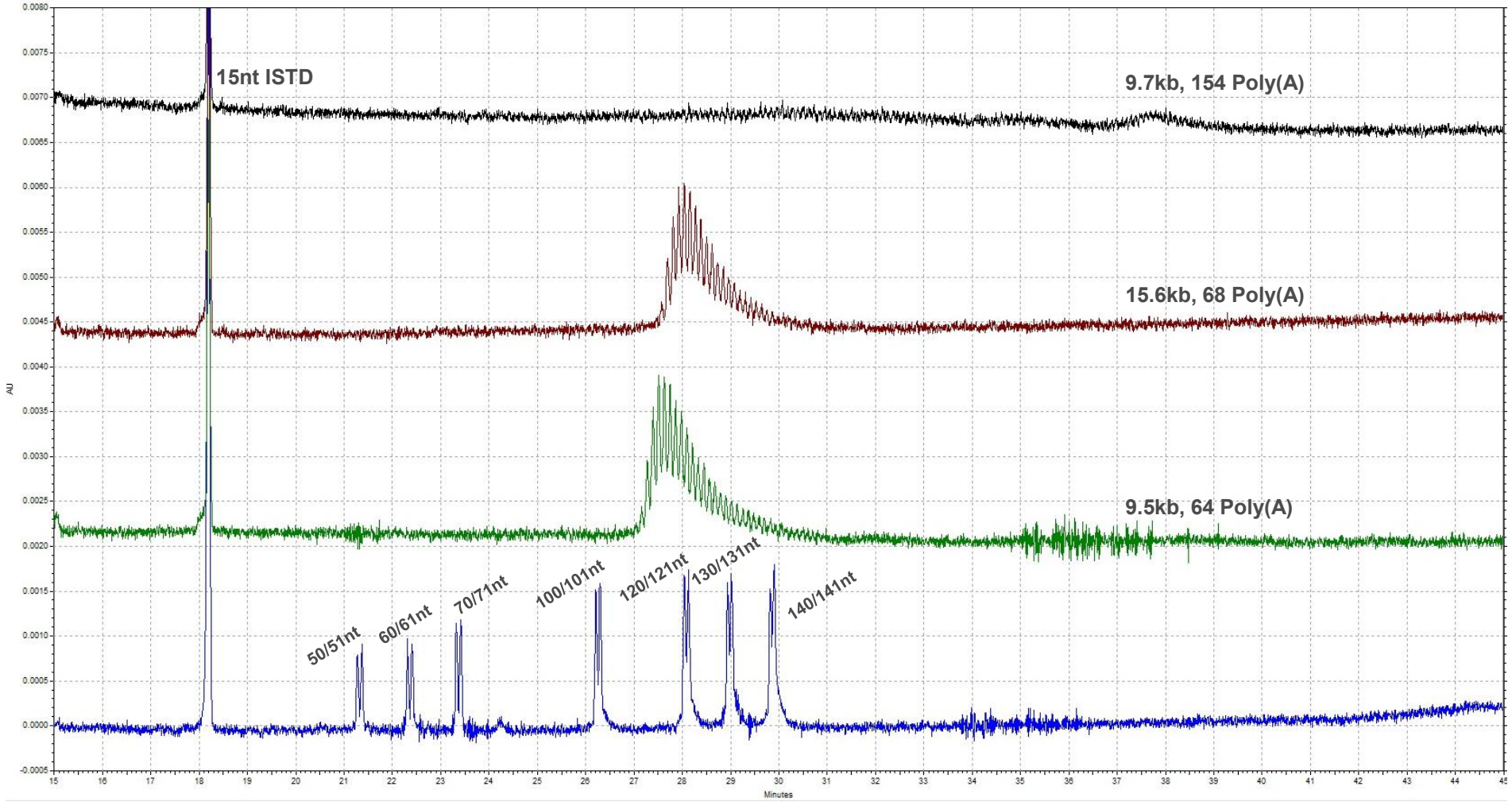
Peak (nt)	Migration Time (min)	Migration Time STDEV (min)	Corrected Peak Area (%)	Corrected Area STDEV (%)
ISTD	18.2	0.1	-	-
50	21.3	0.1	4.4	0.1
51	21.4	0.1	4.6	0.1
60	22.3	0.1	4.9	0.2
61	22.4	0.1	5.0	0.2
70	23.3	0.1	5.8	0.2
71	23.4	0.1	6.3	0.2
100	26.2	0.1	7.1	0.4
101	26.3	0.1	8.9	0.4
120	28.1	0.1	7.8	0.4
121	28.2	0.1	10.0	0.5
130	29.0	0.1	7.1	0.4
131	29.0	0.1	9.7	0.8
140	29.9	0.1	6.7	0.3
141	29.9	0.2	11.8	0.4

- Results are reported as an average of 6 sample separation reps
- Standard deviation for migration time and corrected area is <1.0 for all peaks

*Prototypes were used in the evaluation of this kit

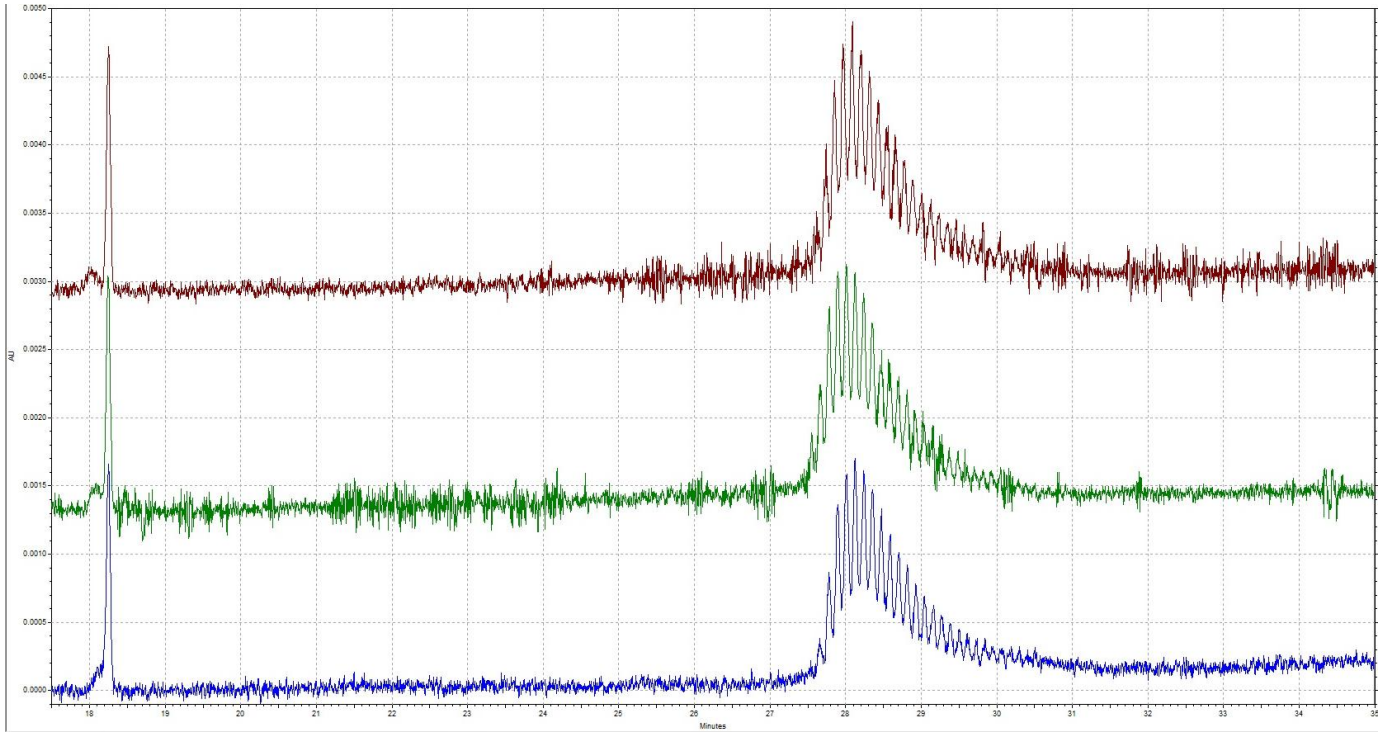
Analysis of Poly(A) tail purified from srRNA by CGE-UV

- "Hill" shape observed for Poly(A) tail separation
  - Low, single-nucleotide resolution achieved for curved portion of the "hill"
  - Individual peaks are more jagged than sharp, difficult to count and integrate
  - No separation for bases of the "hill"
- Migration times reflect theoretical Poly(A) tail length



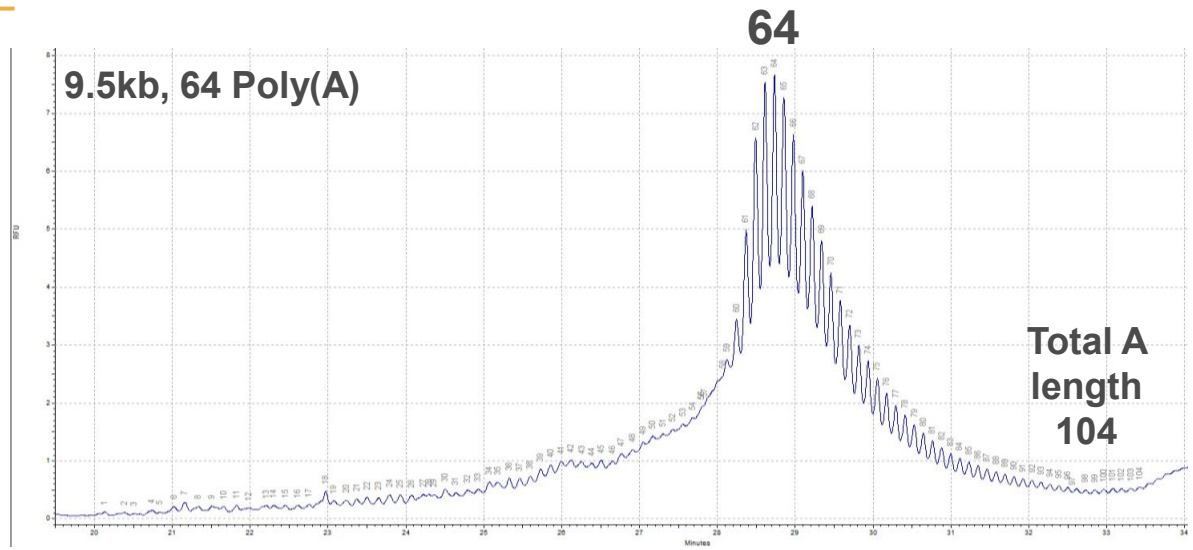
Sample Concentration 3µg/mL

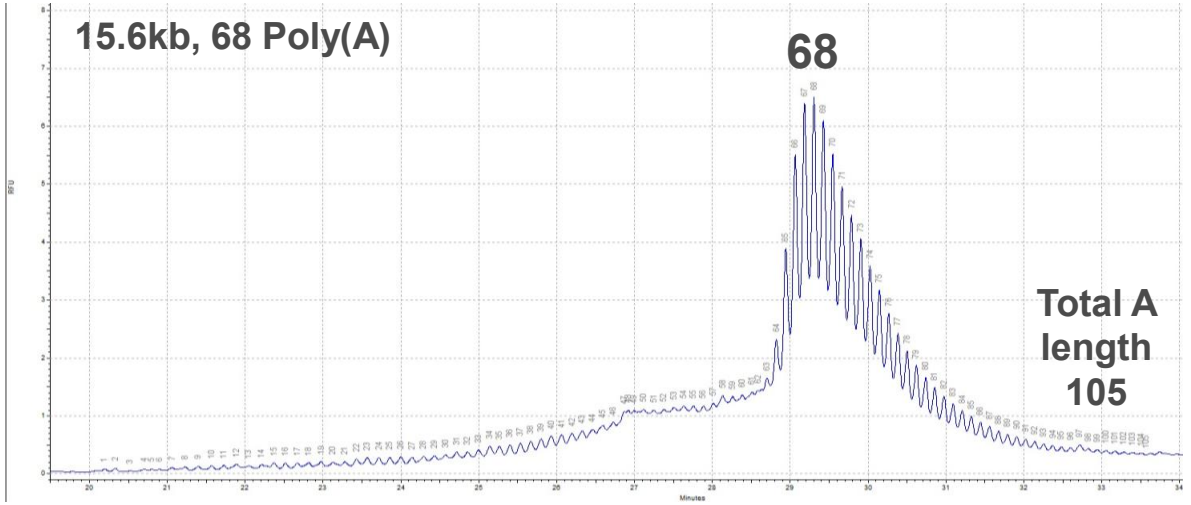
High sample and gel reproducibility across multiple replicates



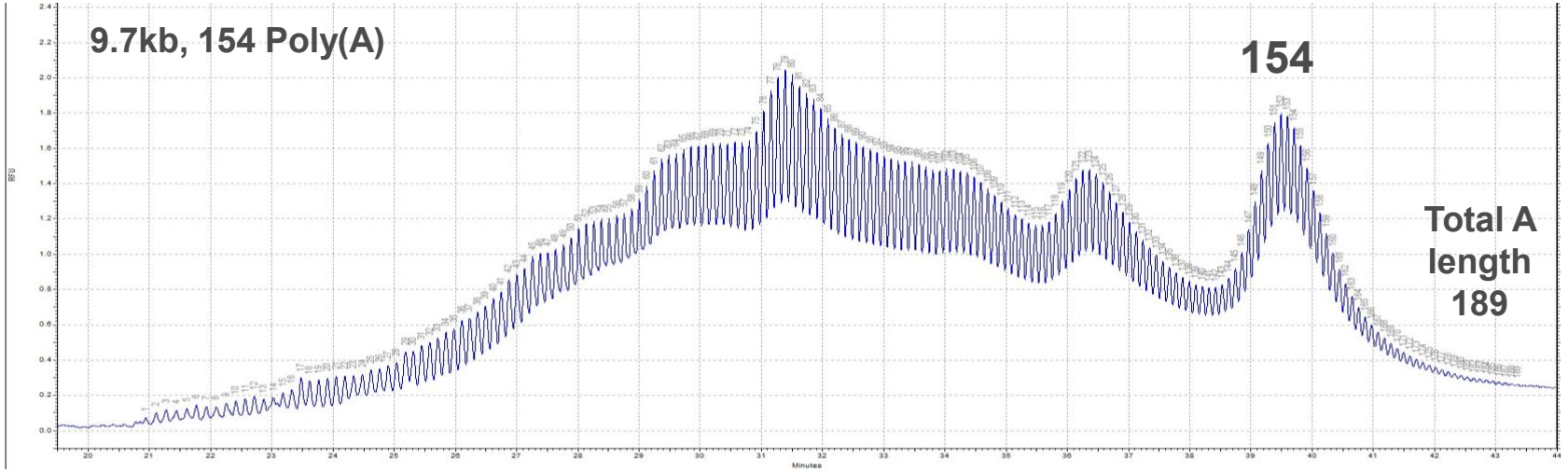
- Purified 64 Poly(A) tail from 9.5kb srRNA
- 3 separation runs at 3µg/mL performed for each sample
- High reproducibility between all replicates for the UV detector

Analysis of Poly(A) tail purified from srRNA by CGE-LIF





- High, single-nucleotide resolution observed for Poly(A) tail separation with LIF detector
  - Allows for easier individual peak integration
- Higher intensity (RFU) compared to UV detector (AU)

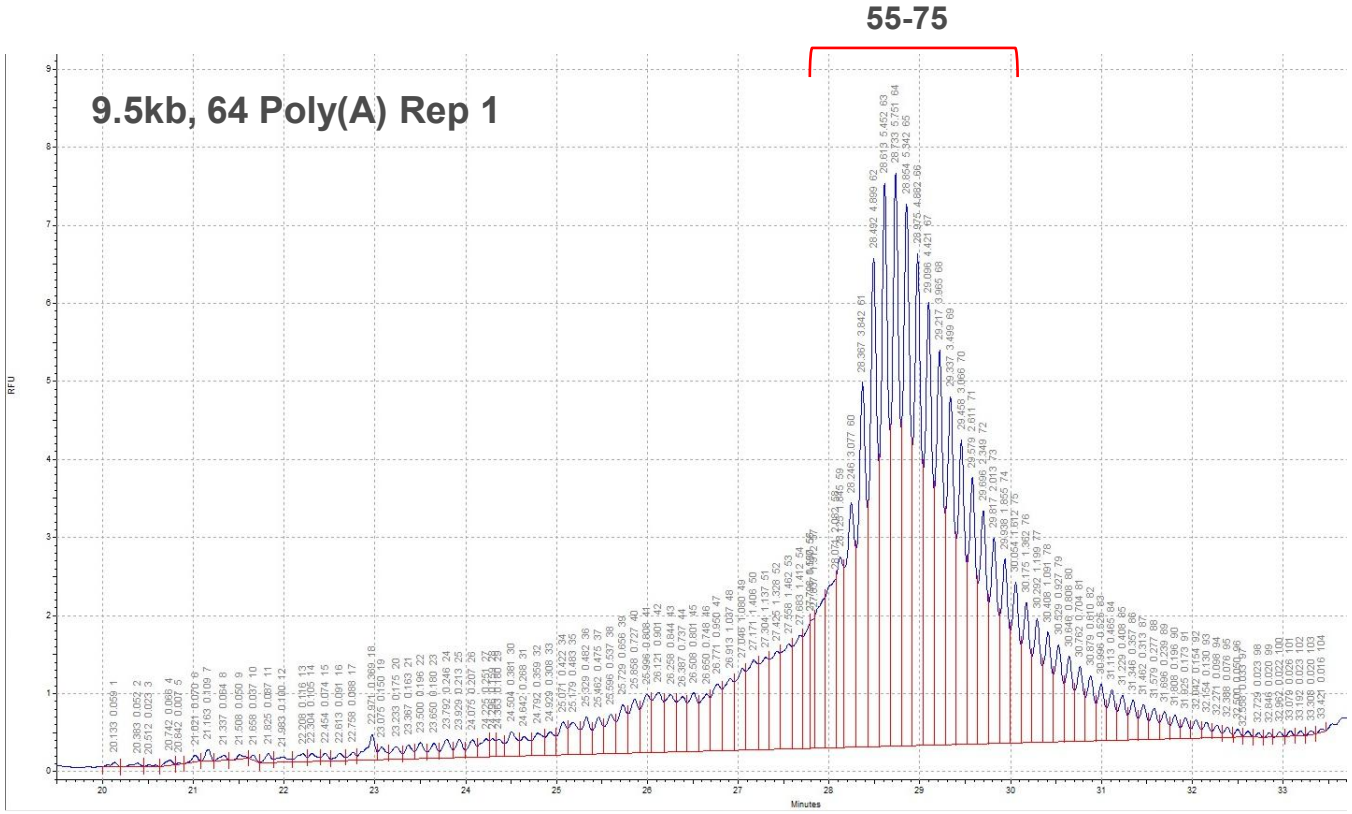


Sample Concentration 3µg/mL

Individual peak identification and integration achieved with CGE-LIF

Peaks (nt)	Migration Time (min)	Migration Time STDEV (min)	Corrected Area (%)	Corrected Area STDEV (%)
55	27.8	0.2	1.1	0.0
56	27.8	0.3	0.9	0.3
57	27.9	0.2	1.6	0.4
58	28.0	0.2	2.2	0.9
59	28.1	0.3	2.0	0.2
60	28.2	0.3	3.1	0.1
61	28.4	0.3	3.9	0.1
62	28.5	0.3	5.0	0.2
63	28.6	0.3	5.6	0.2
64	**28.7**	**0.3**	**5.8**	**0.1**
65	28.9	0.3	5.5	0.2
66	29.0	0.3	5.0	0.2
67	29.1	0.3	4.5	0.2
68	29.2	0.3	4.1	0.1
69	29.3	0.3	3.5	0.1
70	29.5	0.3	3.0	0.0
71	29.6	0.3	2.7	0.1
72	29.7	0.3	2.4	0.2
73	29.8	0.3	2.1	0.1
74	29.9	0.3	1.9	0.1
75	30.1	0.3	1.6	0.0

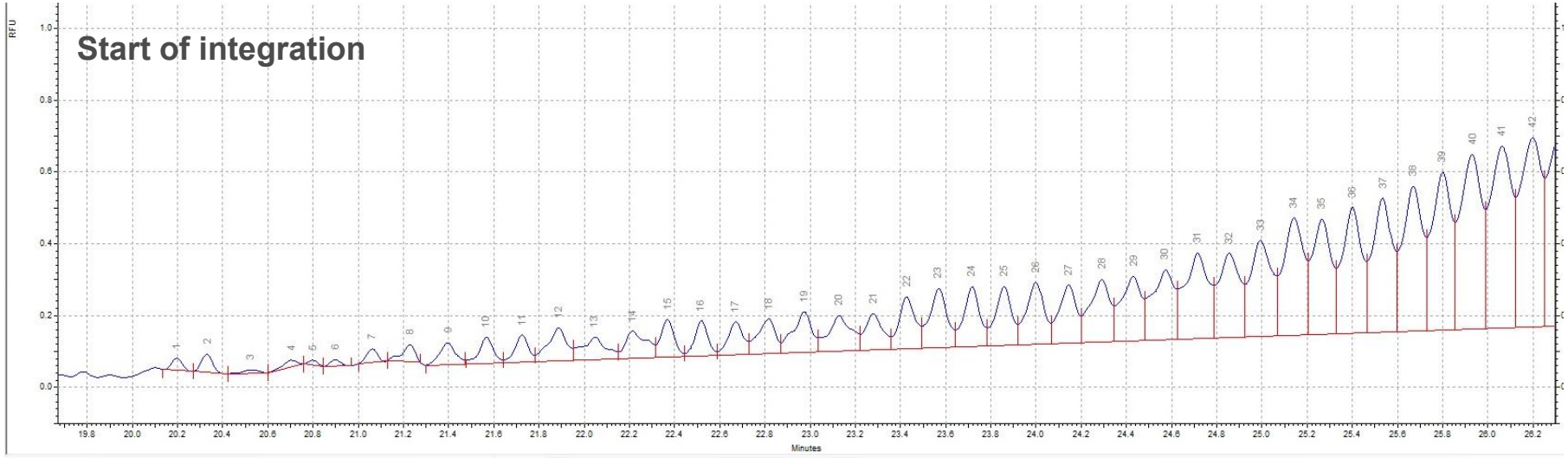
All data from an average of 3 replicates

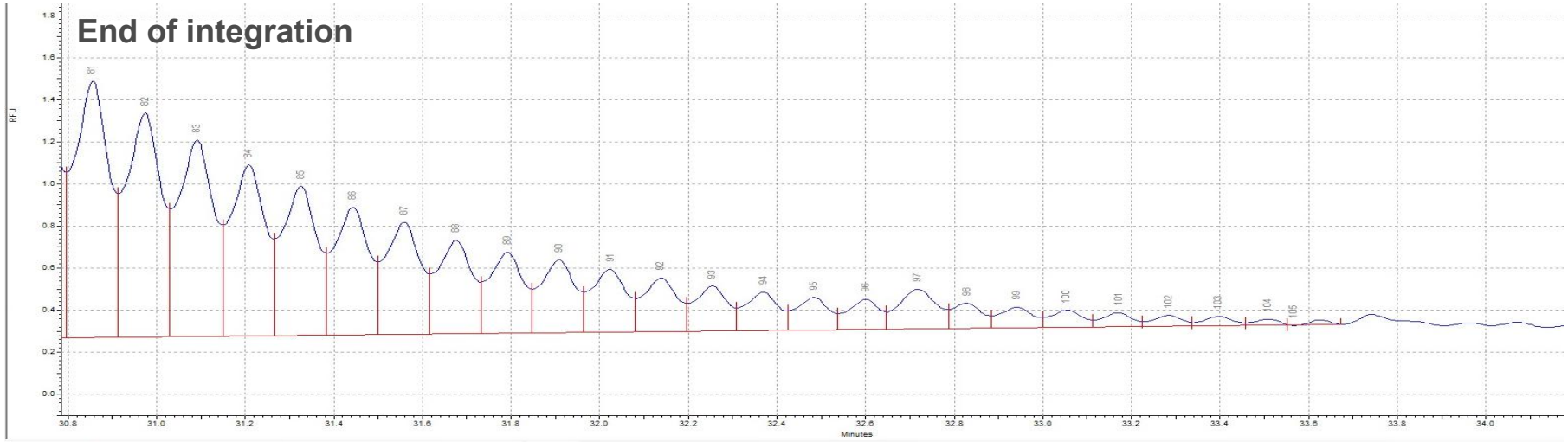


Migration Time (min)					
ISTD	Main Peak	Total Poly(A) tail length	Peak Apex	Relative Migration Time	Intensity (RFU)
18.900	28.732	104	64	1.520	5.5

Manual integration of Poly(A) tail quantification

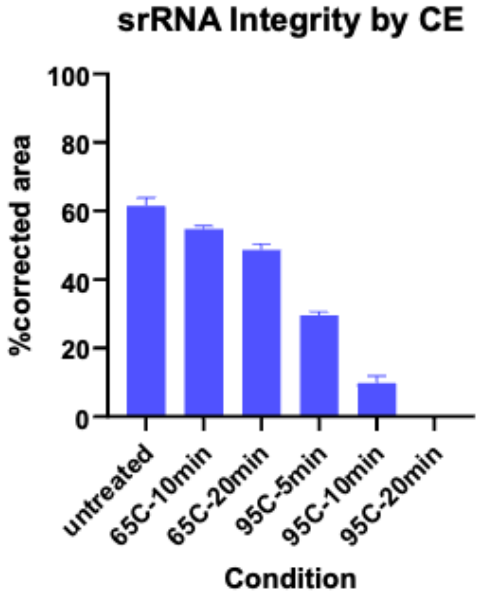
- Manual integration based off first and last detectable peak
  - Determined by difference in signal to noise ratio from baseline
- Allows for some subjectivity in data analysis
  - Additional controls with known nucleotides could be beneficial



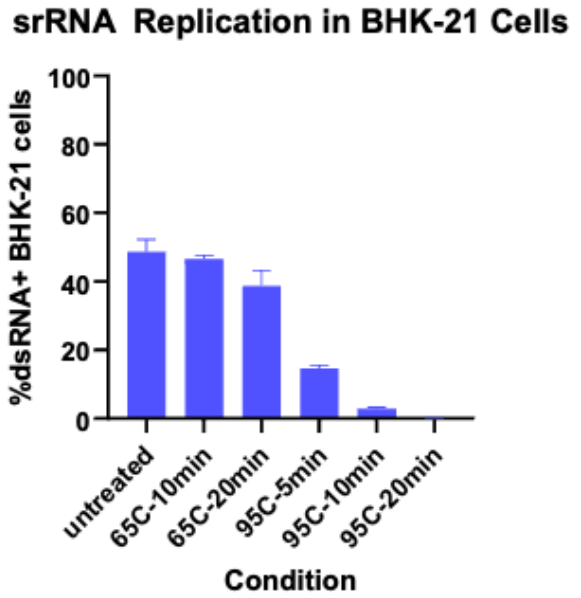


All data from an average of 3 replicates

CGE-LIF method is a stability indicating assay



Condition	Rep 1	Rep 2	Rep 3	Average
untreated	64	60	60	62
10min@65°C	55	56	54	55
20min@65°C	50	46	47	48
5min@95°C	30	28	30	30
10min@95°C	8	12	10	10
20min@65°C	0	0	0	0

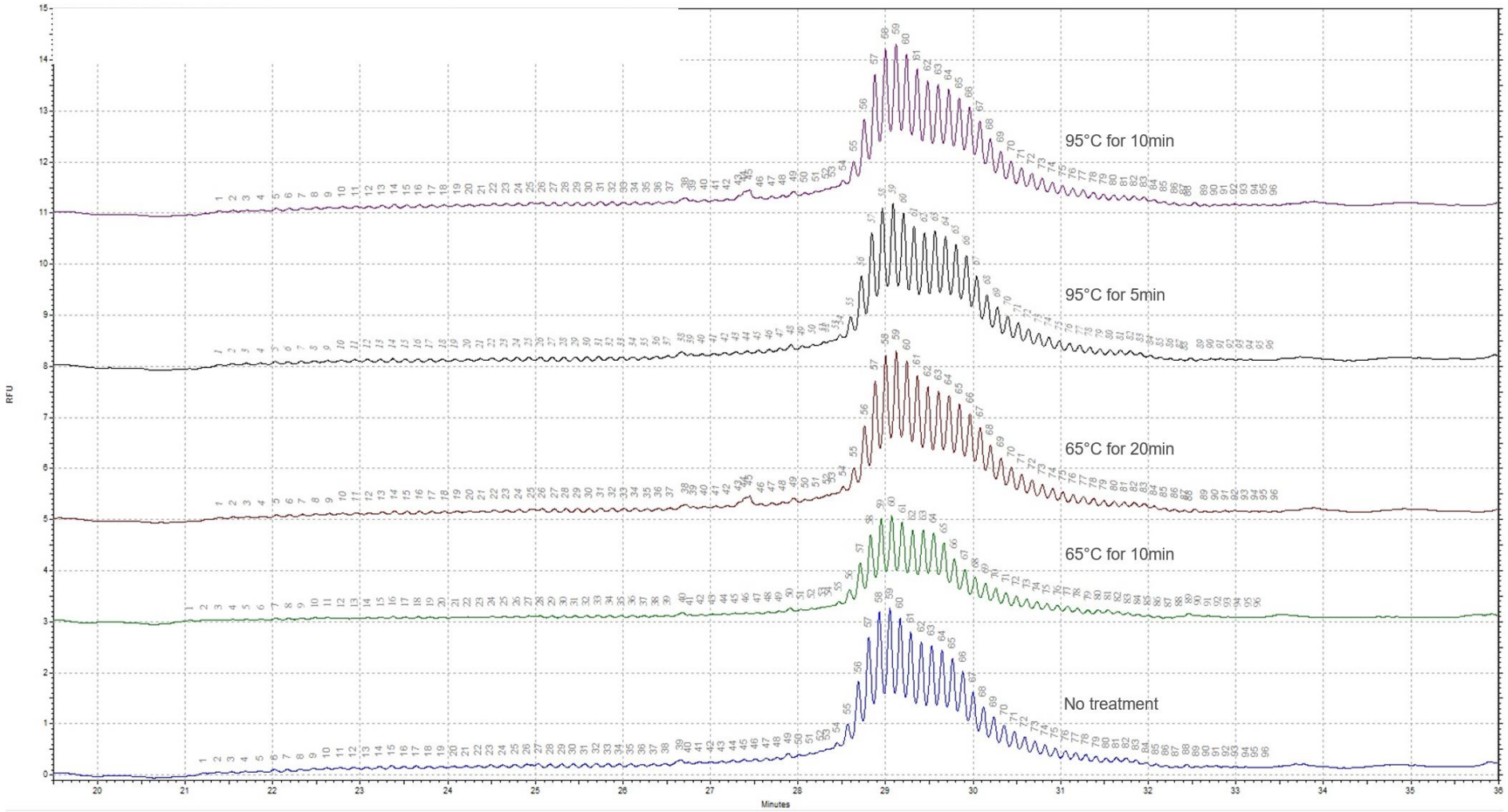


Condition	Rep 1	Rep 2	Rep 3	Average
untreated	46	47	53	49
10min@65°C	47	45	47	47
20min@65°C	43	34	38	39
5min@95°C	15	15	14	15
10min@95°C	3	3	2	3
20min@65°C	0	0	0	0

Similar trend seen with %CPA in capillary electrophoresis and %dsRNA+ detection (srRNA replication) in BHK-21 cells electroporated with a large srRNA sample

Stability of Poly(A) tail unaffected by elevated temperature exposure of srRNA

- Prior to Poly(A) extraction, srRNA was heat treated at several different conditions
  - No treatment
  - 65°C for 10 minutes
  - 65°C for 20 minutes
  - 95°C for 5 minutes
  - 95°C for 10 minutes
- No significant difference between any of the conditions
  - This supports stability of Poly(A) unaffected by heat



15.6kb, 68 Poly(A) tail at 3µg/mL

Conclusions

- New prototype kit with proprietary gel has been developed to separate single-stranded oligonucleotides using a BFS capillary
- Single-nucleotide resolution and high reproducibility achieved using the UV and LIF detector
  - <1.0% relative standard deviations in peak migration time (min) and corrected area (%)
- Accurate individual peak integration possible with LIF detector
- Some minimal drawbacks but not limited to:
  - Large amount of RNA required to extract Poly(A) samples
  - Time required to integrate individual peaks



Acknowledgements

- Replicate Bioscience : Andrew Geall and Shigeki Miyake-Stoner
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