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# Analytical Approaches for Profiling Polyadenylation (PolyA) Tails in mRNA

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# Strategy for Profiling Poly A Tails in mRNA

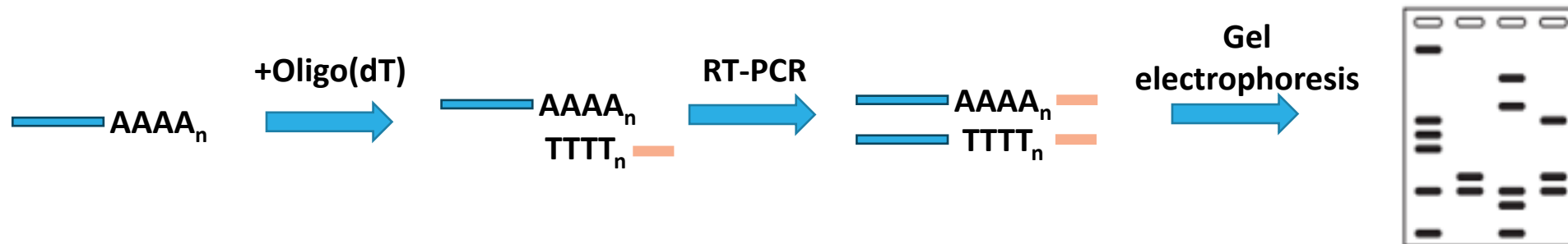
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- Gel electrophoresis
- Sequencing
- Capillary electrophoresis
- Liquid chromatography
- Liquid chromatography-mass spectrometry (LC-MS)

# Poly A Test by Gel Electrophoresis

- Rnase H/Oligo(dT) assay
- PCR amplification based assays
  - Rapid amplification of cDNA ends poly(A) test (RACE-PAT)
  - Ligase-mediated poly(A) test (LM-PAT)
  - Extension poly(A) test (ePAT)
  - Splint-mediated poly(A) test (sPAT)

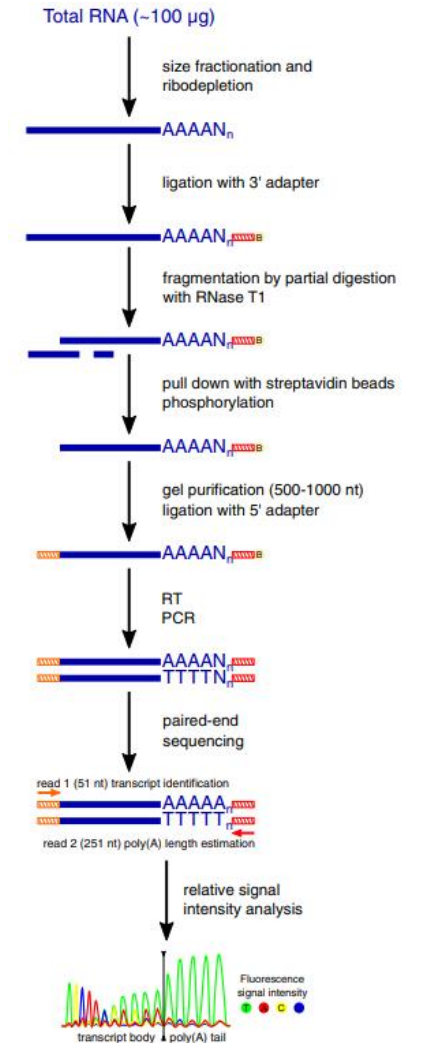
Example work flow of PAT by Gel electrophoresis



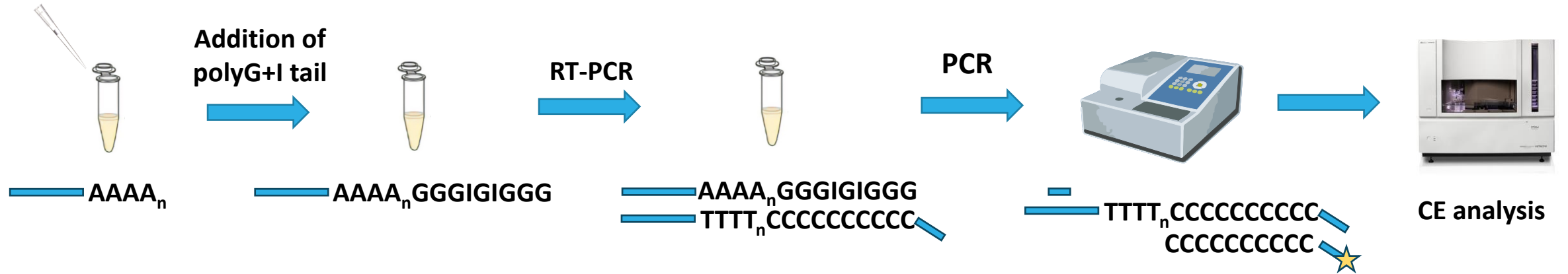
# Poly A Test by Sequencing

| Approach          | Method    | Adapter addition                | Fragmentation | Limitation                                     |
|-------------------|-----------|---------------------------------|---------------|--|
| Illumina          | PAL-Seq   | Splint Ligation                 | Rnase T1      | PCR-bias<br>RT-bias<br>Technically challenging |
|                   | TAIL-Seq  | Ligation                        | Rnase T1      |  |
|                   | mTAIL-Seq | Ligation<br>hairpin DNA adapter | Rnase T1      |  |
|                   | PAT-Seq   | Extension with<br>Polymerase    | Rnase T1      |  |
| PacBio            | FLAM-Seq  | Ligation<br>G/I tailing         | N/A           | PCR-bias<br>RT-bias<br>Technically challenging |
|                   | PAIso-Seq | Extension with<br>Polymerase    | N/A           |  |
| ONT<br>(Nanopore) | DRS       | Splint Ligation                 | N/A           | High error rate                                |

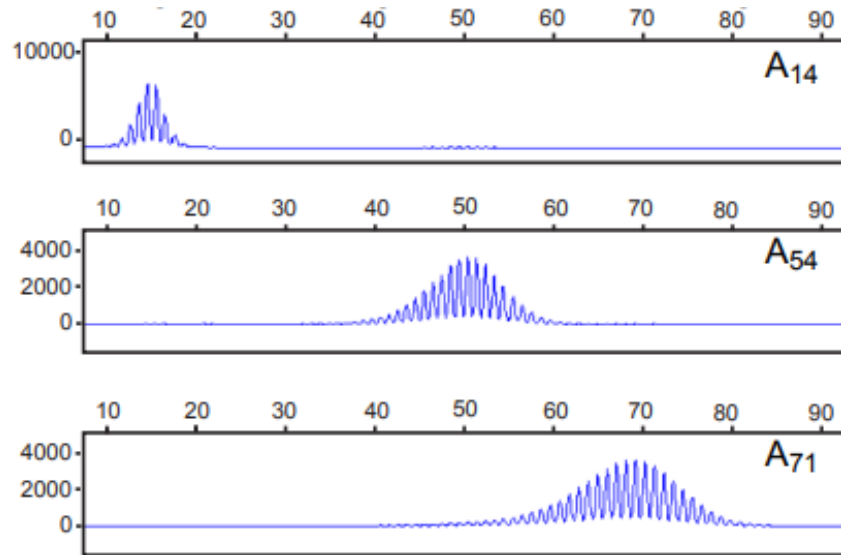
## Example work flow of TAIL-Seq



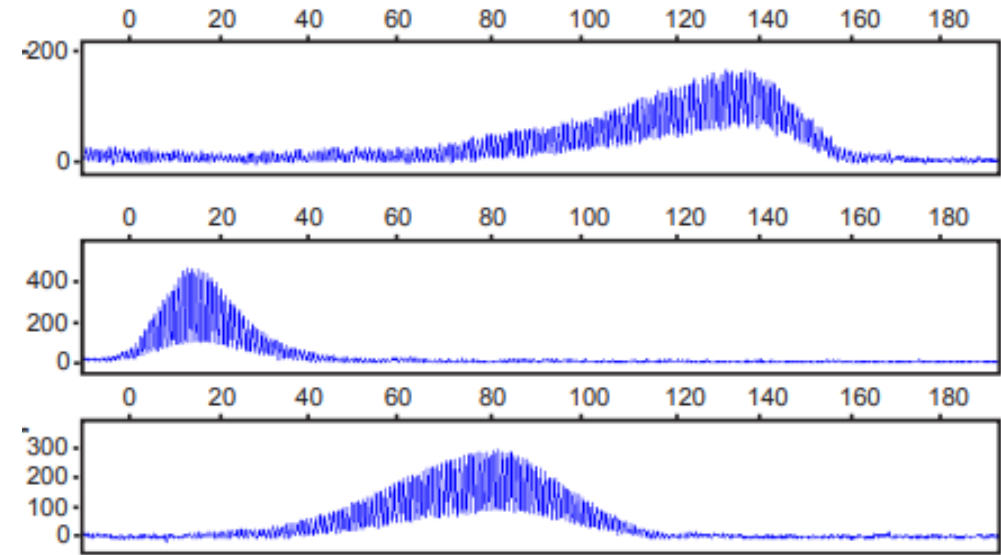
# Poly A Test by Capillary electrophoresis



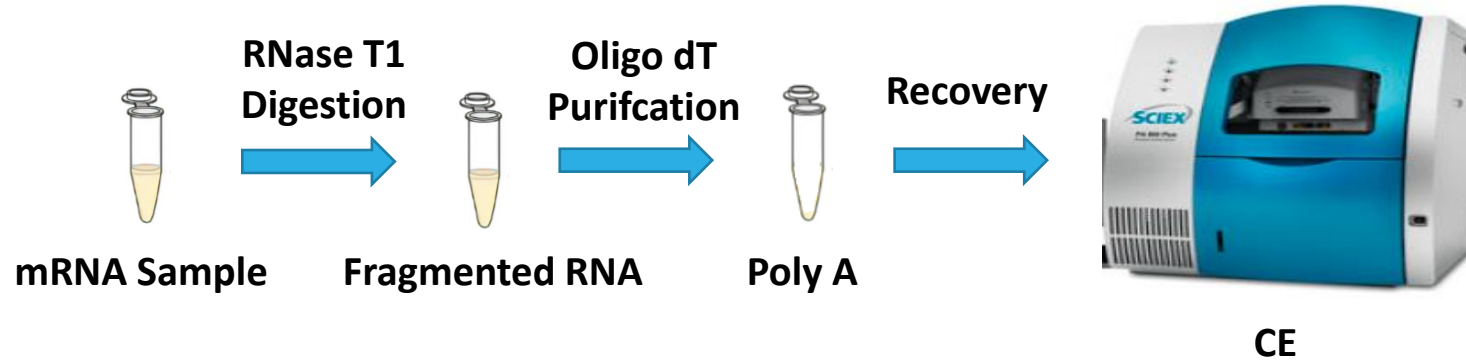
### Calibration with Standards



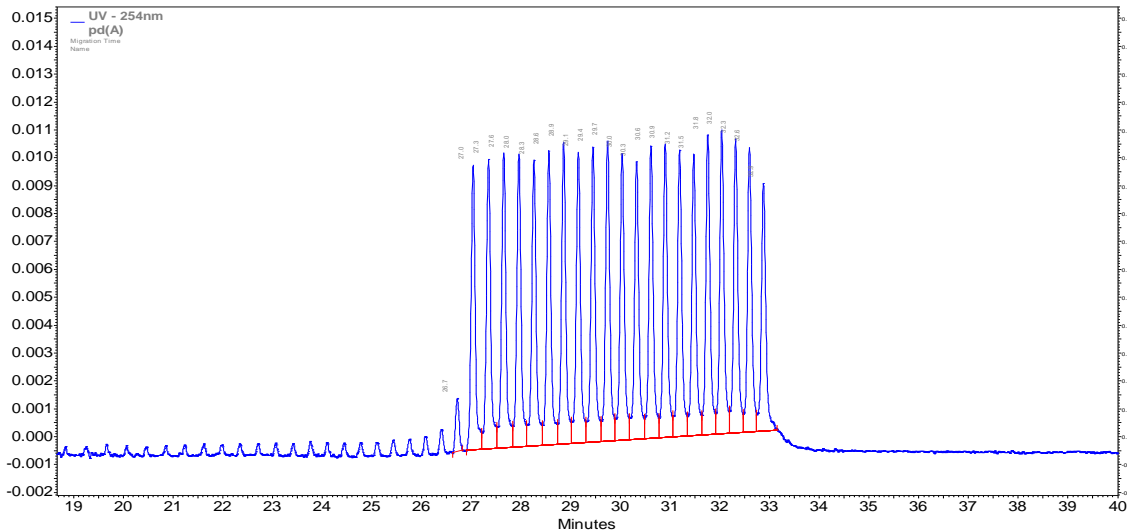
### Sample analysis



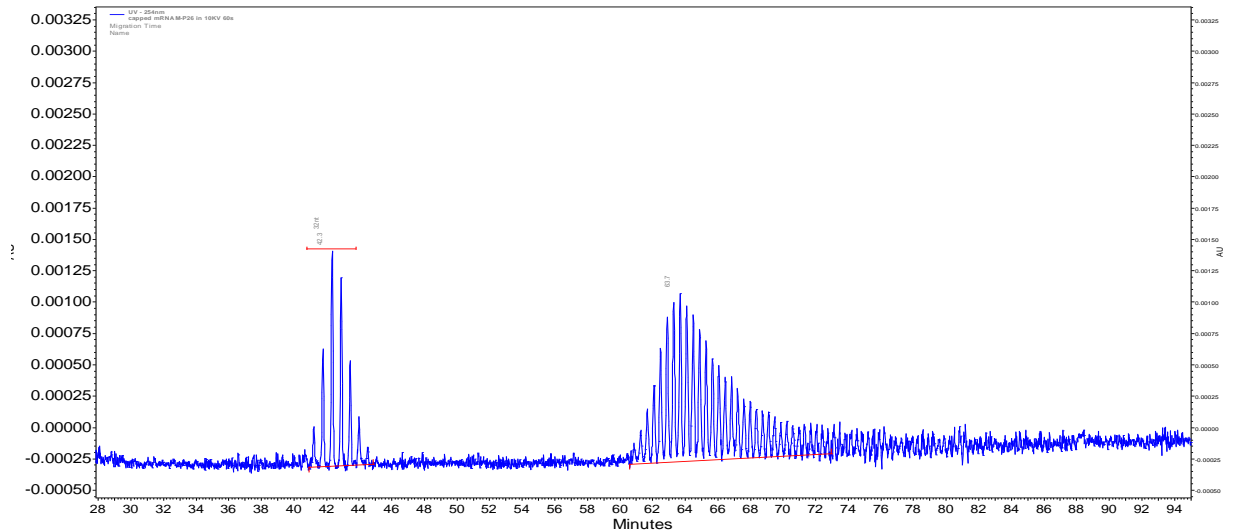
# Poly A Test by Capillary electrophoresis



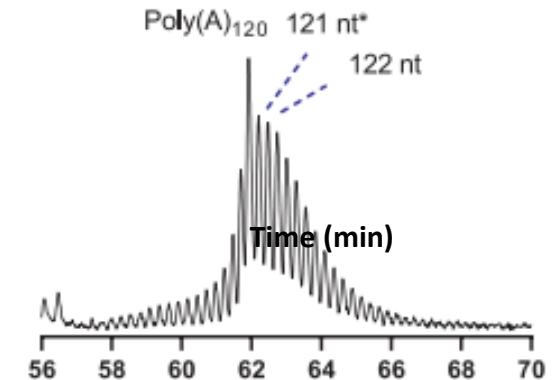
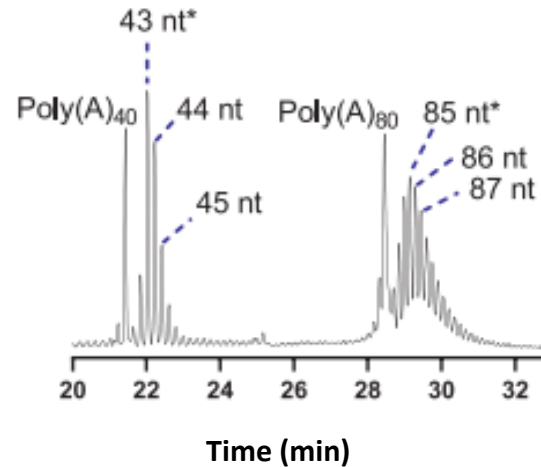
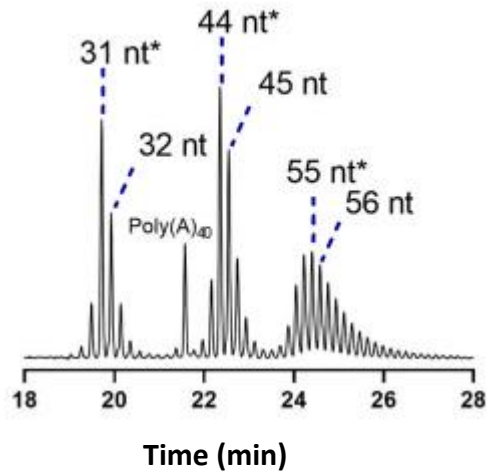
pd(A)40-60 Standard



Sample analysis



# Poly A Test by Capillary electrophoresis



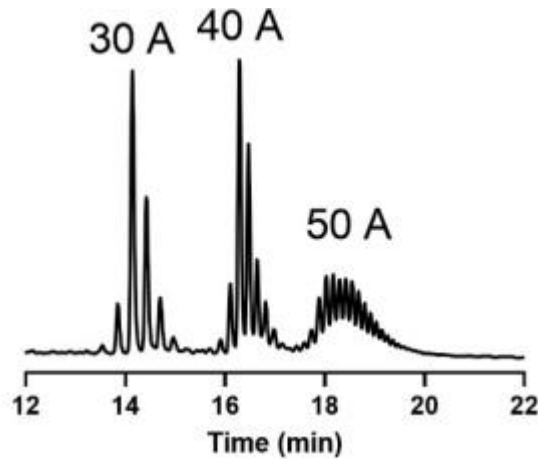
- Need synthetic poly(A) length markers;
- Single nucleotide resolution;
- Result comparable to LC-MS and Nanopore sequencing;
- Readily qualified as a release method.





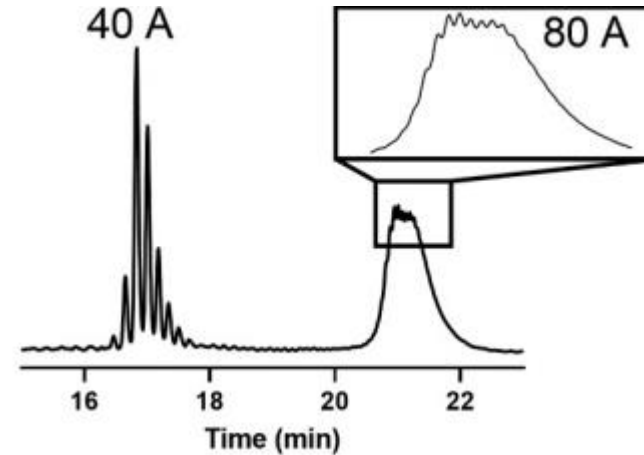
# Poly A Test by AEX

— (AAA...)<sub>30</sub>XGX(AAAA...)<sub>40</sub>XGX(AAAAA...)<sub>50</sub>

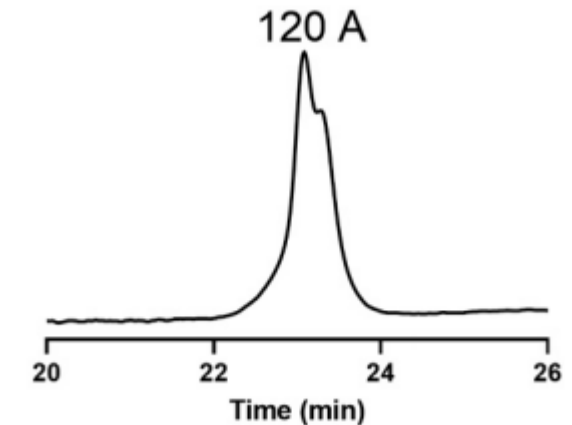


Analyte: negatively charged nucleic acid  
Stationary phase: positively charged anion exchange resins  
Mobile phase: buffer with various ionic strength

— (AAAA...)<sub>40</sub>XGX(AAAAAAAA...)<sub>80</sub>



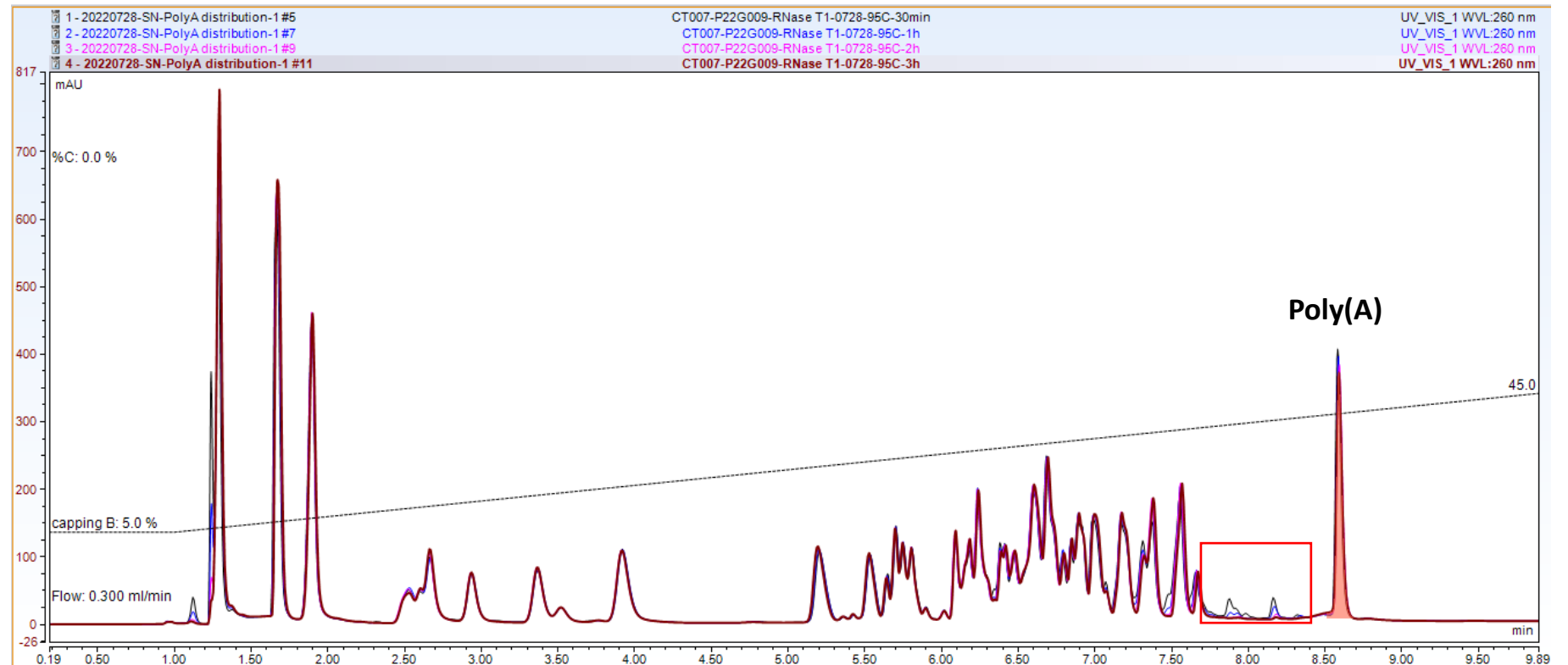
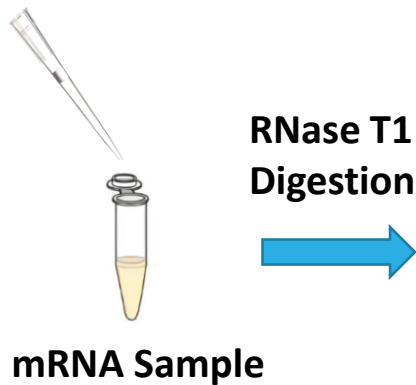
— (AAAAAAAAAAAA...)<sub>120</sub>



- Good resolution for short poly A fragments.
- Hard to achieve single nucleotide resolution for longer poly A fragments.

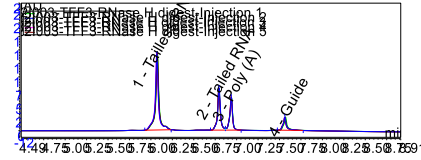
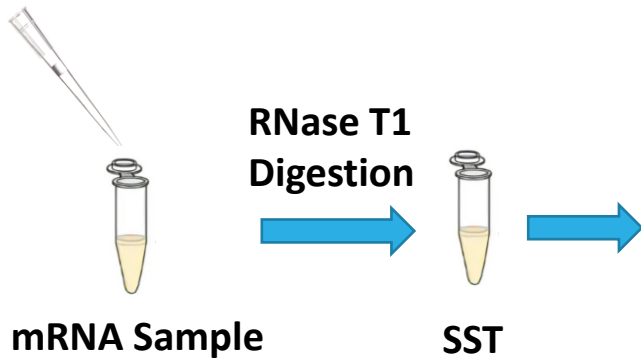
# Poly A Test by RP-LC

Analyte: polarity (hydrophobicity)  
Stationary phase: non-polar medium  
Mobile phase: aqueous solution of polar organic solvent



➤ Separate poly(A) fragments with other oligo fragments.

# Poly A Test by RP-LC



1

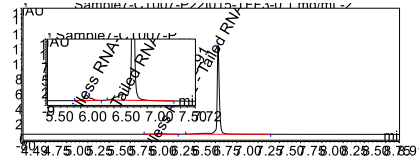
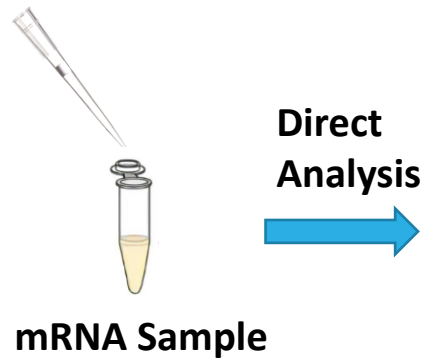
1. Tailless mRNA
2. Tailed mRNA
3. Poly(A)
4. Guide Oligo

2

3

RNase H digestion(SST)

4

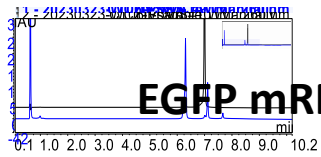


Tailless mRNA

Tailed mRNA

Sample Analysis  
Poly(A) tailed 97.09%

# Poly A Test by RP-LC-mRNA



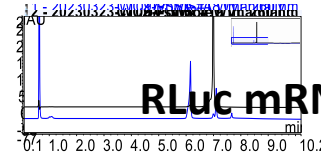
1

1. Tailless mRNA
2. Tailed mRNA
3. Poly(A)
4. Guide Oligo

3

2 4

Sample  
SST



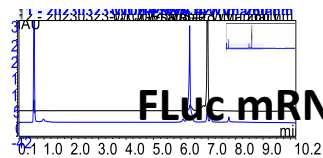
1

1. Tailless mRNA
2. Tailed mRNA
3. Poly(A)
4. Guide Oligo

3

2 4

Sample  
SSST



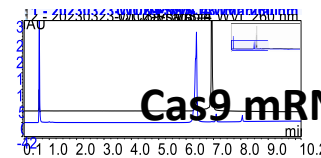
1

1. Tailless mRNA
2. Tailed mRNA
3. Poly(A)
4. Guide Oligo

2 3

4

Sample  
SSST



1

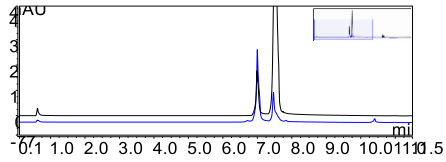
1. Tailless mRNA
2. Tailed mRNA
3. Poly(A)
4. Guide Oligo

2 3

4

Sample  
SSST

# Poly A Test by RP-LC-saRNA



**FLuc saRNA**  
(>5k nt)

1. Tailless mRNA
2. Tailed mRNA
3. Poly(A)
4. Guide Oligo

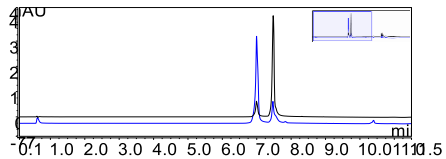
1

2

3

Sample  
SST

4



**EGFP saRNA**  
(>5k nt)

1. Tailless mRNA
2. Tailed mRNA
3. Poly(A)
4. Guide Oligo

1

2

3

Sample  
SST

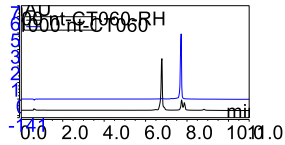
4

- Mobile phases has no strong ion-pairing reagents, such as TEA.
- Applicable to RNAs of various length.

# Poly A Test by RP-LC vs IPRP-LC

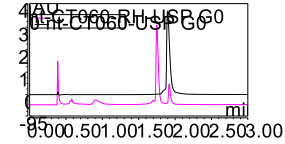
no strong ion-pairing reagents

with strong ion-pairing reagents



Tailed mRNA

Tailless mRNA

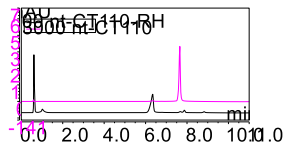


Tailed mRNA

Tailless mRNA

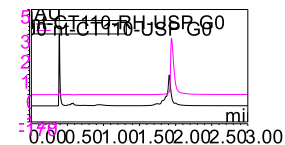
Sample 1  
Sample 1 SST

Sample 1  
Sample 1 SST



Tailed mRNA

Tailless mRNA



Tailed mRNA

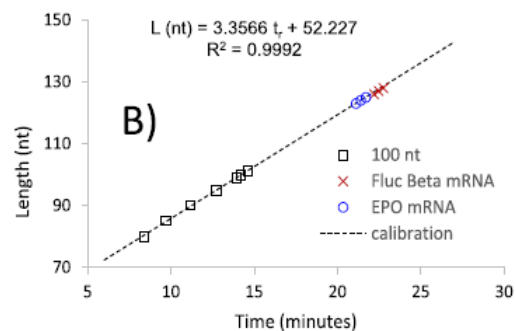
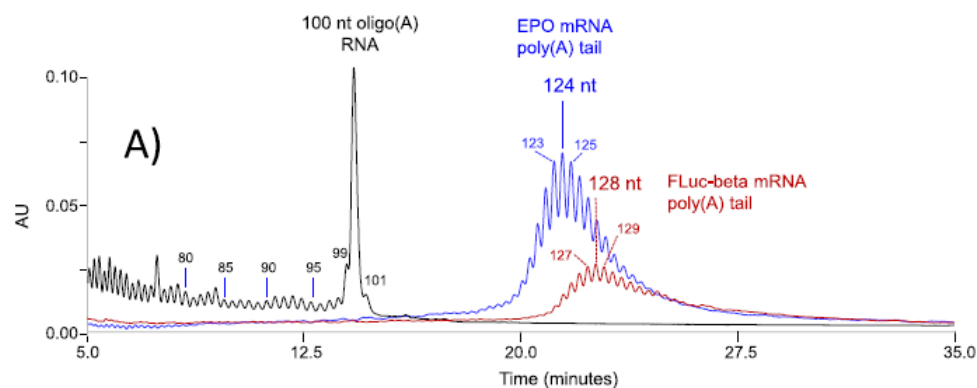
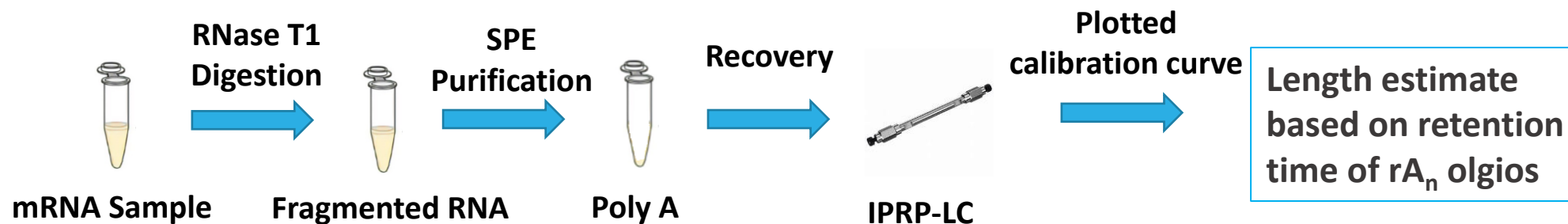
Tailless mRNA

Sample 2  
Sample 2 SST

Sample 2  
Sample 2 SST

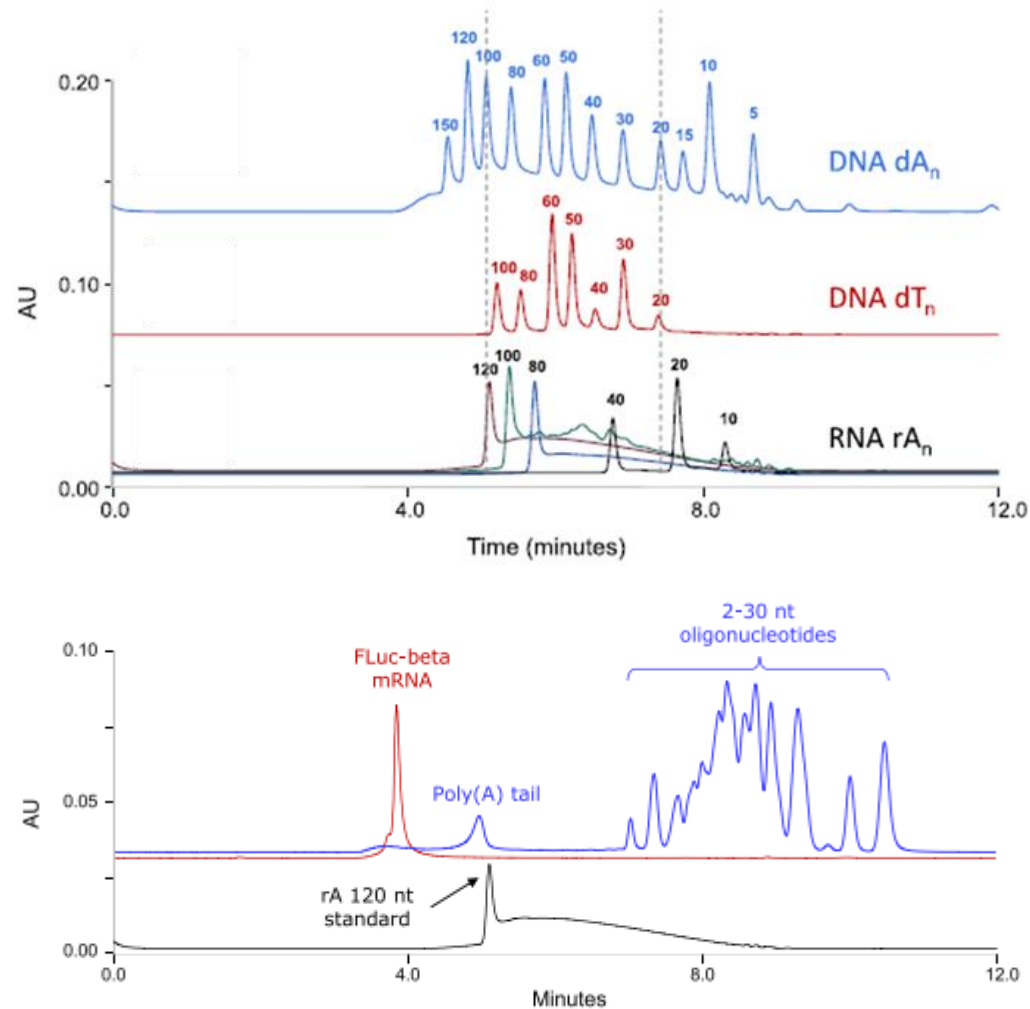
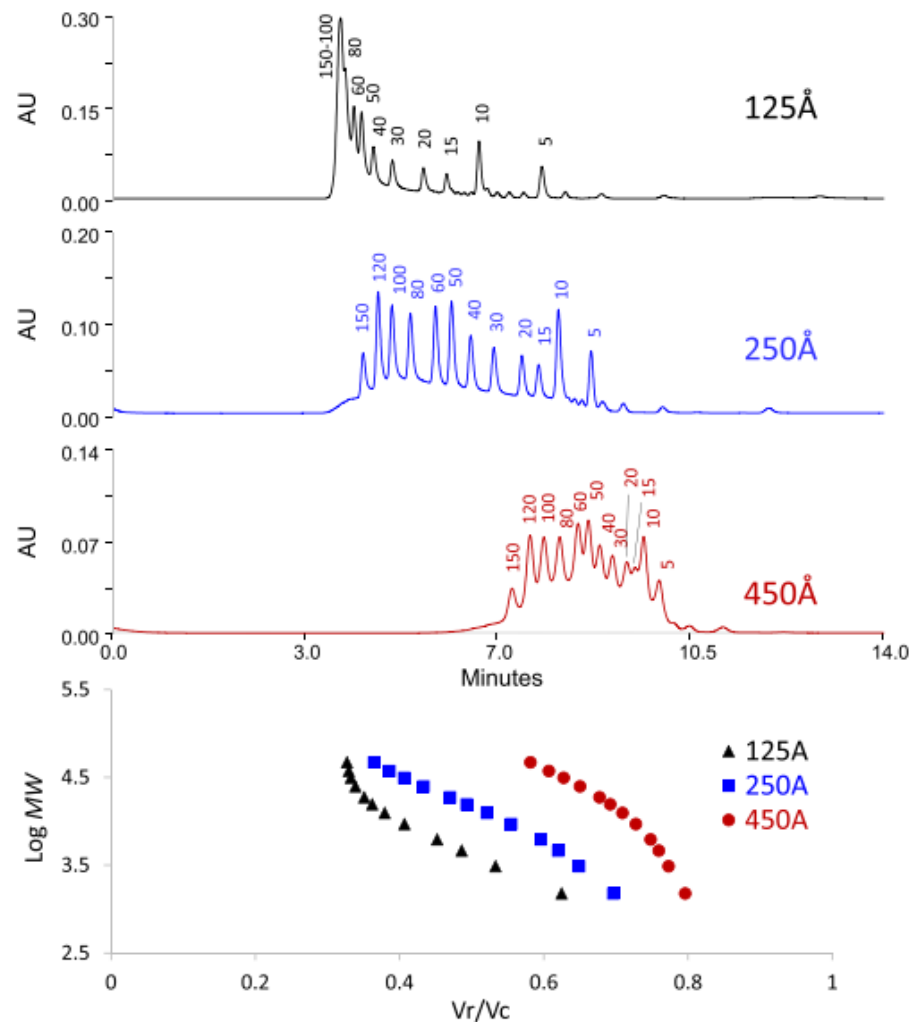
➤ Ion-pairing reagents reduce the separation between tailed and tailless mRNA

# Poly A Test by IPRP-LC



- Hydrophobic OAA ion-pairing mobile phase (not compatible with MS)
- Optimized for the separation of 100–150 nt poly(A) tail oligonucleotides.
- Visualize the poly(A) tail heterogeneity, but cannot archive baseline separation.

# Poly A Test by SEC

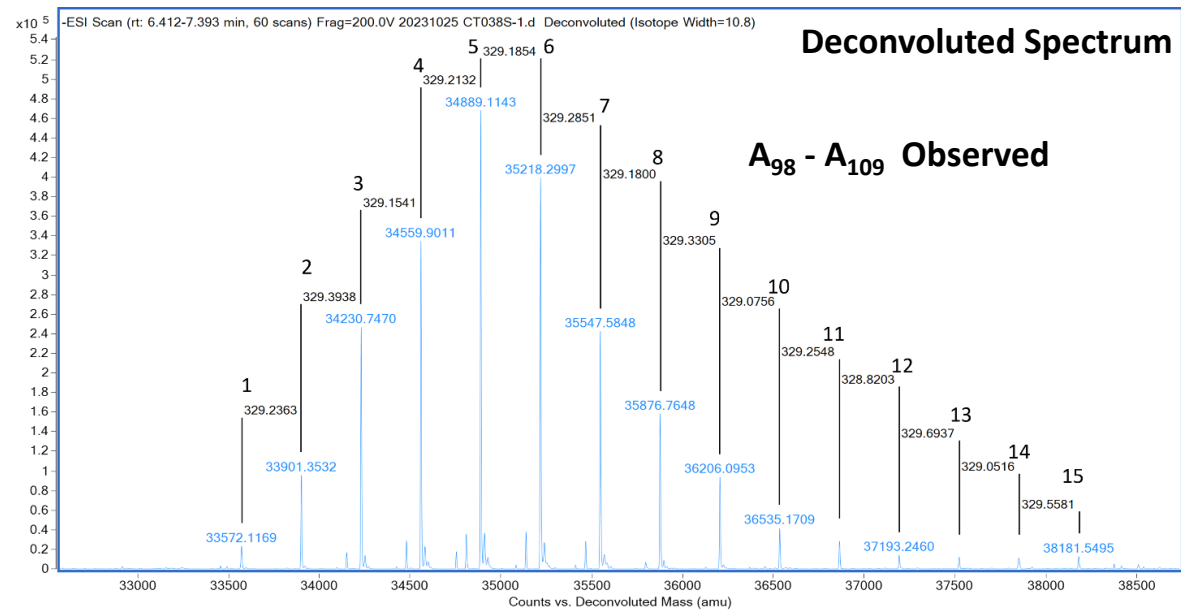
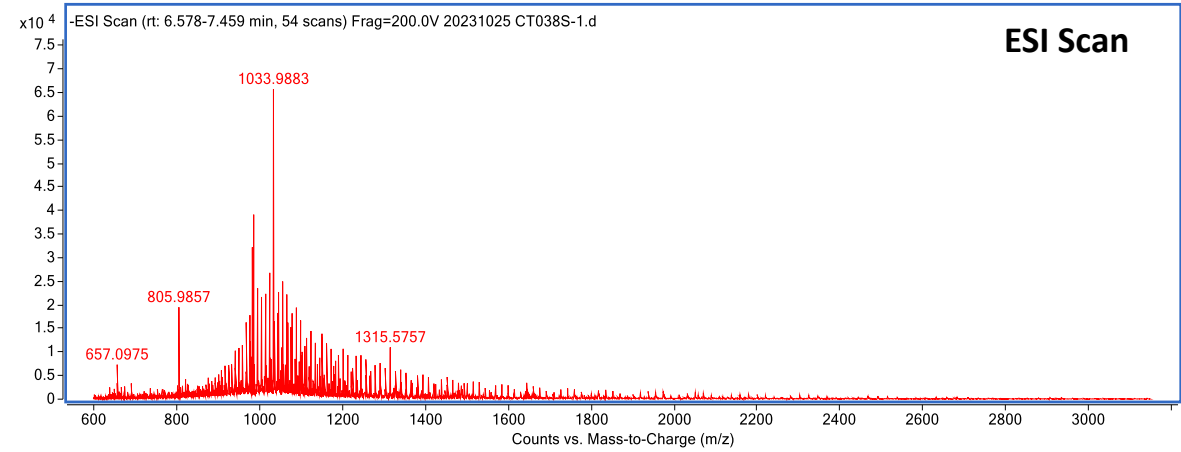
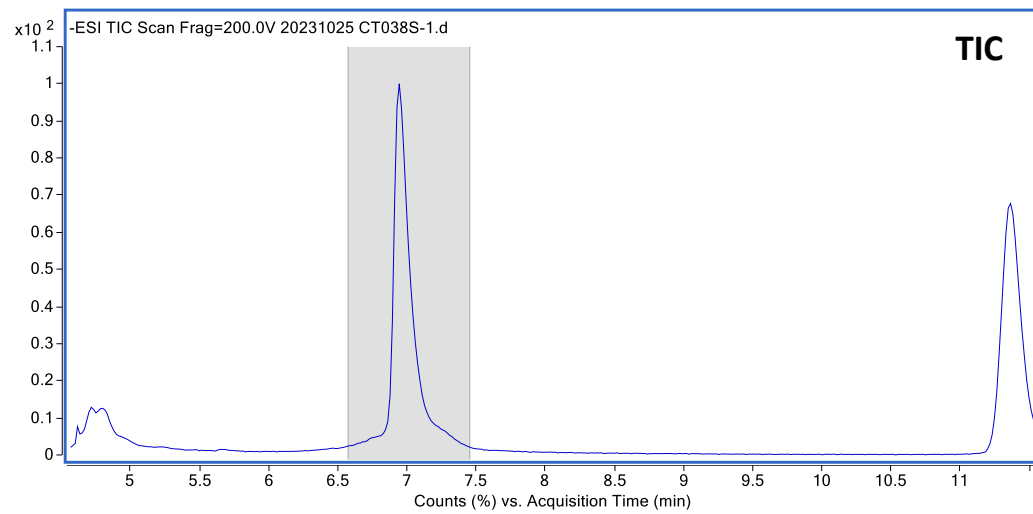
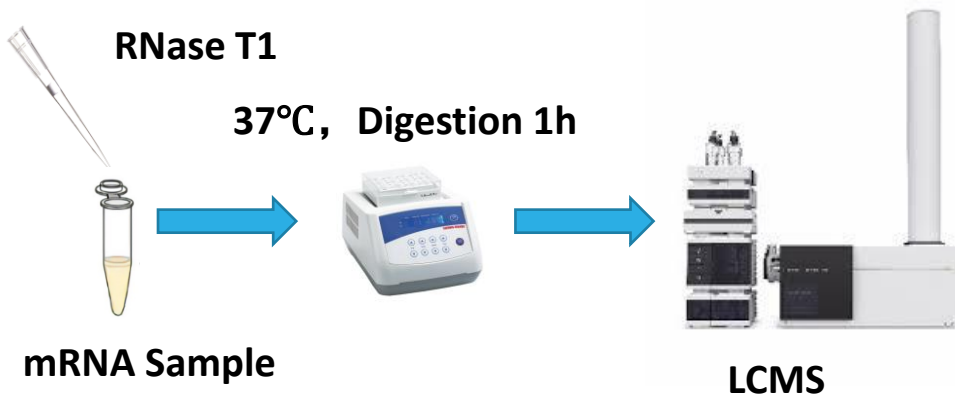


➤ dA<sub>5-150</sub> separation with columns packed with different pore size sorbent

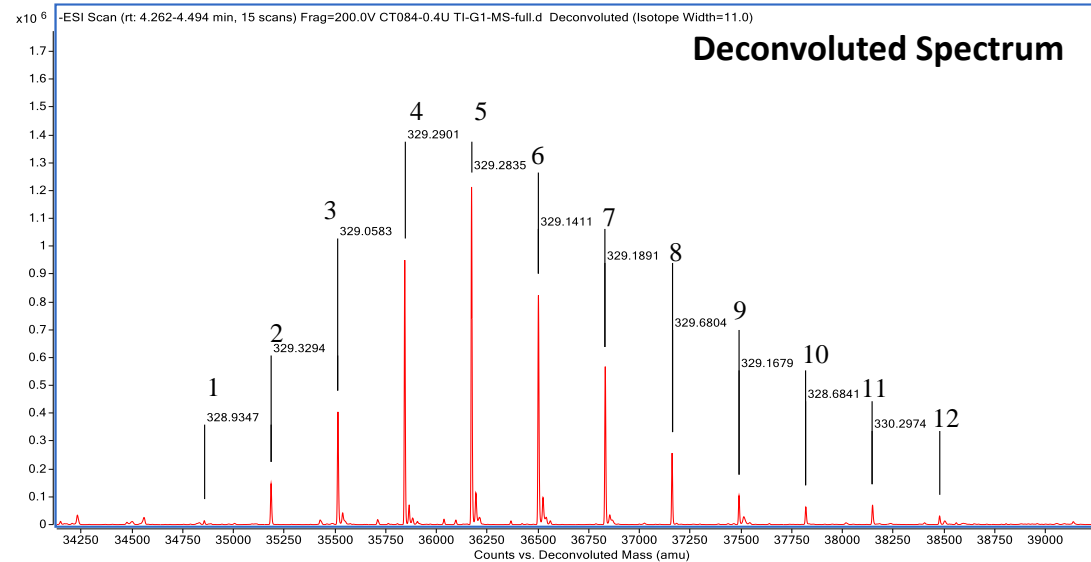
➤ Multiple poly(A) tail species coeluting as a single peak



# Poly A Test by LCMS



# Poly A Test by LCMS



— GXXX(AAAAAAAA...)<sub>n</sub> XXXXG —

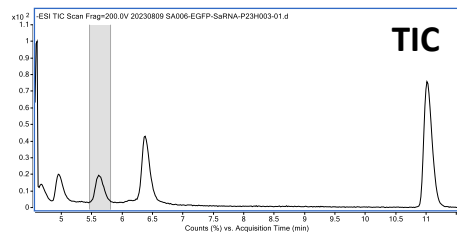
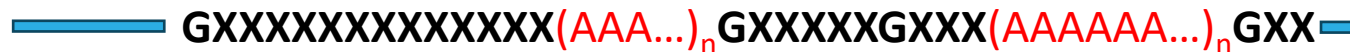
- Linearize pDNA with restriction enzyme that produces blunt or 5' overhanging ends can lead to additional 3' nucleotide sequence from the restriction site in mRNA synthesis.

| #  | Theoretical Sequence       | Observed M.W. | Calculated M.W. | Signal Intensity | Signal Intensity (%) |
|----|----------------------------|---------------|-----------------|------------------|----------------------|
| 1  | XXXA <sub>98</sub> XXXXGp  | 34854.7988    | 34856.3840      | 14093            | 0.3                  |
| 2  | XXXA <sub>99</sub> XXXXGp  | 35184.0051    | 35185.3187      | 147209           | 3.2                  |
| 3  | XXXA <sub>100</sub> XXXXGp | 35513.2115    | 35514.6481      | 404918           | 8.8                  |
| 4  | XXXA <sub>101</sub> XXXXGp | 35842.4179    | 35843.7063      | 950296           | 20.7                 |
| 5  | XXXA <sub>102</sub> XXXXGp | 36171.6243    | 36172.9965      | 1186539          | 25.9                 |
| 6  | XXXA <sub>103</sub> XXXXGp | 36500.8307    | 36502.2800      | 823897           | 18.0                 |
| 7  | XXXA <sub>104</sub> XXXXGp | 36830.0371    | 36831.4211      | 563095           | 12.3                 |
| 8  | XXXA <sub>105</sub> XXXXGp | 37159.2435    | 37160.6101      | 256851           | 5.6                  |
| 9  | XXXA <sub>106</sub> XXXXGp | 37488.4496    | 37490.2906      | 104339           | 2.3                  |
| 10 | XXXA <sub>107</sub> XXXXGp | 37817.6560    | 37819.4584      | 63268            | 1.4                  |
| 11 | XXXA <sub>108</sub> XXXXGp | 38146.8623    | 38148.1425      | 69505            | 1.5                  |
| 12 | XXXA <sub>109</sub> XXXXGp | 34854.7988    | 34856.3840      | 14093            | 0.3                  |

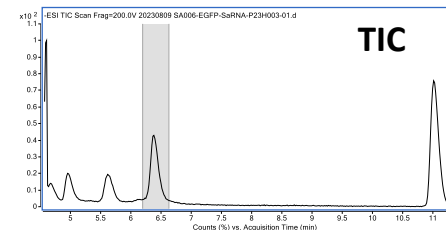
- Rnase T1 digestion results in a 2' or 3' phosphate on G.
- General mass difference between observed and calculated M.W. is around 1-2 Da

# Poly A Test by LCMS

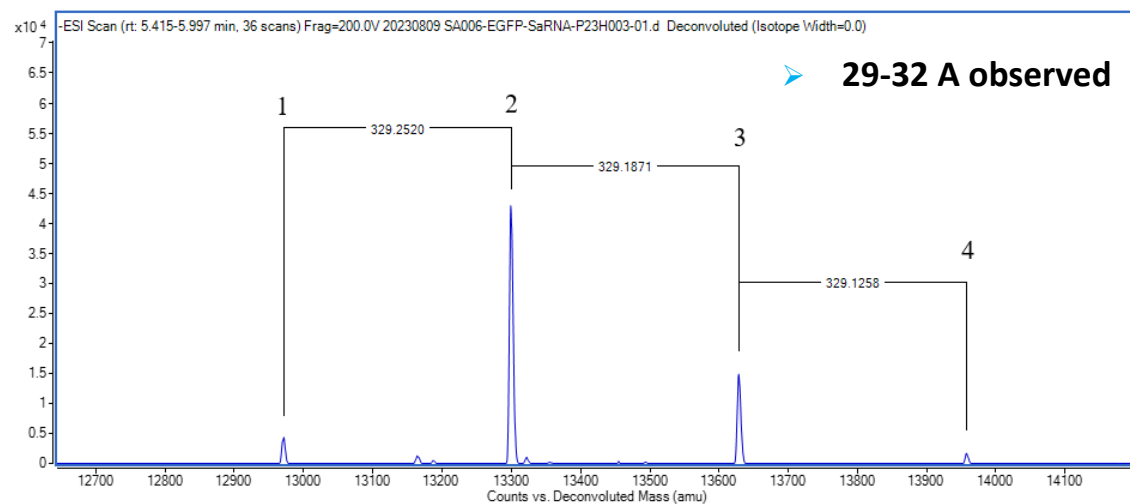
saRNA with two-tail structures connected by a linker sequence:



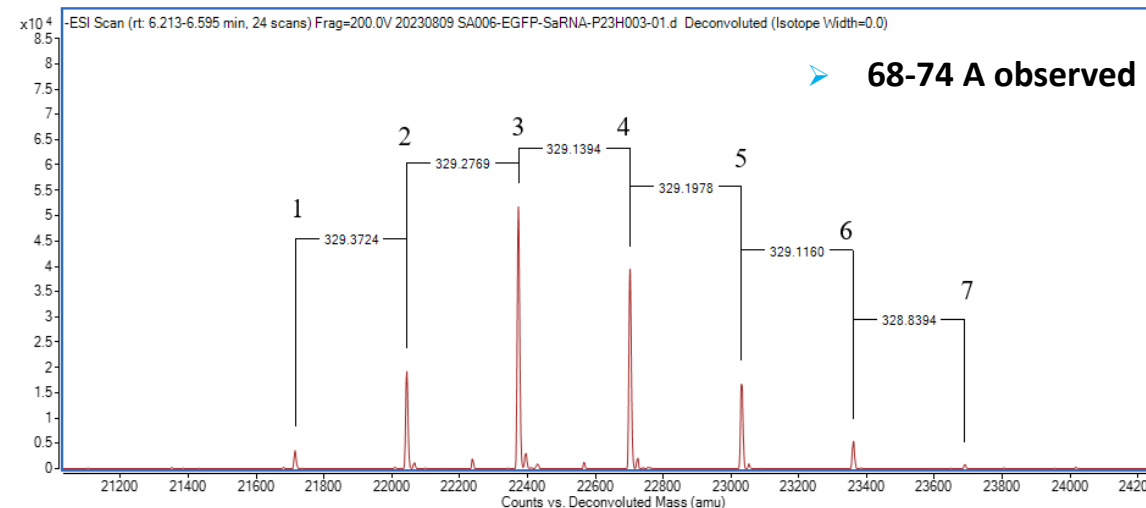
Fragment 1



Fragment 2

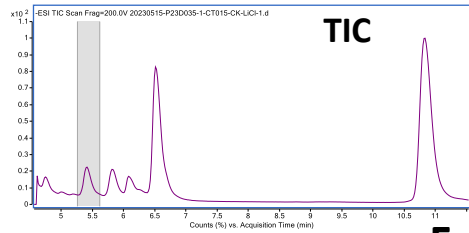


| # | Theoretical Sequence           | Observed M.W. | Signal intensity (%) |
|---|--------------------------------|---------------|----------------------|
| 1 | XXXXXXXXXXXXA <sub>29</sub> Gp | 12970.6990    | 6.6                  |
| 2 | XXXXXXXXXXXXA <sub>30</sub> Gp | 13299.9510    | 66.9                 |
| 3 | XXXXXXXXXXXXA <sub>31</sub> Gp | 13629.1381    | 23.8                 |
| 4 | XXXXXXXXXXXXA <sub>32</sub> Gp | 13958.2638    | 2.6                  |



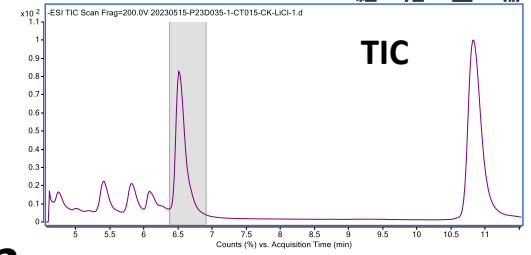
| # | Theoretical Sequence  | Observed M.W. | Signal intensity (%) |
|---|-----------------------|---------------|----------------------|
| 1 | XXXA <sub>63</sub> Gp | 21714.3472    | 2.6                  |
| 2 | XXXA <sub>64</sub> Gp | 22043.7196    | 14.1                 |
| 3 | XXXA <sub>65</sub> Gp | 22372.9965    | 37.9                 |
| 4 | XXXA <sub>66</sub> Gp | 22702.1359    | 28.8                 |
| 5 | XXXA <sub>67</sub> Gp | 23031.3337    | 12.2                 |
| 6 | XXXA <sub>68</sub> Gp | 23360.4497    | 3.8                  |
| 7 | XXXA <sub>69</sub> Gp | 23689.2891    | 0.6                  |

# Poly A Test by LCMS

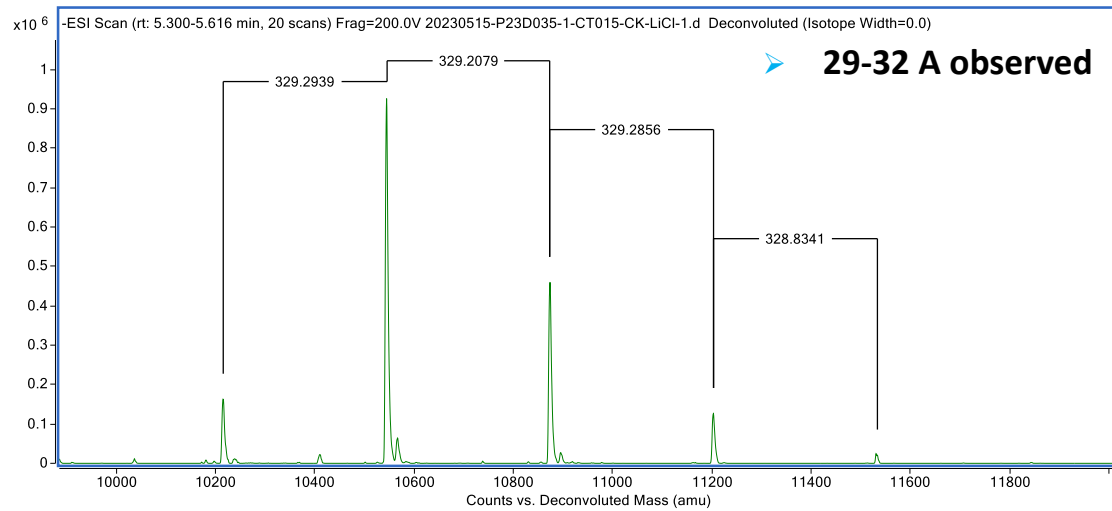


Fragment 1

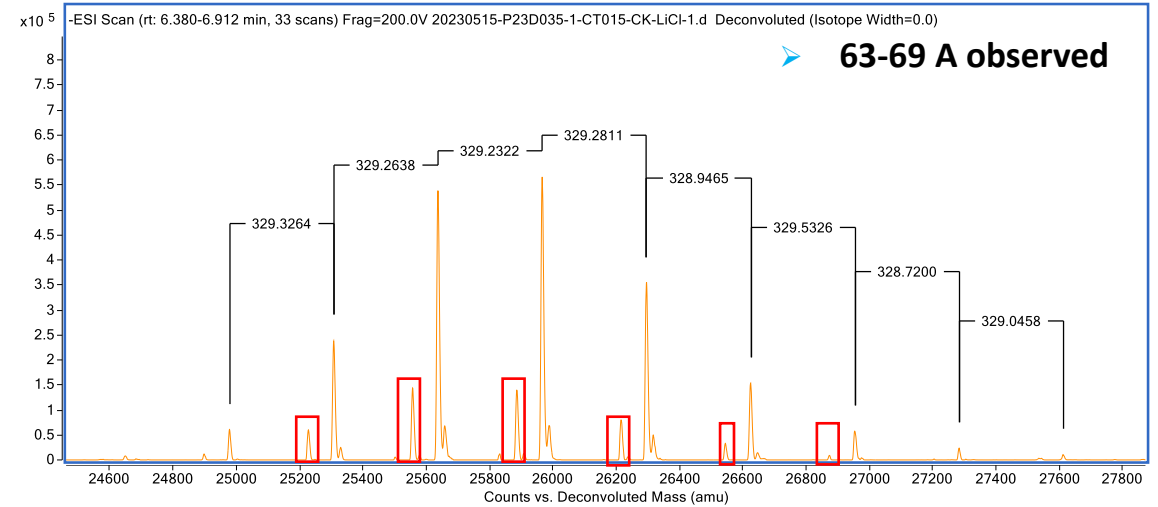
mRNA with two-tail structures connected by a linker sequence:



Fragment 2



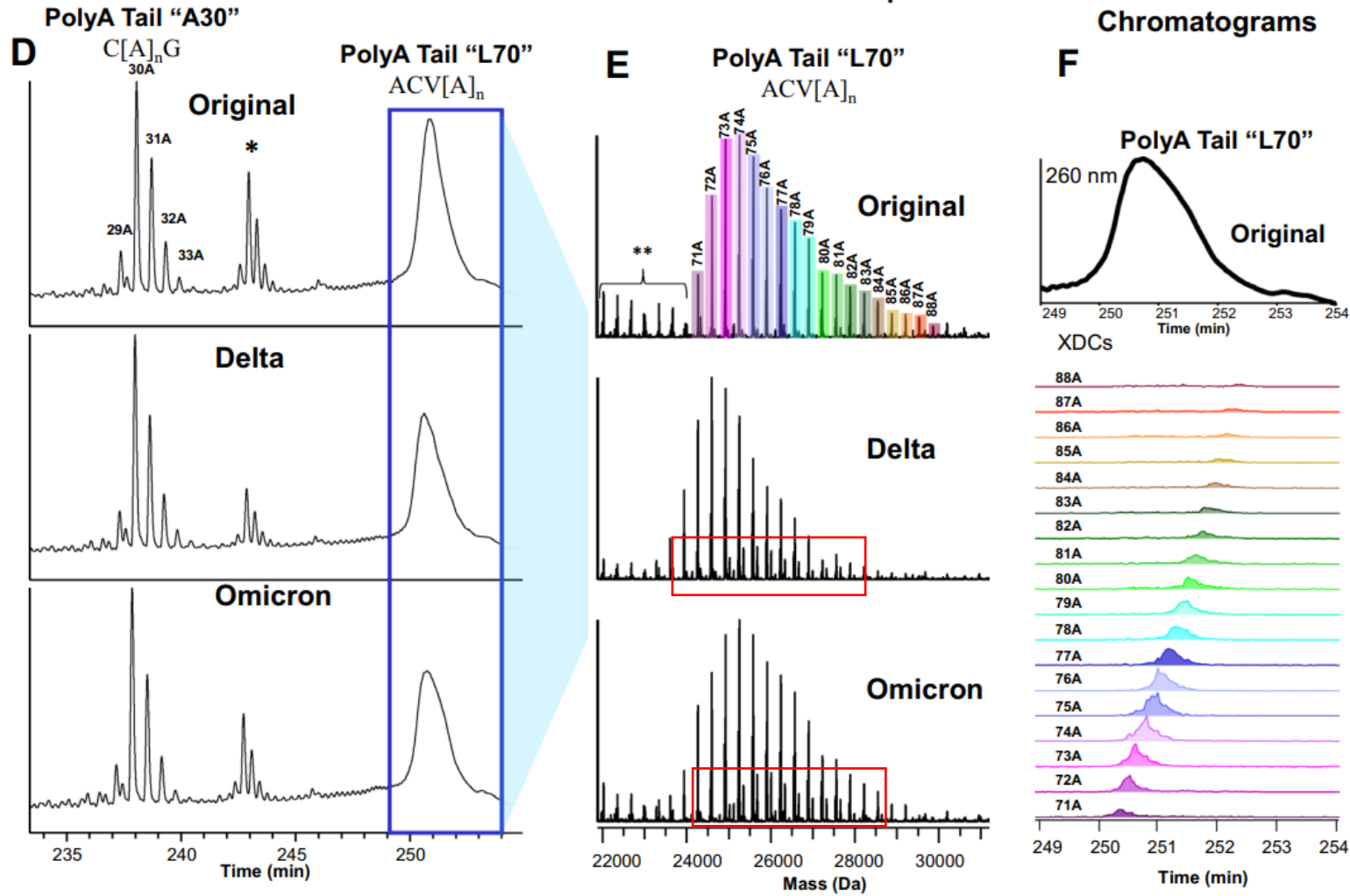
| # | Theoretical Sequence | Observed M.W. | Signal intensity (%) |
|---|----------------------|---------------|----------------------|
| 1 | XA <sub>29</sub> Gp  | 10215.5255    | 8.1                  |
| 2 | XA <sub>30</sub> Gp  | 10544.8193    | 53.0                 |
| 3 | XA <sub>31</sub> Gp  | 10874.0117    | 31.4                 |
| 4 | XA <sub>32</sub> Gp  | 11203.2685    | 7.6                  |



| # | Theoretical Sequence      | Observed M.W. | Signal intensity (%) |
|---|---------------------------|---------------|----------------------|
| 1 | XXXA <sub>68</sub> XXXXGp | 24978.2827    | 3.3                  |
| 2 | XXXA <sub>69</sub> XXXXGp | 25308.6914    | 12.3                 |
| 3 | XXXA <sub>70</sub> XXXXGp | 25637.9059    | 27.6                 |
| 4 | XXXA <sub>71</sub> XXXXGp | 25967.1503    | 28.7                 |
| 5 | XXXA <sub>72</sub> XXXXGp | 26296.3389    | 17.0                 |
| 6 | XXXA <sub>73</sub> XXXXGp | 26625.4378    | 7.9                  |
| 7 | XXXA <sub>74</sub> XXXXGp | 26954.8764    | 3.3                  |

the molecular weight of the discarded phosphate can be observed

# Poly A Test by LCMS



➤ Additional distribution of poly (A) fragments are sometimes observed

**Thanks You!**  
**Q&A**