Rapid in-depth characterization of biologics by microchip CE-MS: mAbs, AAVs and nucleic acids

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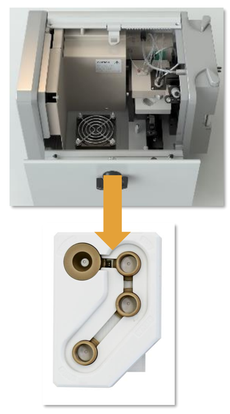
Introduction

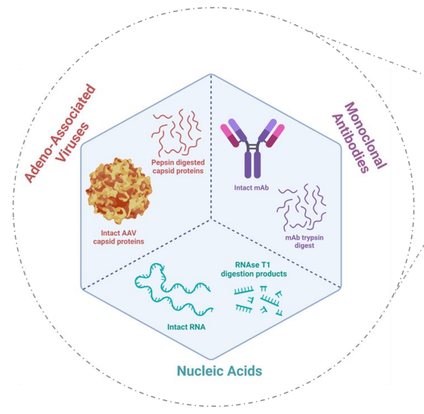
Microchip capillary electrophoresis (CE) coupled with mass spectrometry (MS) has revolutionized the characterization of therapeutic modalities in biopharmaceuticals. Here, we present the applications of an integrated workflow that couples the microfluidic CE system (ZipChip) with MS in characterization of biotherapeutics. The generic workflow is rapid, simple, with minimum method development and sample prep. We will showcase 3 distinct application examples using the ZipChip CE-MS workflow:

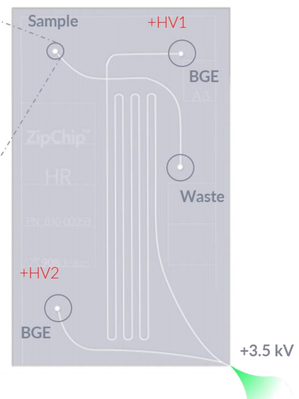
Multi-level characterization of monoclonal antibodies (mAbs) including charge heterogeneity, glycosylation profiles, and critical quality attributes. The combination of microchip CE and MS provides efficient analysis, enhancing understanding of mAb structure.

Characterization of adeno-associated viruses (AAVs) including intact denatured Viral Protein (VP) characterization as well as peptide mapping to further characterize PTMs of the VP. Examples of the serotype AAV6, AAV8, and AAV9 are shown in this poster

Analysis of nucleic acids covering FLP determination of Oligos as well as Oligo mapping of biological RNAs. Apart from benefits stated above, the workflow requires NO ION PAIRING Reagents, eliminating system contamination.







ZipChip – A microfluidic CE-based front end for mass spectrometry

Characterization of Monoclonal Antibodies: Charge Variant Analysis[1] and Peptide Mapping[2]

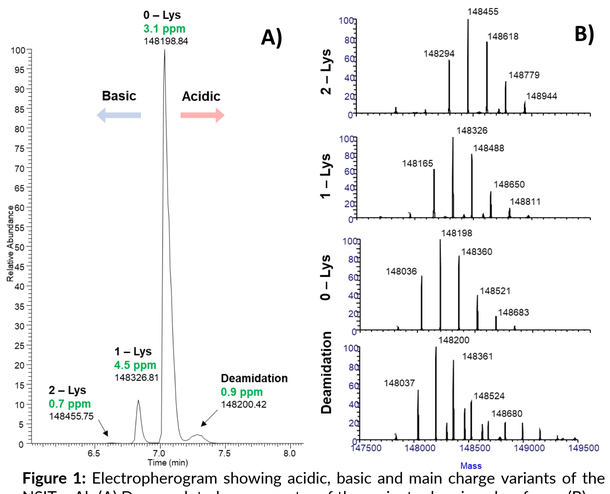


Figure 1: Electropherogram showing acidic, basic and main charge variants of the NSIT mAb (A) Deconvoluted mass spectra of the variants showing glycoforms (B)

Charge variant analysis (CVA) is crucial for identifying critical quality attributes (CQAs) of mAbs. ZipChip provides ready-to-use consumables for a simplified CVA protocol, requiring only dilution and injection. Figure 1 shows CVA results for NIST mAb. When coupled with High Resolution MS (HRMS), this generic approach enables rapid and highly sensitive detection of charge variants and glycoforms of mAbs for a variety of mAbs and bispecific antibodies without further method development.[3] Furthermore, the peak profiles align with iCIEF, allowing direct MS identification of unknown species.[3] The ZipChip/MS workflow tolerates matrices like detergents and salts, eliminating the need for species removal through cleanup procedures.

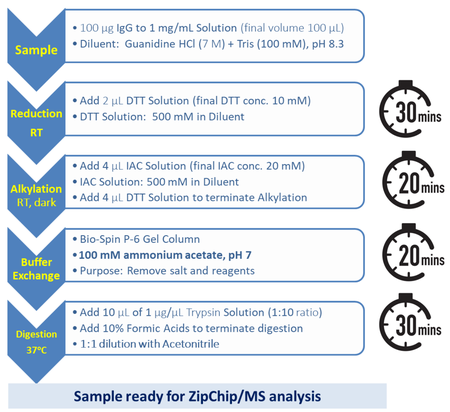


Figure 2: Workflow showing the steps involved in sample preparation procedure for peptide mapping of a mAb for ZipChip analysis

The **Peptide Mapping** sample preparation protocol is shown in Figure 2. Ammonium acetate is used to replace Tris buffer for the trypsin digestion, making it directly compatible with ZipChip/MS analysis with no further sample prep steps. When compared with LCMS protocol using TRIS buffer, this workflow demonstrated similar performance for CQAs identified with only 8nL of 1mg/mL of material needed per injection. Figure 3 shows the result of NIST mAb peptide mapping showing excellence sequence coverage and accurate identification of main PTMs with in less than 20 minutes per run. The workflow was applied to different biopharmaceutical formats (IgG4, IgG1, Fc fusion proteins) with great results.[4]

CHAIN	RESIDUE MODIFICATION	RELATIVE ABUNDANCE (%)	RSD (%)
HC	M255+Oxidation	1.99	7.1
HC	N300+A1G0	0.52	14.3
HC	N300+A1G0F	3.09	4.6
HC	N300+A2G0F	44.45	0.2
HC	N300+A2G1F	41.39	0.5
HC	N300+A2G2F	9.68	2.0
HC	N300+A1G1F	2.70	1.9
HC	N300+Unglycosylated	1.69	7.1
HC	M361+Oxidation	0.79	5.8
HC	N387+Deamidation	0.94	4.3
HC	N387+Succinimide1	0.71	1.0
HC	M431+Oxidation	1.27	7.4
LC	M4+Oxidation	2.05	18.7

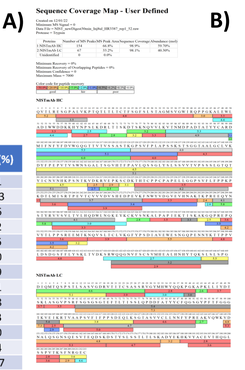


Figure 3: A) Base peak electorpherogram (BPEs) of the peptide mapping analysis on NIST mAb. Green and red bars indicate identified peptide for light and heavy chains. Embedded table shows quantitation of most relevant PTMs. B) Sequence coverage obtained for NIST mAb tryptic digest analyzed through CE-MS/MS analysis.

Characterization of Viral Capsid Proteins (VP) for AAVs: Intact Denatured and Peptide Mapping[5]

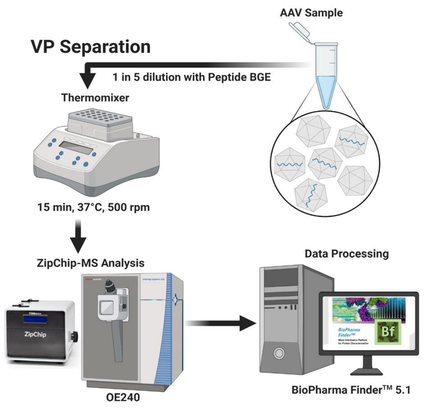


Figure 4: Schematic showing the workflow for analysis of AAV viral capsid proteins.

Intact Denatured Analysis is performed using a ZipChip™ (908 Devices) coupled to an Orbitrap Exploris™ 240 MS (Thermo Scientific). The analysis time was 5 minute using a standard High Resolution (HR) chip (908 Devices) along with a Peptide Background Electrolyte (BGE) containing 4% DMSO. The workflow is shown in Figure 4.

Figure 5 (right): BPEs of the 5 replicates analysis (overlaid) performed for AAV8 (3A), AAV6 (3B) and AAV9 (3C). Data show separation of the three VPs during the analysis with capability to detect multiple proteoforms for each VP, including phosphorylation and acetylation. LOD testing showed that all Identified proteoforms were detected at concentrations 8.80E6 viral particles, with at least one proteoform of each VP detected at concentrations as low as 2.64E6 viral particles.

AAV8 Full

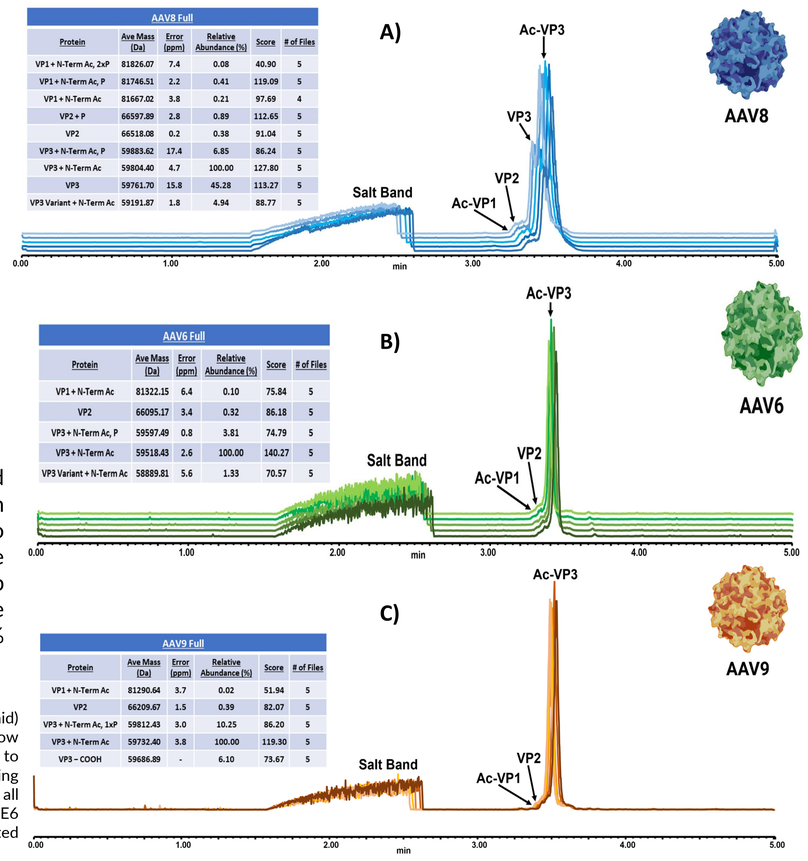
Protein	Ave Mass (Da)	Error (ppm)	Relative Abundance (%)	Score	# of Files
VP1 + N-Term Ac, 2xP	81826.07	7.4	0.08	40.90	5
VP1 + N-Term Ac, P	81746.51	2.2	0.41	119.09	5
VP1 + N-Term Ac	81667.02	3.8	0.21	97.69	4
VP2 + P	66597.89	2.8	0.89	112.65	5
VP2	66518.08	0.2	0.38	91.04	5
VP3 + N-Term Ac, P	59883.62	17.4	6.85	86.24	5
VP3 + N-Term Ac	59804.40	4.7	100.00	127.80	5
VP3	59761.70	15.8	45.28	113.27	5
VP3 Variant + N-Term Ac	59191.87	1.8	4.94	88.77	5

AAV6 Full

Protein	Ave Mass (Da)	Error (ppm)	Relative Abundance (%)	Score	# of Files
VP1 + N-Term Ac	81322.15	6.4	0.10	75.84	5
VP2	66095.17	3.4	0.32	86.18	5
VP3 + N-Term Ac, P	59597.49	0.8	3.81	74.79	5
VP3 + N-Term Ac	59518.43	2.6	100.00	140.27	5
VP3 Variant + N-Term Ac	58889.81	5.6	1.33	70.57	5

AAV9 Full

Protein	Ave Mass (Da)	Error (ppm)	Relative Abundance (%)	Score	# of Files
VP1 + N-Term Ac	81290.64	3.7	0.02	51.94	5
VP2	66209.67	1.5	0.39	82.07	5
VP3 + N-Term Ac, 1xP	59812.43	3.0	10.25	86.20	5
VP3 + N-Term Ac	59732.40	3.8	100.00	119.30	5
VP3 – COOH	59686.89	-	6.10	73.67	5



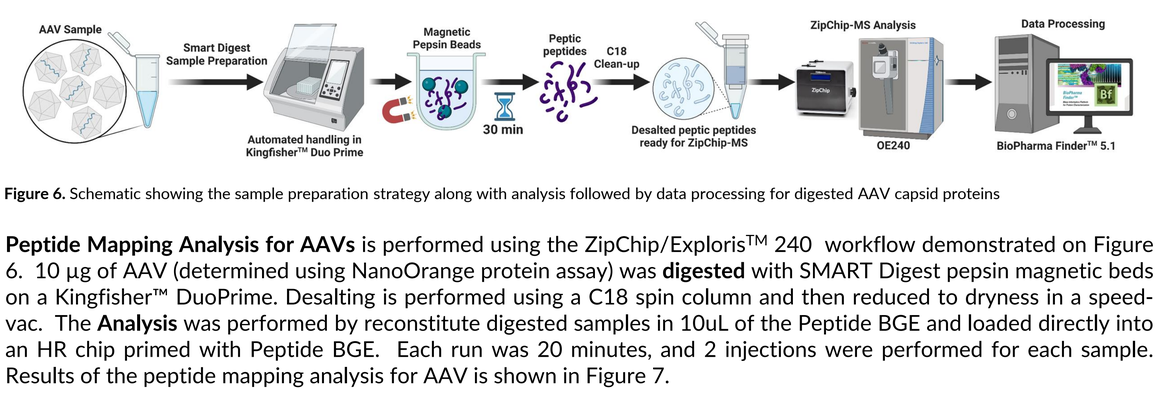
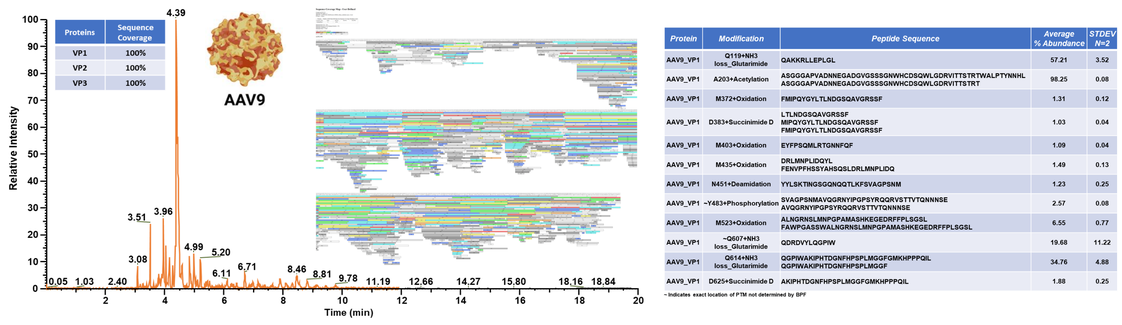


Figure 6. Schematic showing the sample preparation strategy along with analysis followed by data processing for digested AAV capsid proteins

Peptide Mapping Analysis for AAVs is performed using the ZipChip/Exploris™ 240 workflow demonstrated on Figure 6. 10 µg of AAV (determined using NanoOrange protein assay) was **digested** with SMART Digest pepsin magnetic beds on a Kingfisher™ DuoPrime. Desalting is performed using a C18 spin column and then reduced to dryness in a speed-vac. The **Analysis** was performed by reconstitute digested samples in 10uL of the Peptide BGE and loaded directly into an HR chip primed with Peptide BGE. Each run was 20 minutes, and 2 injections were performed for each sample. Results of the peptide mapping analysis for AAV is shown in Figure 7.



Protein	Modification	Peptide Sequence	Average % Abundance	STDEV N=2
AAV9_VP1	Q119+NH3 loss_Glutarimide	QAKKRLLEPLGL	57.21	3.52
AAV9_VP1	A203+Acetylation	ASGGGAPVADNNEGADGVGSSSGNWHCDSQWLGDRVITTSTRTWALPTYNNHL ASGGGAPVADNNEGADGVGSSSGNWHCDSQWLGDRVITTSTRT	98.25	0.08
AAV9_VP1	M372+Oxidation	FMIPQYGYLTLNDGSQAVQRSSF	1.31	0.12
AAV9_VP1	D383+Succinimide D	LTLNDGSQAVQRSSF MIPQYGYLTLNDGSQAVQRSSF FMIPQYGYLTLNDGSQAVQRSSF	1.03	0.04
AAV9_VP1	M403+Oxidation	EYFPSQMLRTGNNFQF	1.09	0.04
AAV9_VP1	M435+Oxidation	DRLMNPLIDQYL FENVPFHSSYAHSQSLDRLMNPLIDQ	1.49	0.13
AAV9_VP1	N451+Deamidation	YYLSKTINGSGQNQQTLKFSVAGPSNM	1.23	0.25
AAV9_VP1	–Y483+Phosphorylation	SVAGPSNMAVQGRNYIPGPSYRQQRVSTTVTQNNNSE AVQGRNYIPGPSYRQQRVSTTVTQNNNSE	2.57	0.08
AAV9_VP1	M523+Oxidation	ALNGRNSLMNPGPAMASHKEGEDRFFPLSGSL FAVPQASSNWALNGRNSLMNPGPAMASHKEGEDRFFPLSGSL	6.55	0.77
AAV9_VP1	–Q607+NH3 loss_Glutarimide	QDRDVYLQGPW	19.68	11.22
AAV9_VP1	Q614+NH3 loss_Glutarimide	QGPIWAKIPHTDGNFHPSPLMGGFGMKHPPPQIL QGPIWAKIPHTDGNFHPSPLMGGF	34.76	4.88
AAV9_VP1	D625+Succinimide D	AKIPHTDGNFHPSPLMGGFGMKHPPPQIL	1.88	0.25

– Indicates exact location of PTM not determined by BPF

Figure 7: Microchip CE-MS separation for peptide mapping and identification of PTMs. CE-MS base peak electropherograms of AAV9 serotype (left) illustrating high sequence coverage. On the left the table of identified PTMs.

- Rapid microchip CE-MS can be utilized for the in-depth characterization of AAVs including viral capsid protein analysis and peptide mapping.
- VP separation is performed in as little as 5 minutes while detecting multiple proteoforms of each viral capsid protein.

Characterization of Nucleic Acids: Oligonucleotide Analysis[6] and Bottom-up Oligo Mapping of RNAs[7]

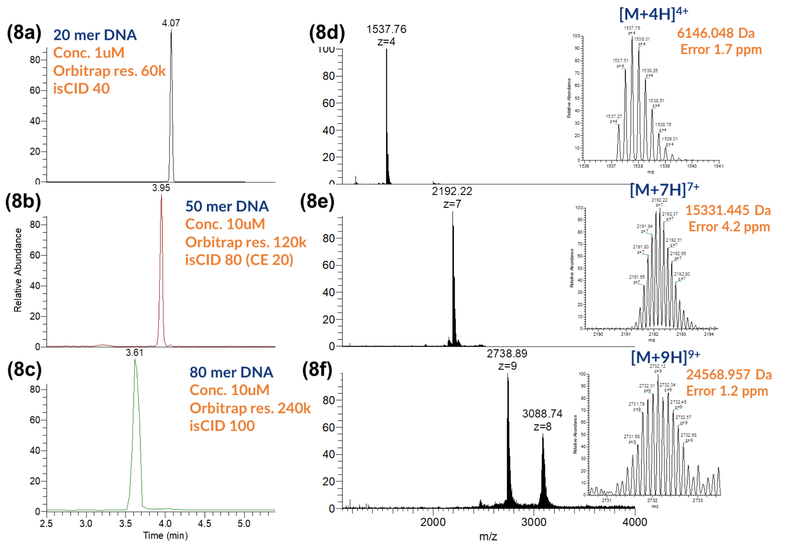


Figure 8. *Extracted Ion Electropherograms and raw mass spectra of DNAs 20mer, 50mer, 80mer (8a-8c), mass spectra generated by averaging the peaks of interest ((8d-8f) inserts show M+H adduct in the dominant charge state for each of the Oligo.*

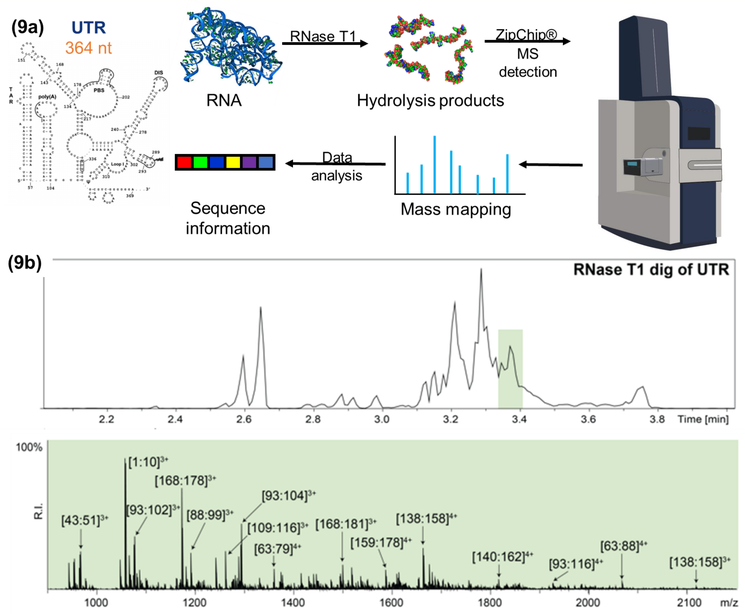


Figure 9. *Protocol for digestion and analysis workflow (**9a**); Bottom-up analysis of UTR on ZipChip-timsTOF (**9b**)*

The **Intact Oligos Analysis** can be easily done by the ZipChip CE/MS platform. The method is rapid with NO need for ion-pairing reagents. In this protocol, the ready to use consumables are a High-Resolution Bare Glass (HRB) chip and the ZipChip Oligos BGE. A generic protocol can be sued for the analysis of oligos full length product (FLP) 10-80 mers. Figure 8 presents the example electropherograms and corresponding mass spectra for 20mer, 50mer, and 80mer DNA sample.

Oligo Mapping of a larger biological RNA by a partial digestion was also performed using similar CE-MS protocol (Figure 9a) with analysis time to be 4 minutes. The sample used was HIV-1 5'-UTR (untranslated region) RNA (UTR), with 364 nucleotides and was partially digested using RNase T1. Figure 9b shows the electropherogram and an example mass spectrum for one of the peaks of interest. 100% sequence coverage of UTR was achieved.

Conclusion

- ZipChip CE/MS is a versatile platform for analysis of a variety of therapeutic and pharmaceutically important molecules such as mAbs, AAVs and nucleic acids and both intact and partially digested levels.
- This microfluidic technology offers a rapid method of analysis for all formats; ready-to-use consumable kits eliminate the need for method development.
- Due to its mass spec friendly BGE solutions and lack of ion-pairing reagents, switching between methods is extremely simple, thereby reducing critical bottlenecks in analytical workflows in a biopharmaceutical mass spectrometry laboratory.

References

1. **Application Note 7.5** "Native charge variant analysis of biotherapeutics using ZipChip CE-ESI-MS: From comprehensive characterization to monitoring product degradation characteristics." 908 Devices.
2. **Application Note** "Microchip CE/MS Peptide Mapping Workflow with Trypsin Digestion for the Analysis of Critical Quality Attributes of Monoclonal Antibodies" 908 Devices.
3. Wu, Z. et al. *J. Pharm. Biomed. Anal. 2023, 223, 115147.*
4. Carillo, S.; Kulkarni, A. (2023, April). Direct Microchip CE/MS Peptide Mapping Workflow for the Analysis of mAb CQAs [Webinar] https://908devices.com/resource/zipchip-webinar-ls-zc-wb-0017/
5. **2023** *In-depth Characterization of Adeno-Associated Viruses (AAVs) using Microchip CE-MS* (Poster); ASMS, Houston, TX, USA.
6. **Application Note 9.5** "Rapid characterization of oligonucleotides using microfluidic capillary electrophoresis - mass spectrometry by ZipChip. 908 Devices
7. **2023** *Continuing the investigation of microchip capillary electrophoresis coupled with mass spectrometry in the bottom-up characterization of progressively larger RNAs* (Poster); ASMS, Houston, TX, USA.

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