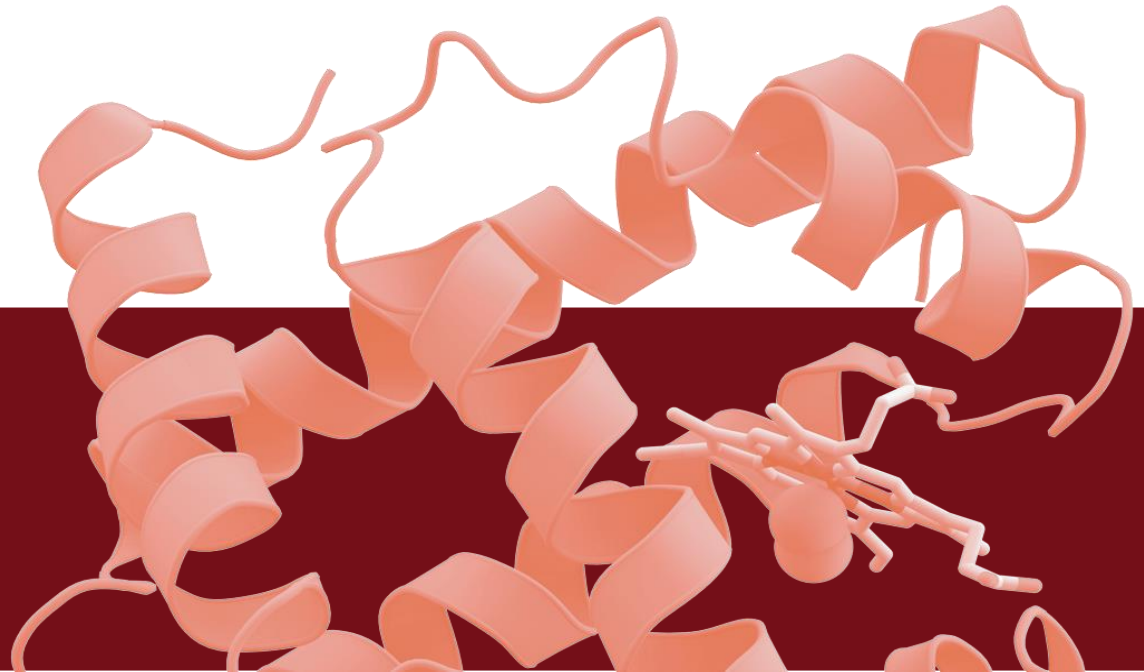
REDSHIFTBio®

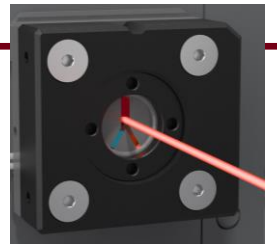
Analyzing RNA Structure using Microfluidic Modulation Spectroscopy (MMS) and Measuring Structural Changes in Riboswitches

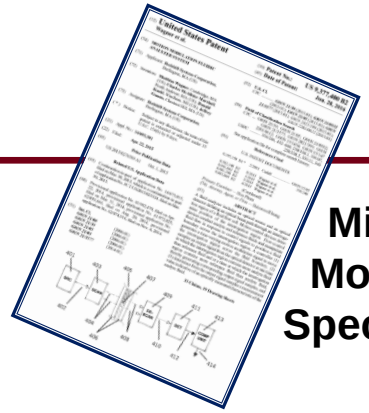
Dave Sloan, PhD
Senior VP - LifeSciences



About RedShift BioAnalytics, Inc.

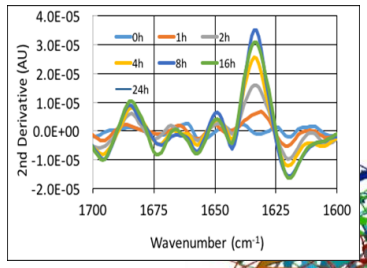
- **RedShiftBio®:** Massachusetts-based biotech company backed by two of the largest life science instrumentation companies, one of which is Waters.
- **MMS:** Microfluidic Modulation Spectroscopy, a powerful new technology for characterizing biomolecules in solutions.
- **Apollo & Aurora TX:** Automated and high-throughput instruments for enhanced characterization and monitoring of biomolecules in their native state
- **delta:** Proprietary analytical software for both automated and streamlined data analysis.



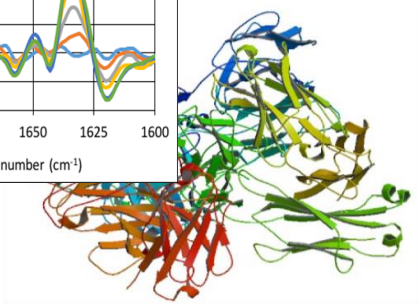


Microfluidic Modulation Spectroscopy

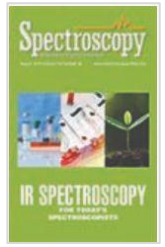
Infrared Optics



Biomolecule Analytics Software



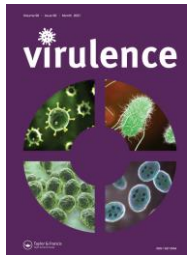
Growing List of Peer-Reviewed Publications



Spectroscopy (Aug 2019)

BioPharm International (May 2020)

Analytical Biochemistry (Feb 2022, Dec 2023)



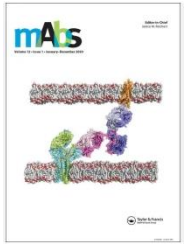
Virulence (July 2021)

Biological Macromolecules (Feb 2022)

Journal of Pharmaceutical Sciences (Jan 2020)

American Pharmaceutical Review (July 2020)

PNAS (March 2019)



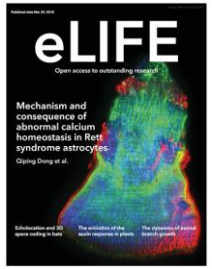
mAbs (Dec 2021)

Journal of Pharmaceutical Sciences (Aug 2020)

American Pharmaceutical Review (Oct 2018)

Food Bioscience (Feb 2023)

Journal of Biomolecular Structure and Dynamics (Aug 2023)



eLife(Sep 2023)

Analyst (Jul 2024)

AuroraTX – 2nd Generation System Powered By MMS

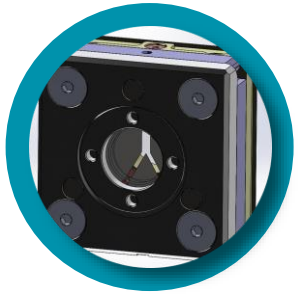
It's not just for proteins anymore!

The One-Drop ~~Protein~~ Characterization Solution

Precise, Automated, and Ultra-Sensitive Biomolecule Characterization

High Power Quantum Cascade Laser

Provides 30x sensitivity to detect small changes in structure over wide concentration ranges



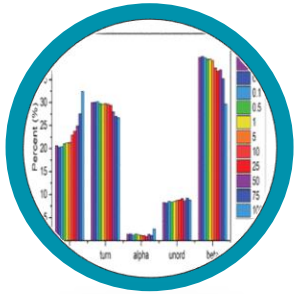
High Precision Microfluidic Flow Cell

Enables extreme reproducibility and allows use of complex buffers

Fully Automated Sample Handling System

Reduces hands-on time and human error

TX upgrade offers thermal ramping capabilities and extended spectral range for RNA/DNA analysis.



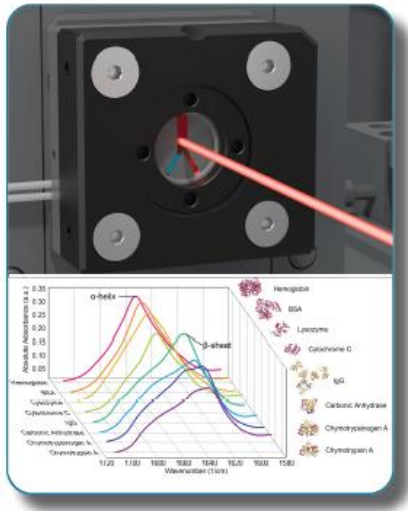
Simple Operation, Integrated touchscreen, and Advanced Analytics

Ease of use with minimal training and high-quality data

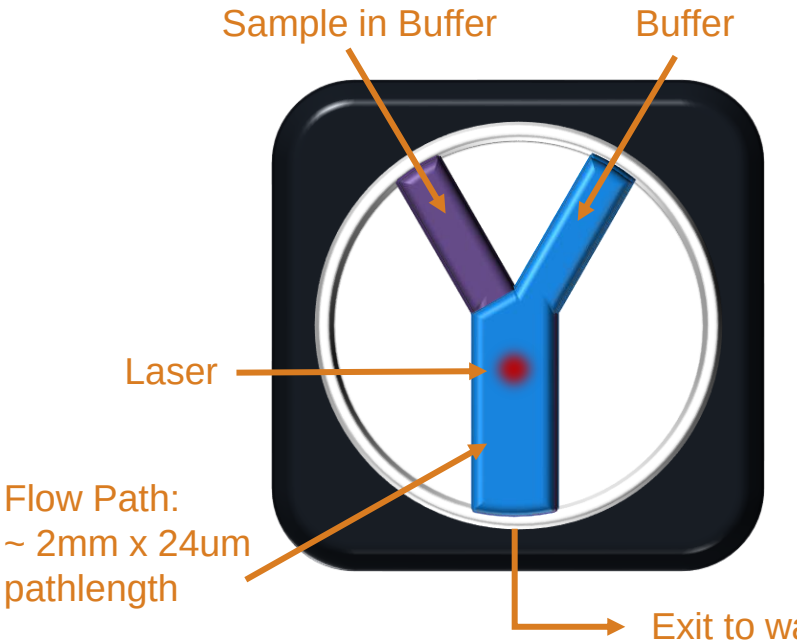
Key Enhancements: Fast Analysis, Low Volume, High Resolution

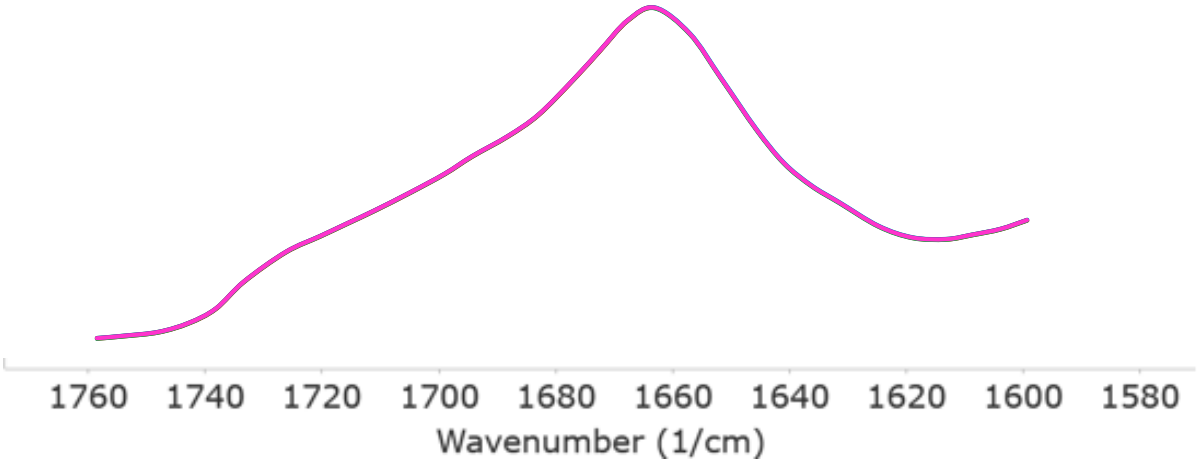
REDSHIFTBio®

Microfluidic Modulation Spectroscopy (MMS)

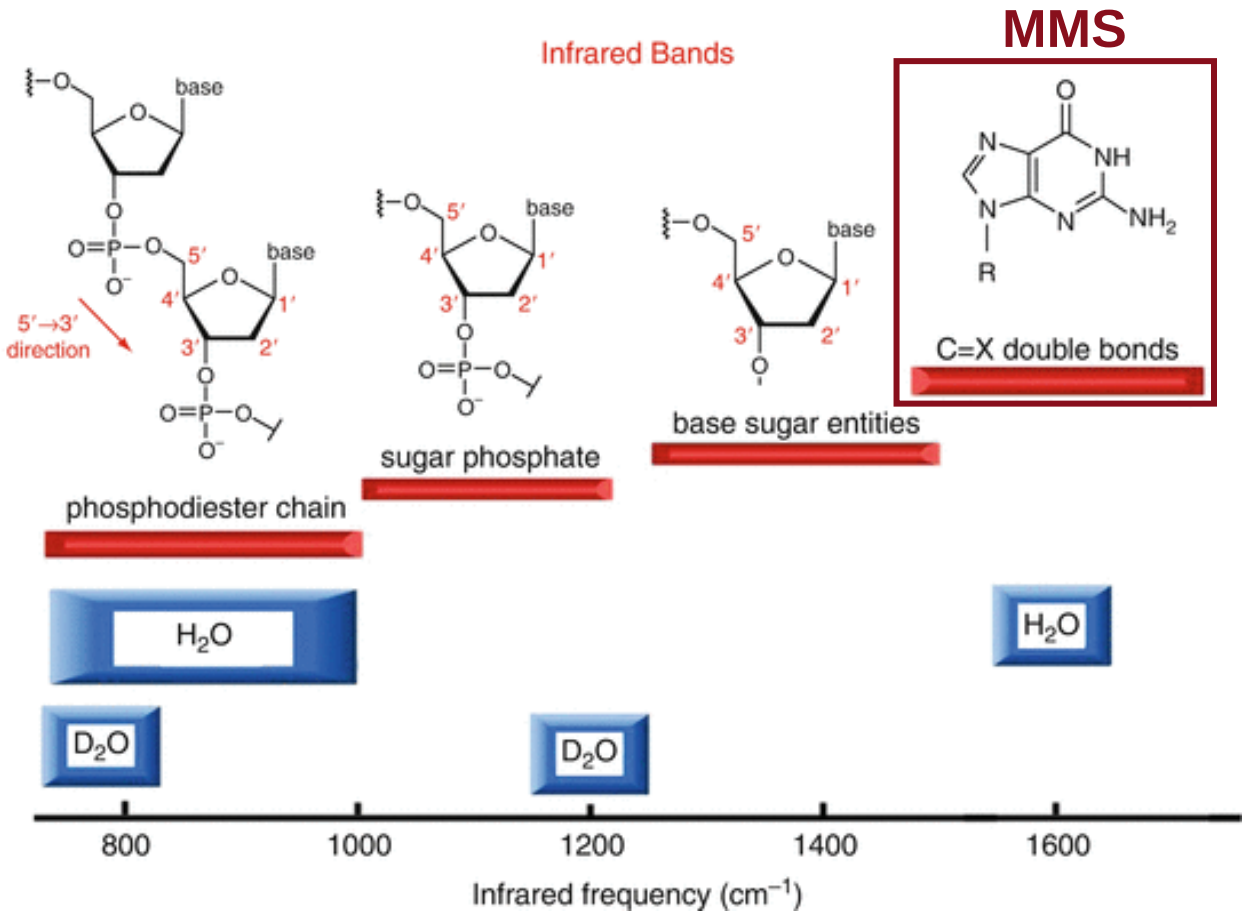


- Unique microfluidics alternates sample and buffer in flow cell
  - The absorbance of the sample and buffer are alternately measured across the Amide I band
  - Differential Absorbance (DiffAU) is recorded
  - Rapid sample-buffer referencing without cell movement provides >98% system repeatability.





Nucleic Acid IR Bands – What Does MMS Measure?



Assignment	Wave number (cm-1)
Base vibrations	1800-1500
Double-helical structures	1673-1660 to 1689-1678
Thermal denaturation	1696-1684, 1677-1653
Triple-helical structures	1800-1500
Base-sugar vibrations	1500-1250
Interaction involving the N7 sites of purines	1495-1476
Anti/syn conformation	1381-1369
Sugar conformation	1344-1328
Sugar-phosphate vibrations	1250-1000
Backbone conformation, PO_2^- stretching band	B-form double helix ~1225 A-form ~1240 Z-form ~1215
Sugar vibrations	1000-800
Sensitivity to sugar conformation	N-type sugars, 882-877, 865-860 S-type sugars, 842-820
Contribution from POP vibration	840-800

Biophysical Chemistry Banyay *et al.* (2003) A library of IR bands of nucleic acids in solution. *Biophys Chem.*

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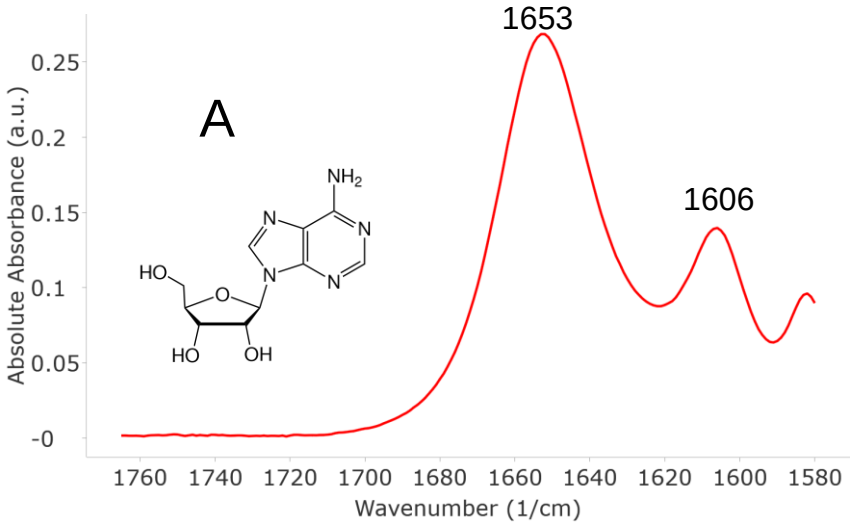
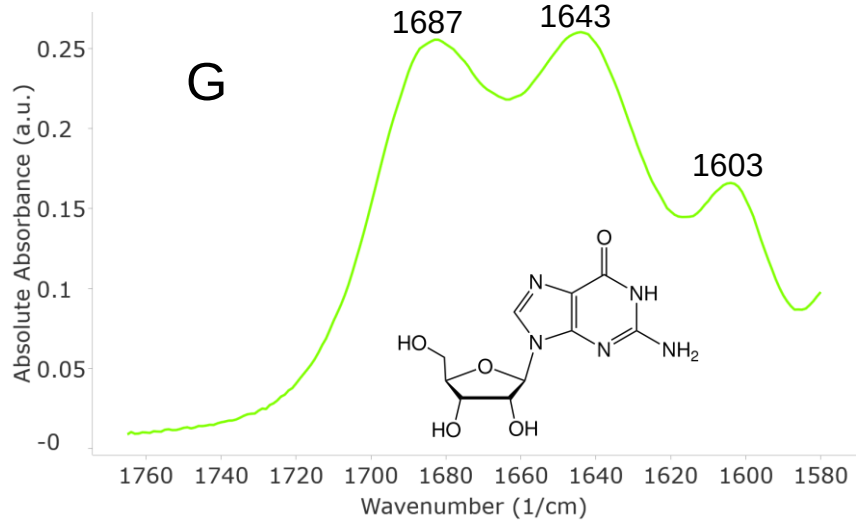
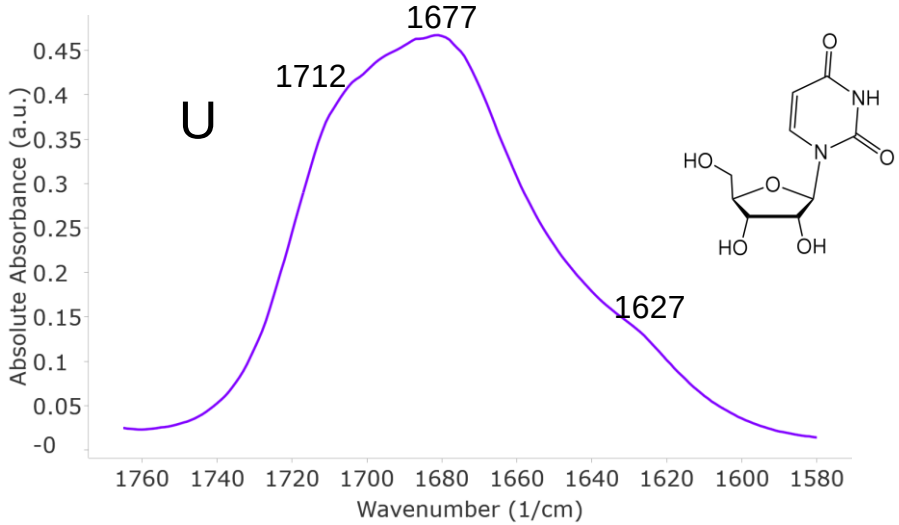
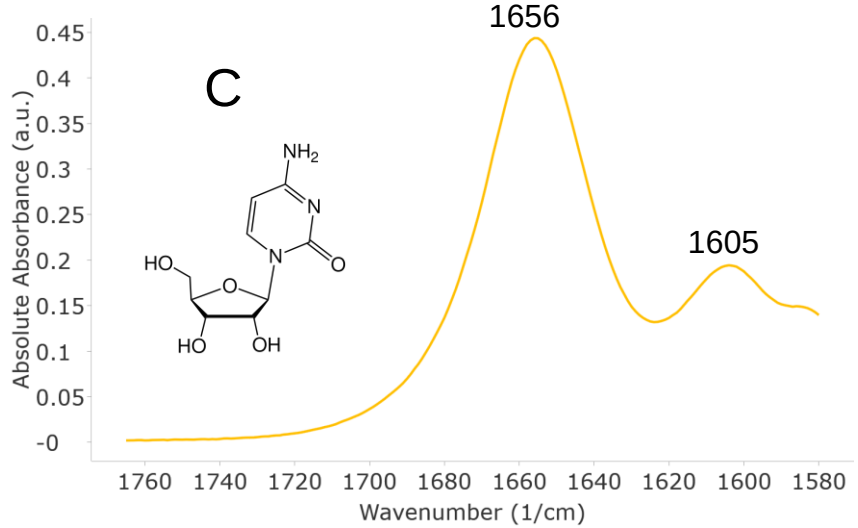
Extended Range Building Blocks: Nucleosides MMS data

The nucleosides are the building blocks for RNA and DNA (we're showing A,U,C, and G, but we've also measured T!) and have signature peaks in the amide I band. Using these building blocks, we can predict what sequences will look like and compare to experimental data to observe base-pairing, Hoogsteen pairing, and other higher order structures like triple strands.









Bonds responsible for these absorption bands:
C=O stretch
C=C and C=N ring vibrations

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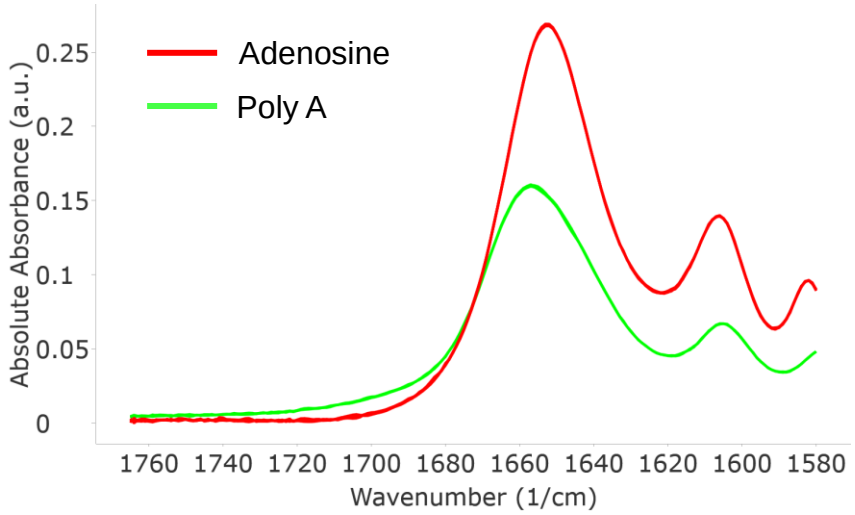
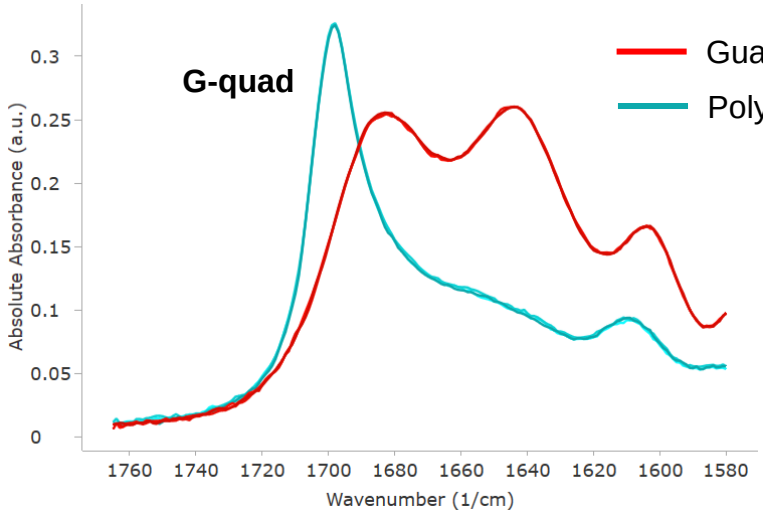
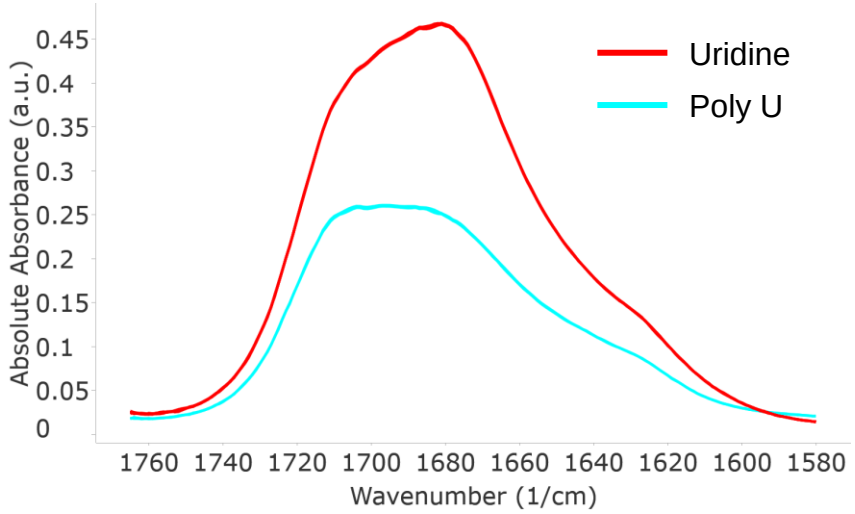
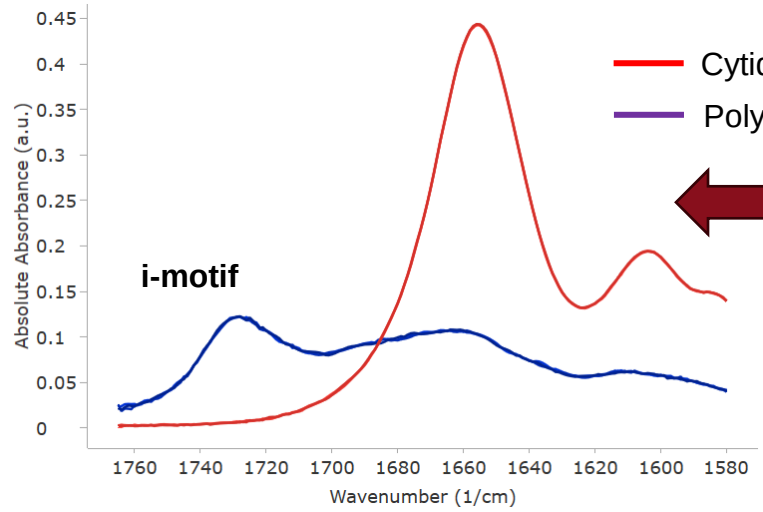
Nucleosides vs Polynucleotides









Higher order structures are changing the spectra for these polynucleotides

Bonds responsible for these absorption bands:
C=O stretch (strong, 1640-1720 cm^{-1})
C=C and C=N ring vibrations (weak, 1580-1630 cm^{-1})

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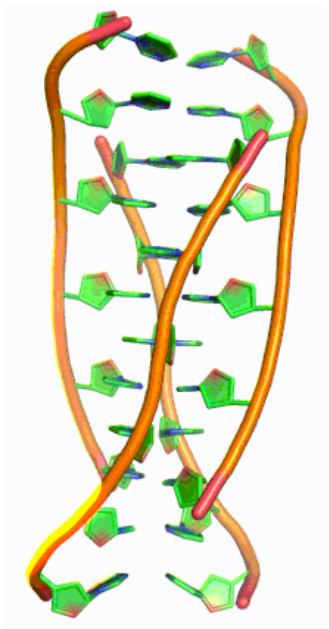
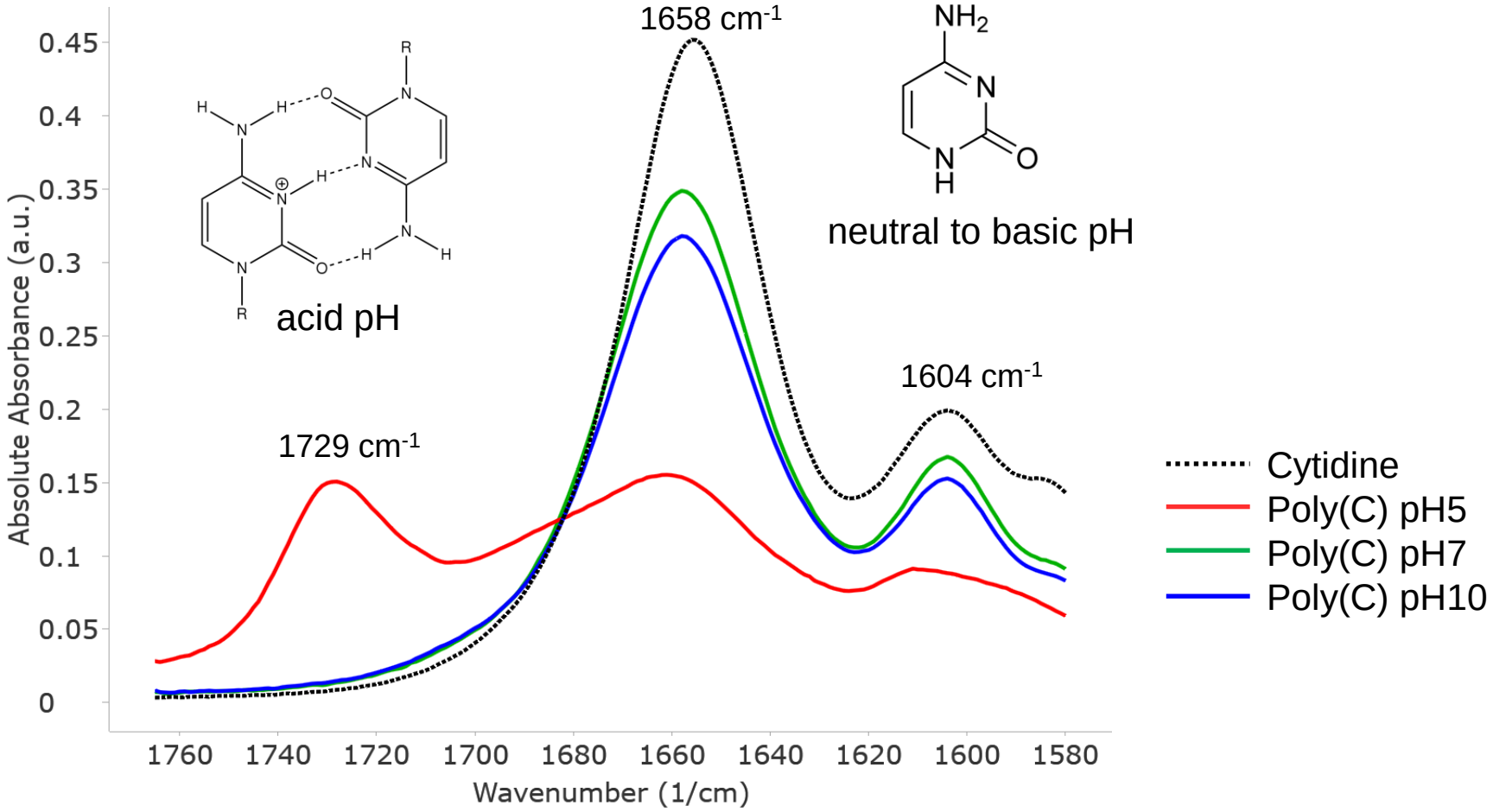
Poly(C): i-motif vs Monomer, Structure vs Identity



i-motif

MMS absolute absorbance



MMS detects unique spectral feature for i-motif due to C-C$^+$ base pairing

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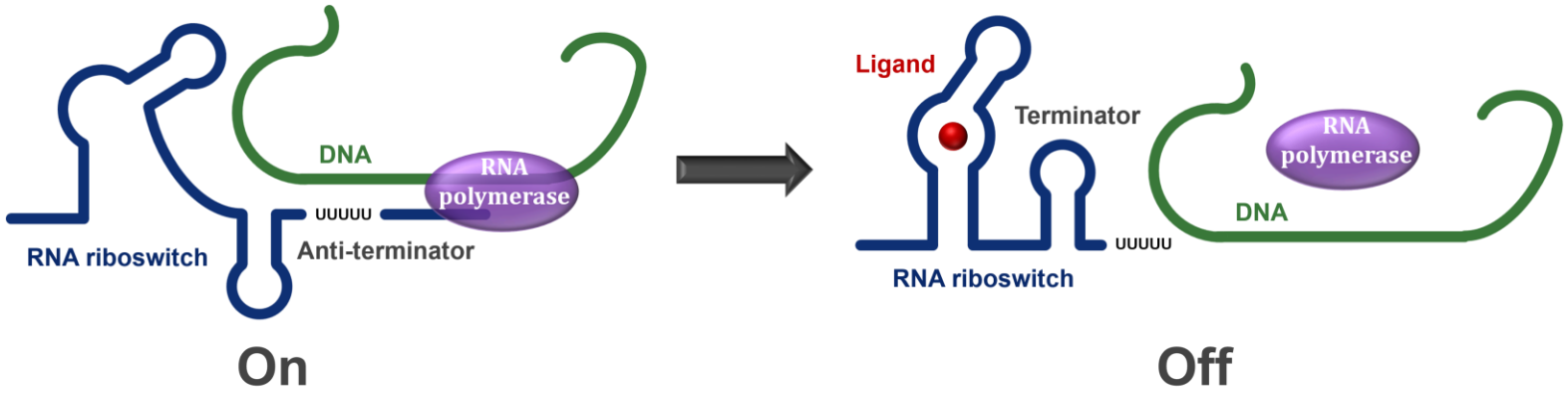
Today's topic: riboswitches and ligand binding

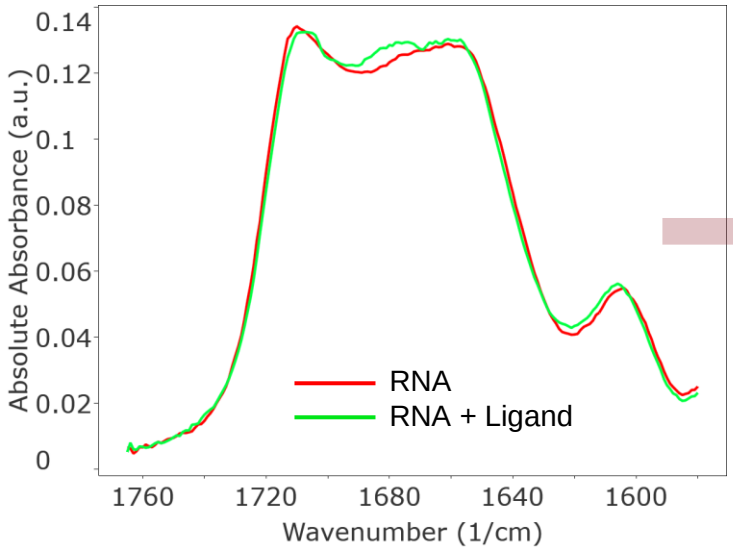
- Riboswitch
  - mRNA regulatory domains that bind metabolites and modulate gene expression.
  - Acts at all levels of gene expression: transcription, splicing, translation
- Riboswitch-ligand binding
  - Serves as a feedback to control gene expression
  - Structural rearrangement on the RNA



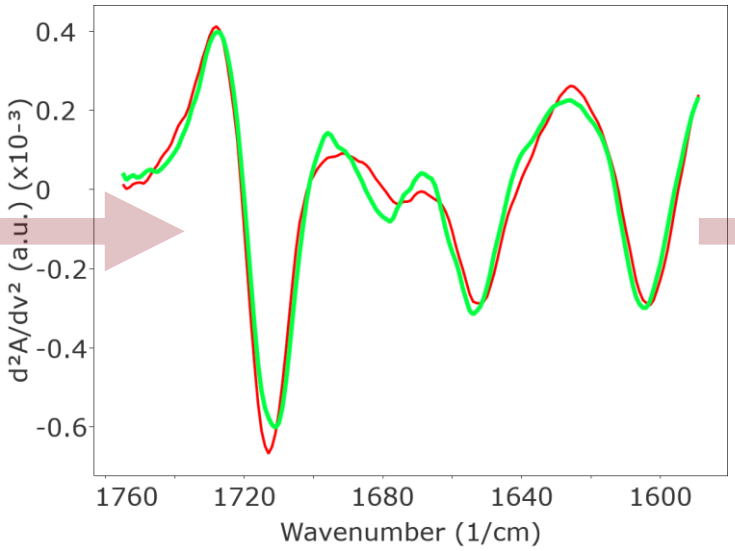
Schematic showing a riboswitch that **regulates** transcription

PreQ1 riboswitch: MMS data output

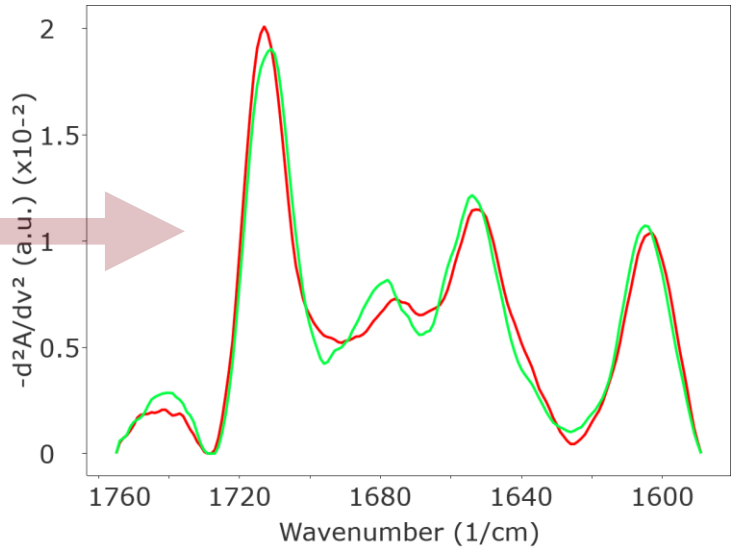
Absolute absorbance spectra



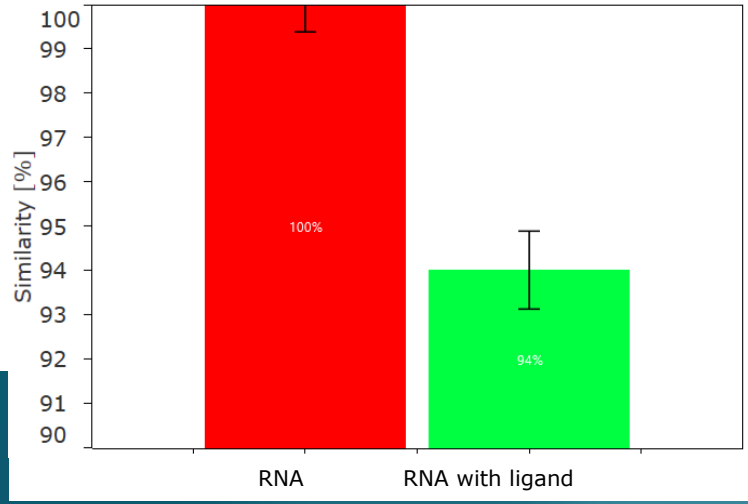
Second derivative spectra



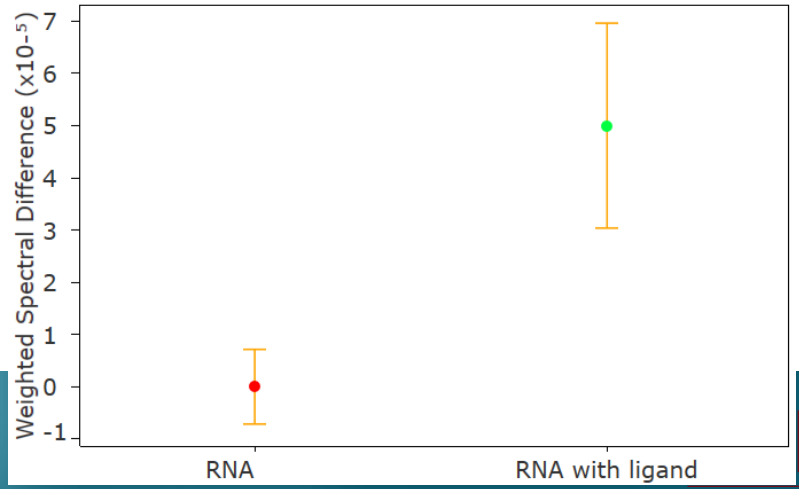
Similarity spectra



Similarity score by area of overlap

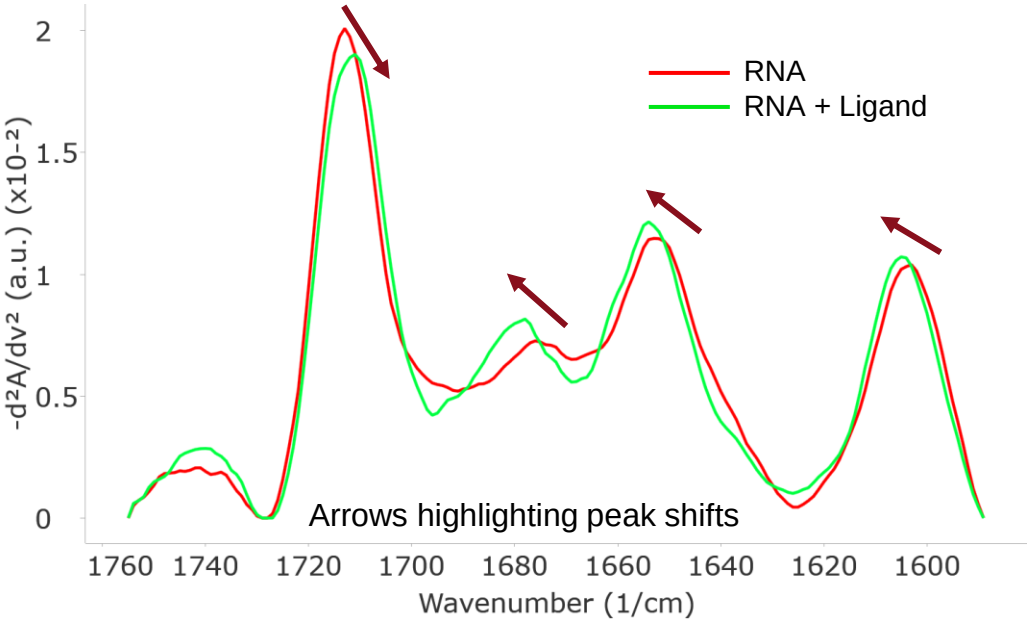


Weighted spectral difference



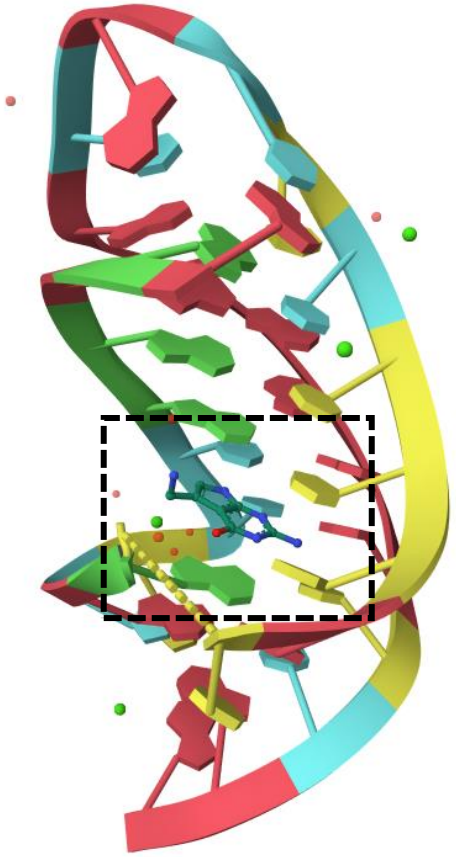
PreQ1 riboswitch-ligand binding

PreQ1 riboswitch with and without ligand



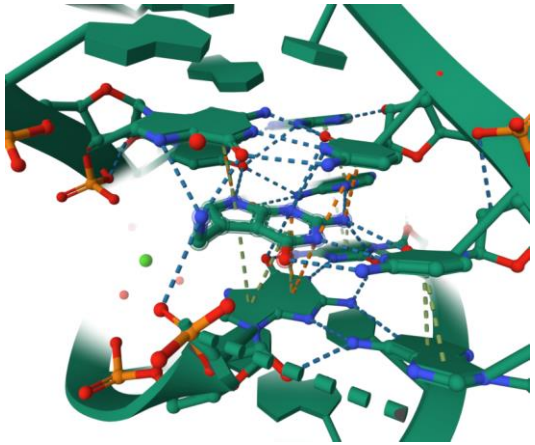
Sample	Repeatability	Similarity
RNA	97.6%	100%
RNA+Ligand	98.4%	**94.0%**

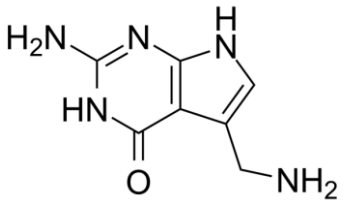
Ligand binding detected



PreQ1 riboswitch crystal structure (3FU2)

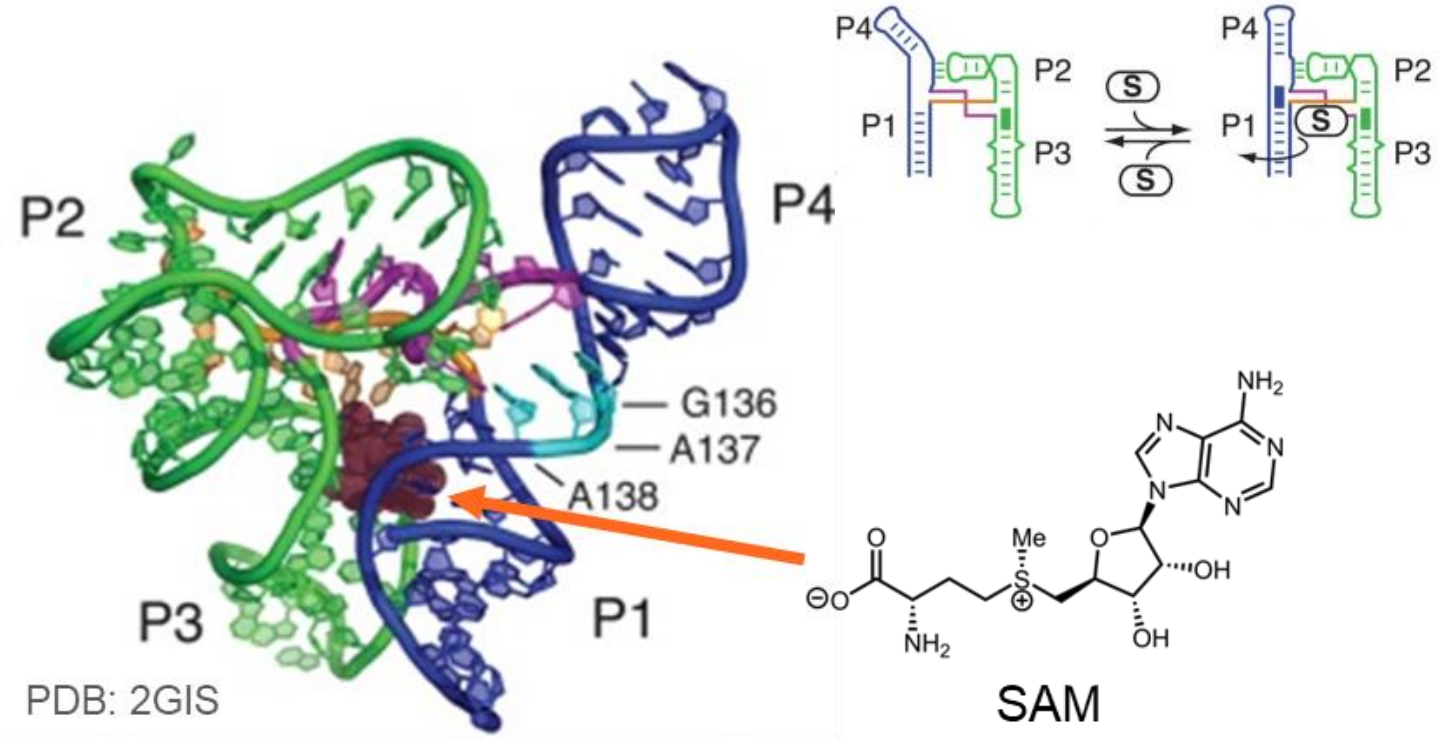
Ligand-base interactions



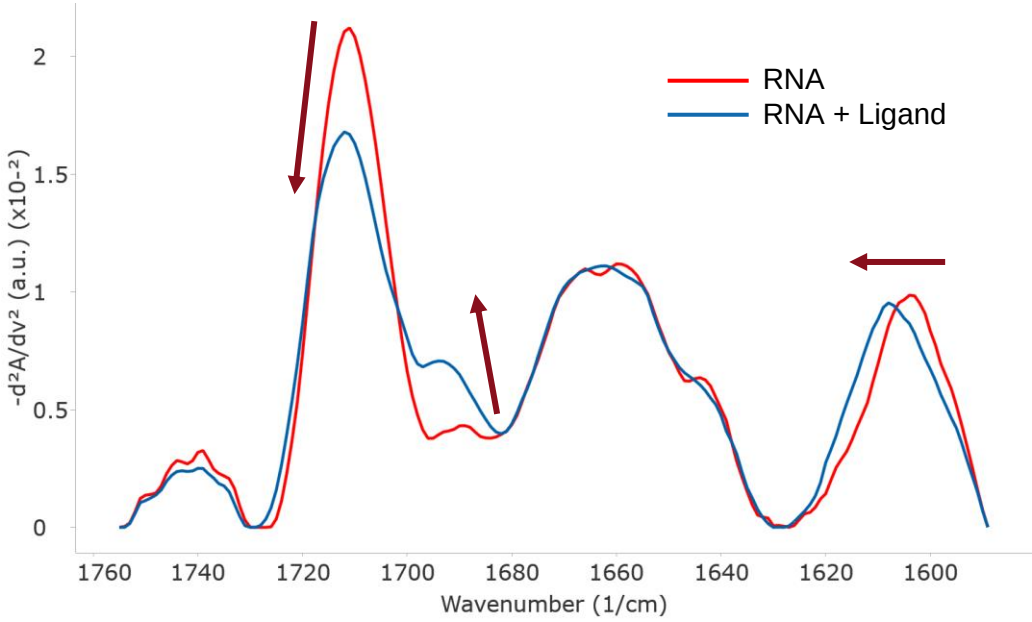


PreQ1

SAM-I Riboswitch Data



SAM-I riboswitch with and without ligand

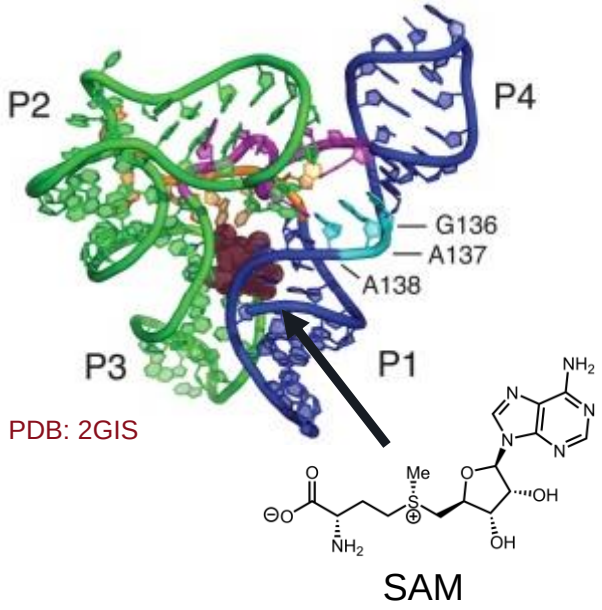


Riboswitches are small, well-folded RNA that bind tightly to small molecular ligands. When bound, the RNA undergoes conformational change that controls whether the riboswitch is active or inactive.

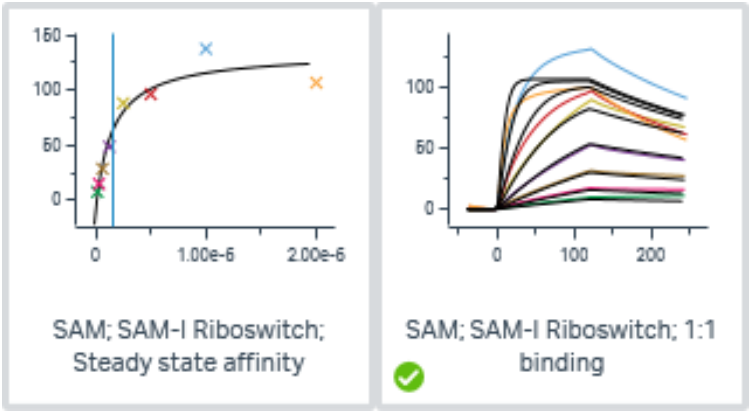
Example:
SAM-I riboswitch

- The SAM-I riboswitch is an RNA regulatory element in bacteria that terminates transcription in response to SAM binding.
- SAM binds the riboswitch with ~150 nM affinity (determined by SPR) to form a kinetically long-lived complex.
- Upon binding, SAM contacts many riboswitch bases and induces extensive conformational rearrangements in P4 and P1.

SAM-I 3D structure with SAM:

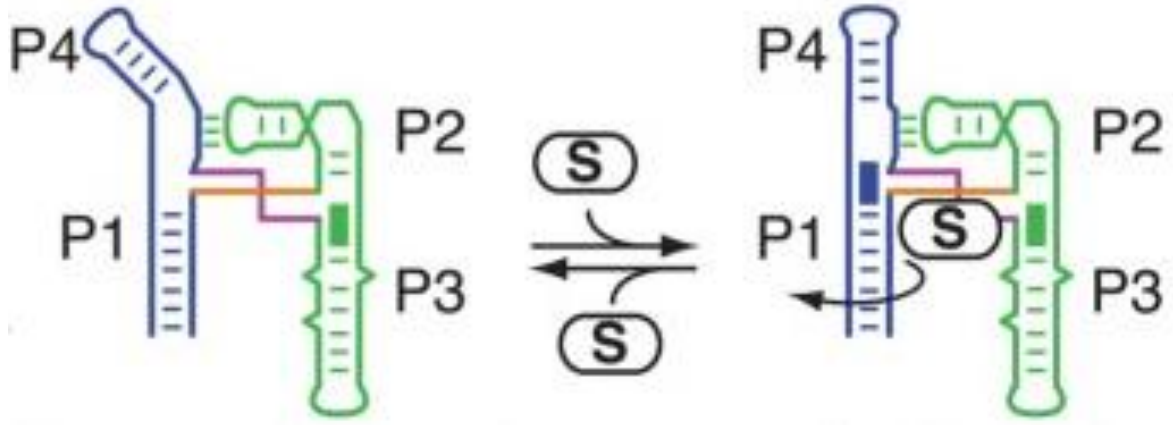


SPR binding data from Arrakis Tx:



KD (nM)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
150 ± 80	84000	0.0030

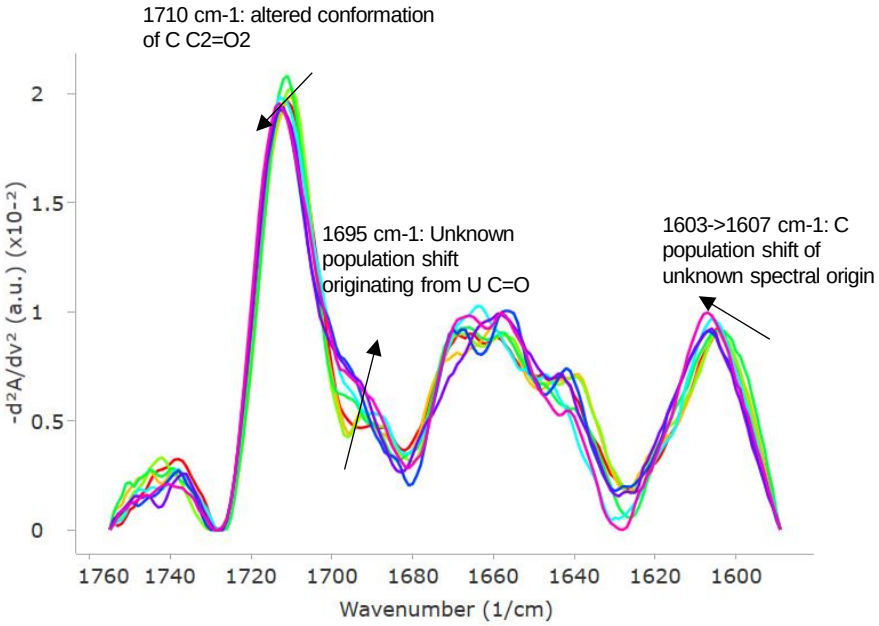
Binding-induced conformational changes in SAM-I:

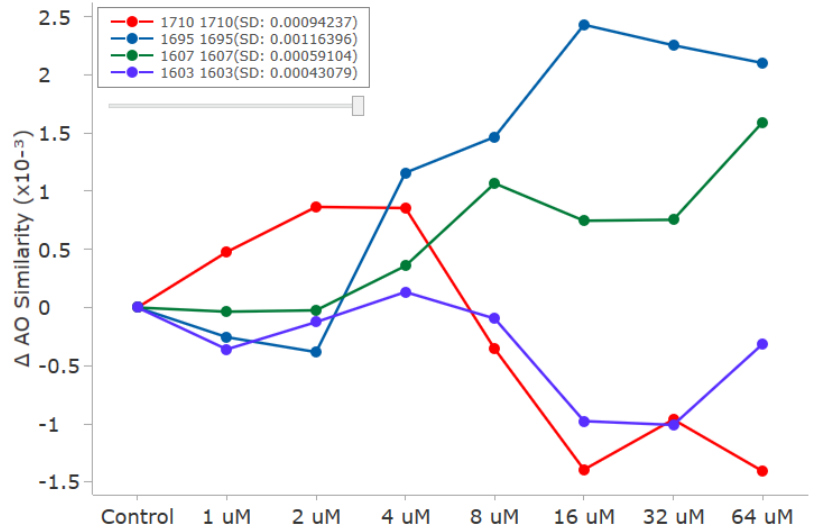


Riboswitch Figures: Dussault AM, Dubé A, Jacques F, Grondin JP, Lafontaine DA. Ligand recognition and helical stacking formation are intimately linked in the SAM-I riboswitch regulatory mechanism. RNA. 2017 Oct;23(10) 1539-1551. doi:10.1261/rna.061796.117. PMID: 28701520; PMCID: PMC5602112.

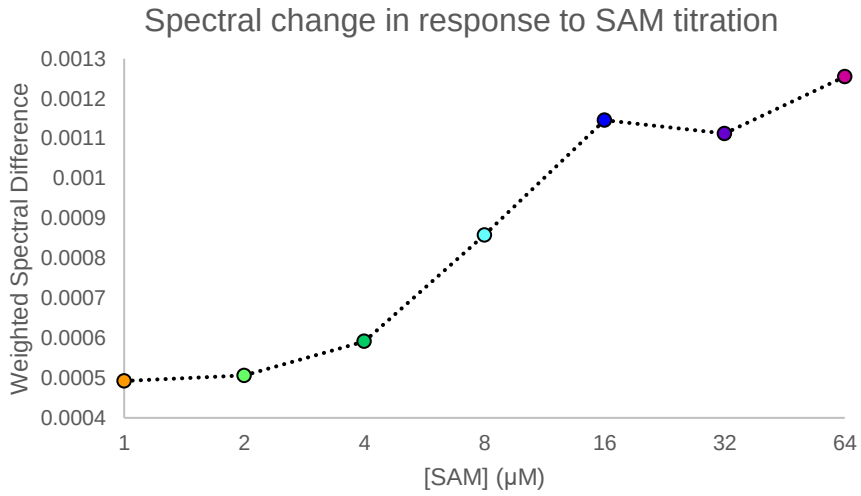
Dose-dependent spectral changes were observed upon titration of SAM into SAM-I

- Spectral changes were observed in the 1710, 1695, and 1607 cm-1 regions primarily corresponding to C and U residues.
- Required ~0.5 mg of RNA per spectrum compared to NMR, which would require roughly 1.5 mg to collect a 1D proton spectrum.
- RNA concentration was ~22 uM (0.67 mg/mL), so a KD was not determined due to significant free ligand depletion.

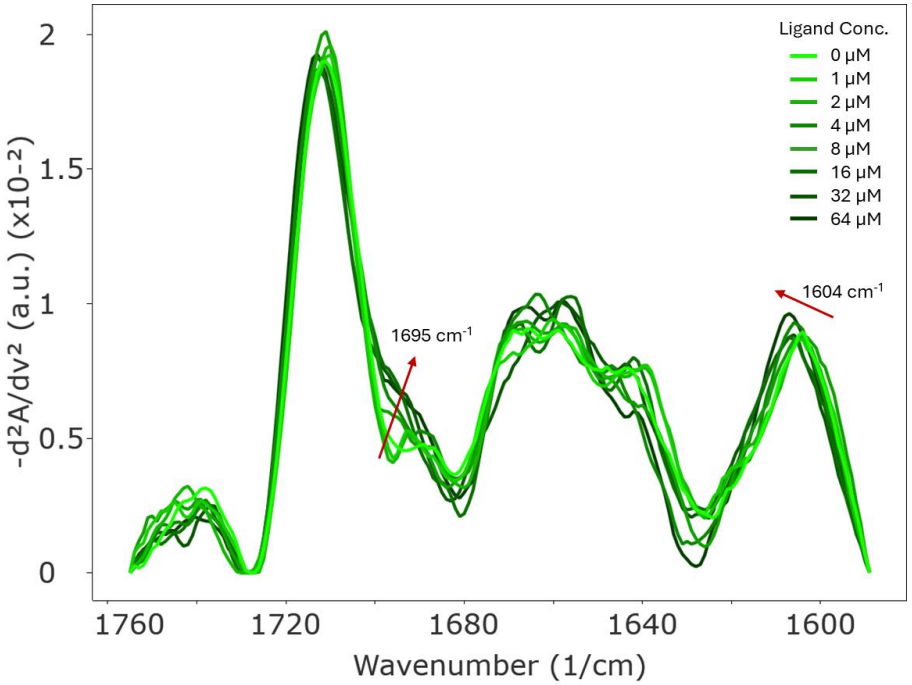


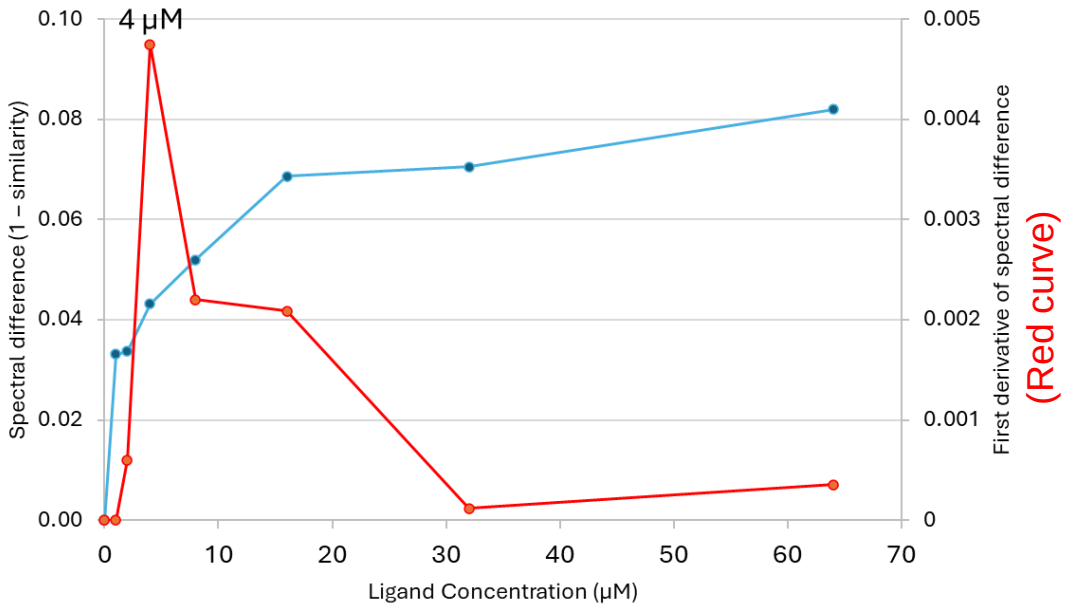


Ligand Concentration (μM)	% Repeatability	% Similarity
0	96.6	100
1	96.5	96.6
2	96.2	96.5
4	97.1	95.5
8	96.6	94.6
16	96.0	92.9
32	96.8	92.7
64	96.7	91.8



SAM-I Riboswitch: Ligand Binding Study

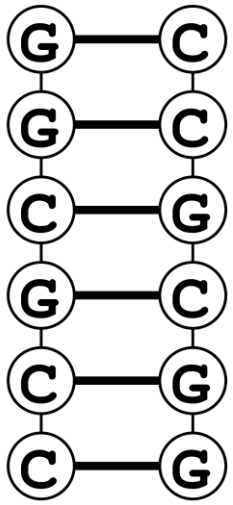




There are clear peak shifts observed in MMS due to ligand binding and by running a titration series, we can see the change and plateau that can give an indication of apparent Kd.

GC base pairing model RNA construct: CCGCGG self-complementary duplex

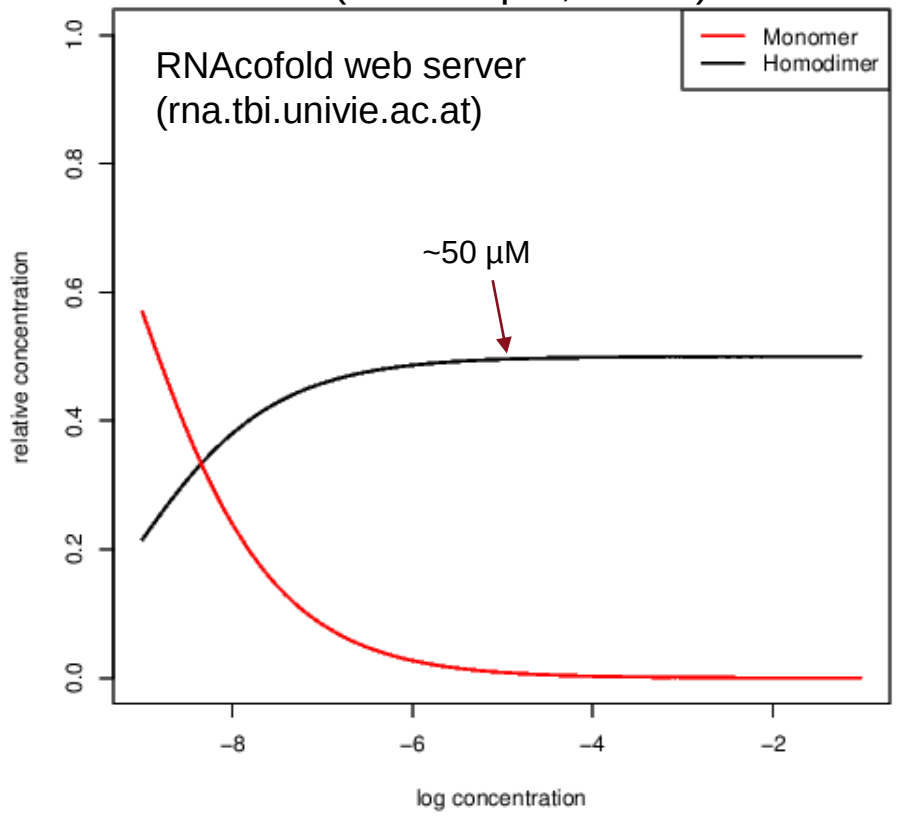
100% Watson-Crick helix predicted at 295 K:



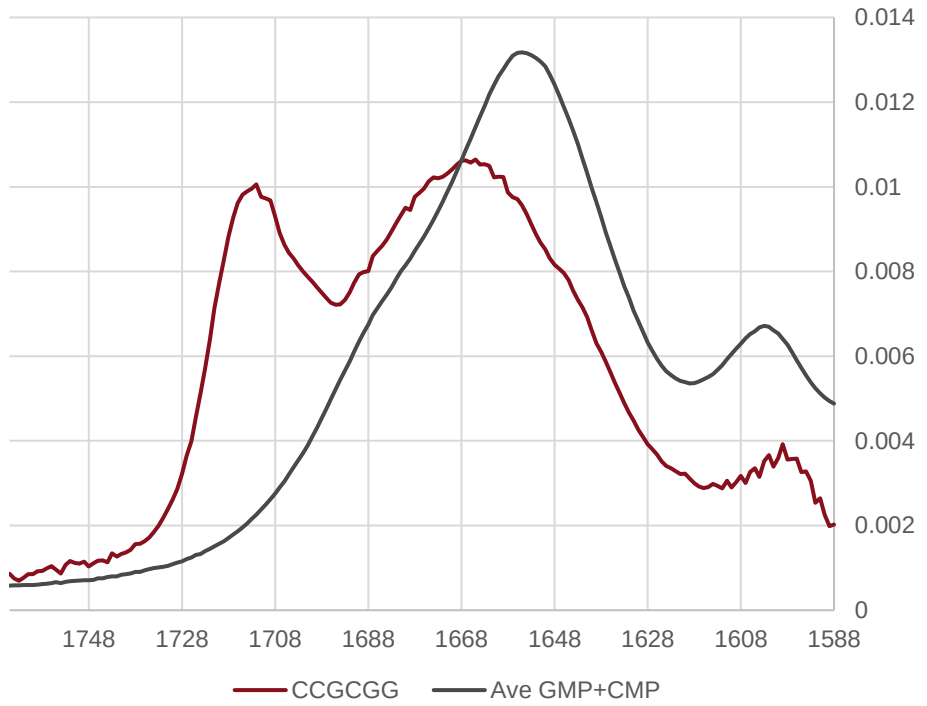
ENERGY = -13.1 CCGCGG_ CCGCGG

Duplexfold Web Server (https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/DuplexFold/Example.php)

Very little monomer present under MMS conditions (i.e. >50 µM, 295 K)

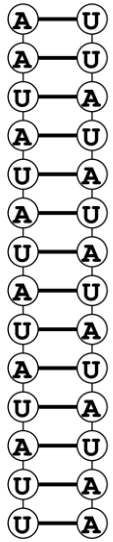


All spectral integrals normalized to 1



AU base pairing model RNA construct: 1RNA (UUAUAUAUAUAUAA) self-complementary duplex

100% Watson-Crick helix predicted at 295 K:

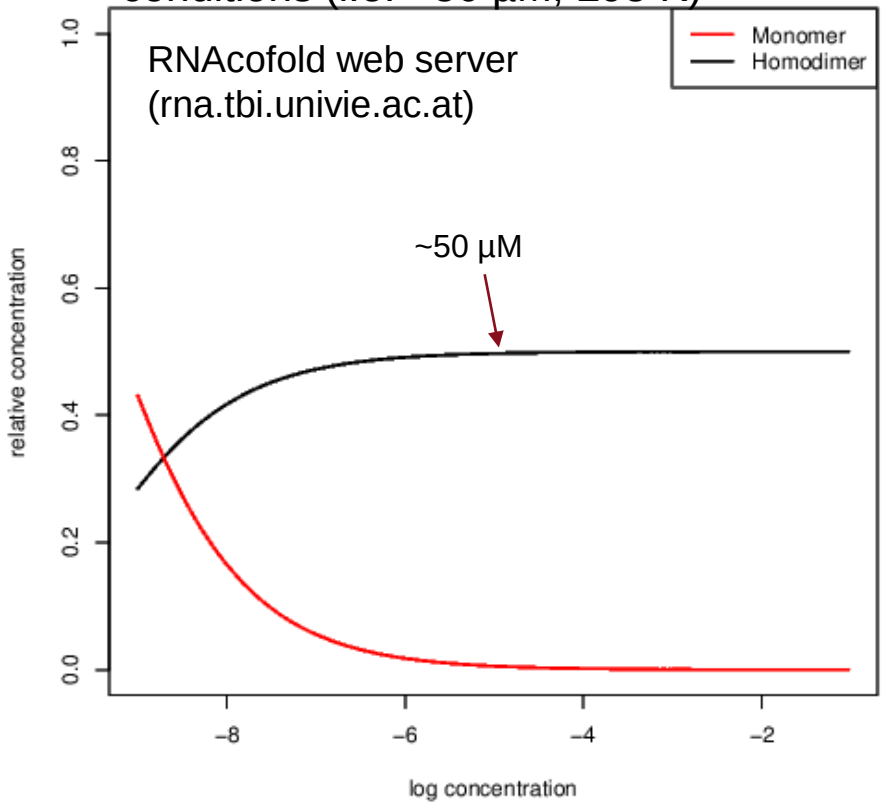


PDB: 1RNA

ENERGY = -14.0 1RNA_ 1RNA

Duplexfold Web Server (https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/DuplexFold/Example.php)

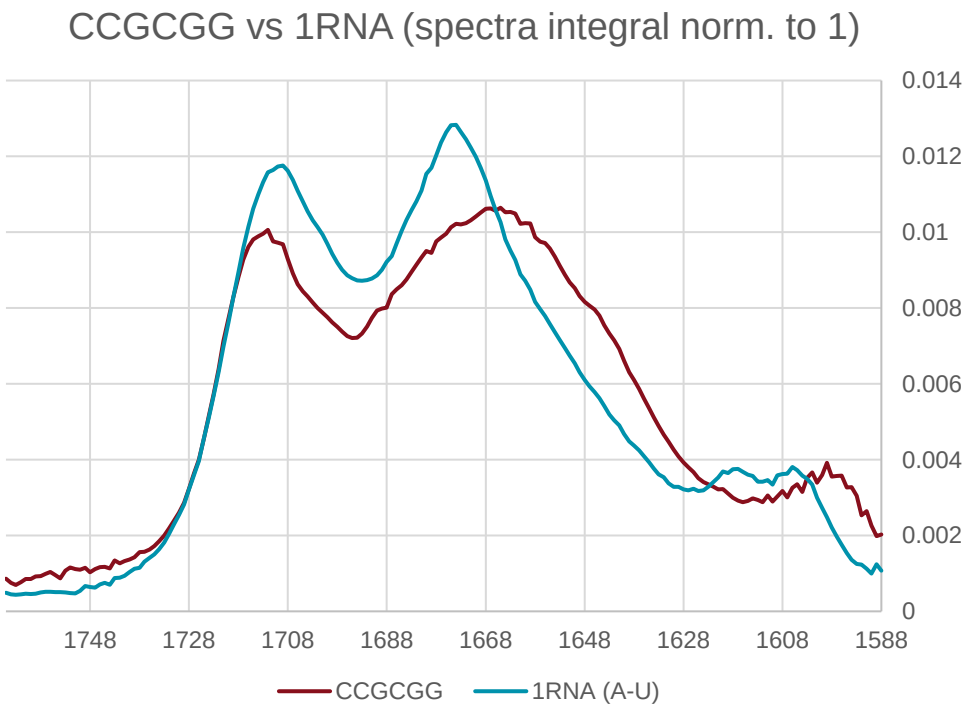
Very little monomer present under MMS conditions (i.e. >50 µM, 295 K)



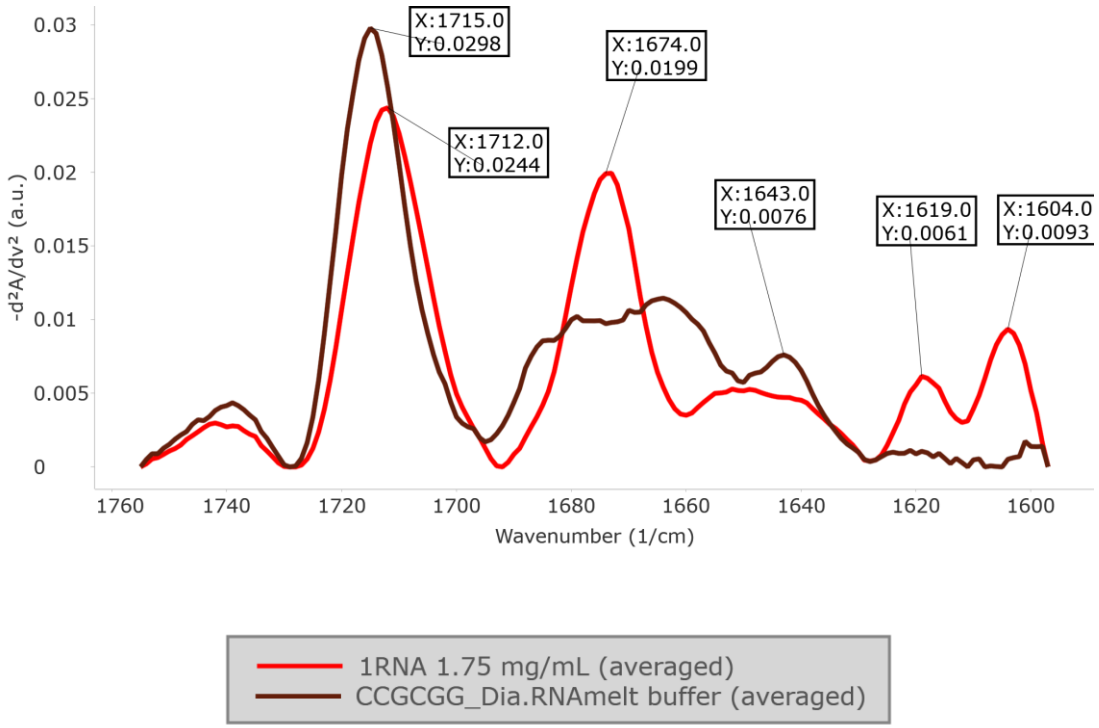
All spectral integrals normalized to 1

Comparison of 1RNA A-U duplex vs CCGCGG duplex

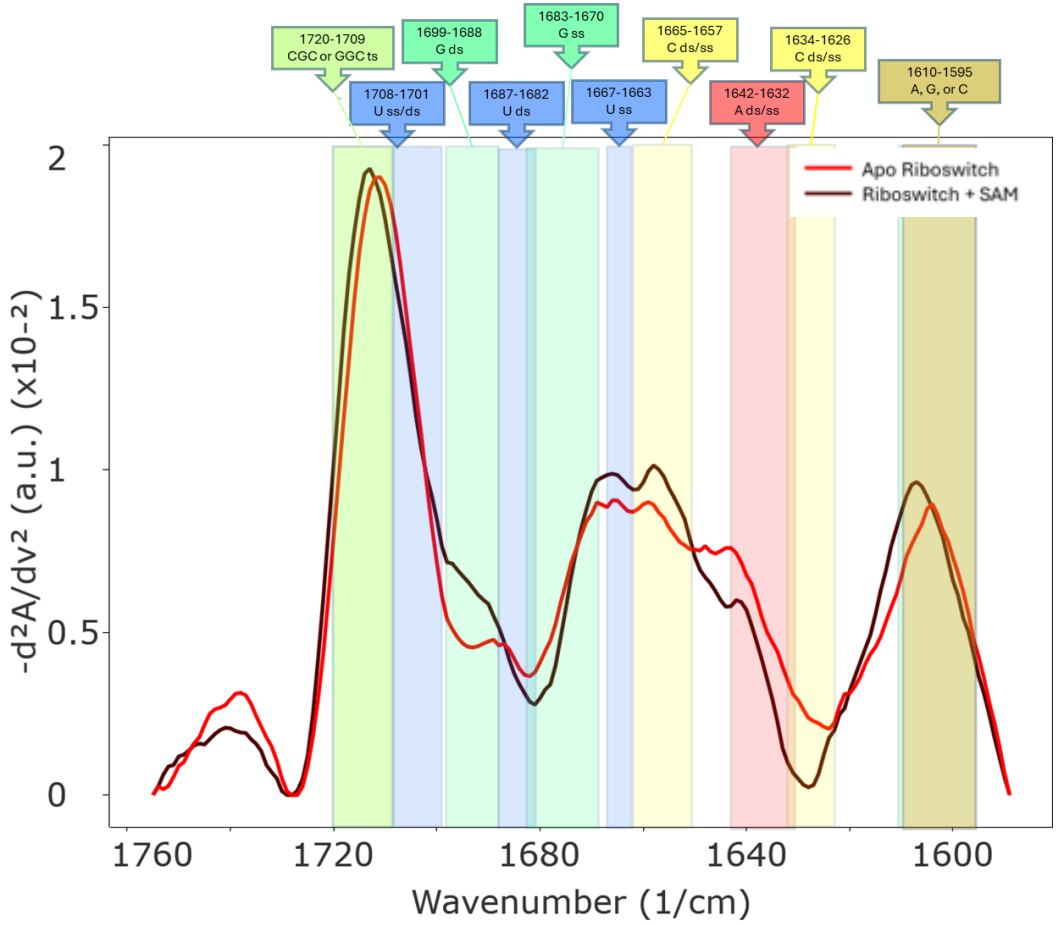
Absolute Spectra Comparison:



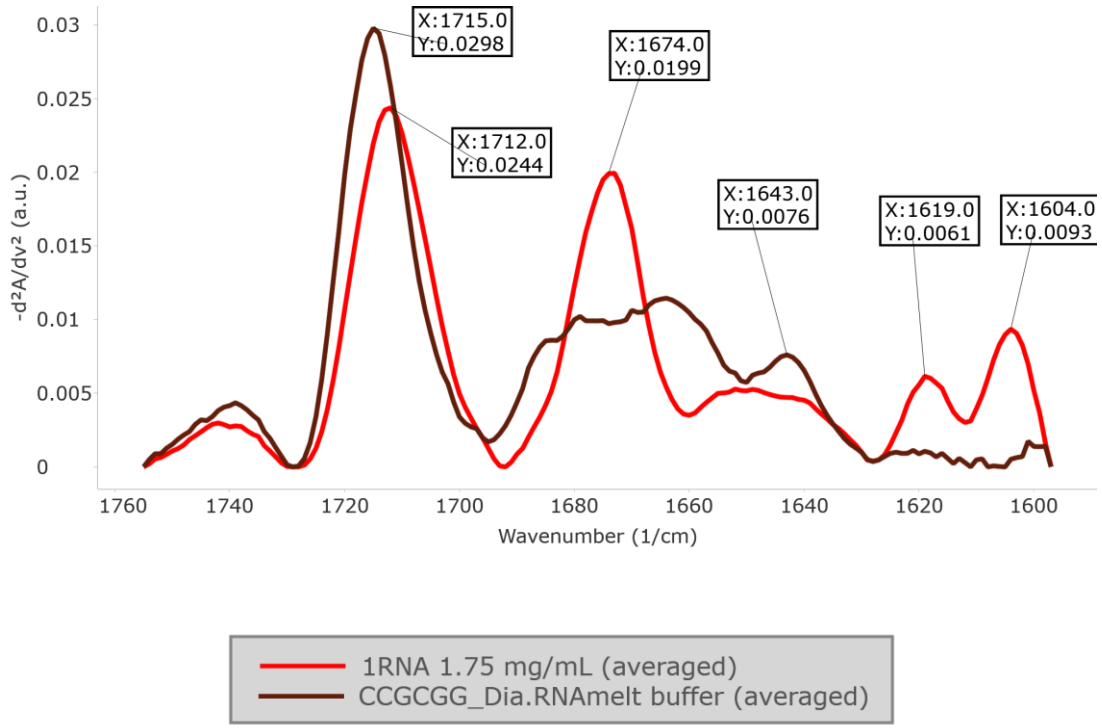
Similarity Plot Comparison:



Comparison of 1RNA A-U duplex vs CCGCGG duplex



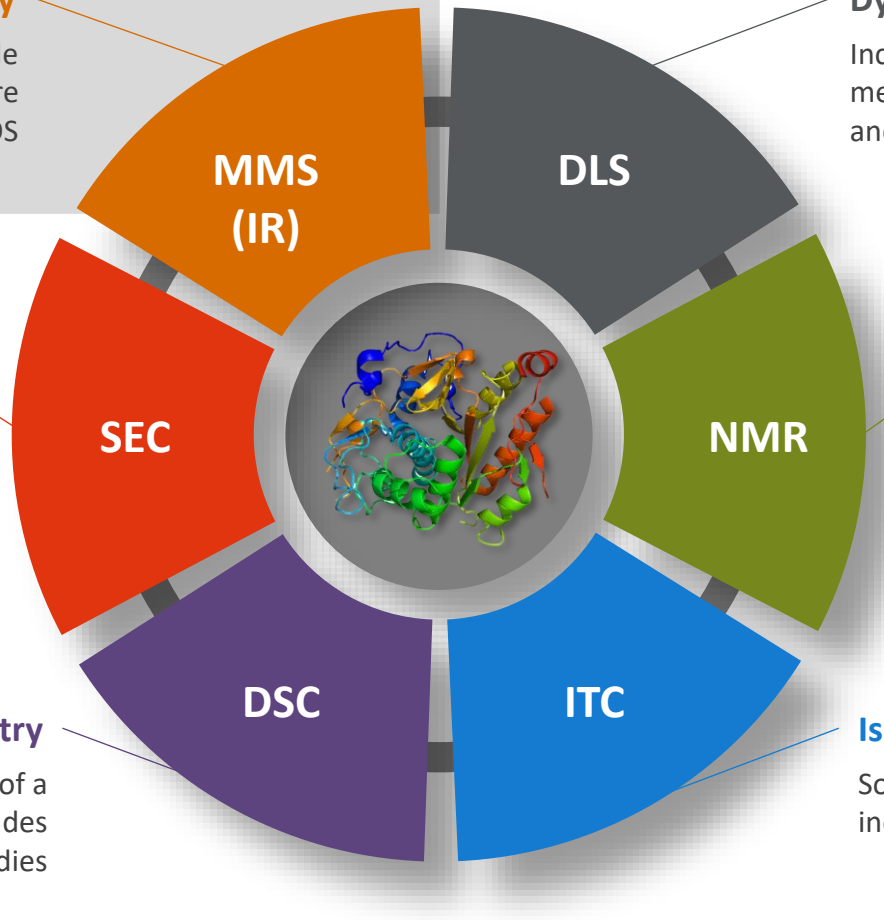
Similarity Plot Comparison:



MMS Is An Essential Part Of Your Biophysical Tool Kit

Microfluidic Modulation Spectroscopy

Automate IR technique compatible wide concentration range, adjuvants, buffers are additional excipients provides a fingerprint of HOS



Dynamic Light Scattering

Incumbent technology for particle size measurement of proteins, aggregation detection, and colloidal stability studies

Size Exclusion Chromatography

Size Exclusion Chromatography with advanced detection (MALS) provides molecular weight assessment of oligomeric species in a formulation.

Nuclear Magnetic Resonance

High-resolution structural and spatial information of the biomolecule in solution. The ultimate tool for solving the crystal structure in three dimensions.

Differential Scanning Calorimetry

Measure the conformational stability of a protein based on Melting point. Provides thermal fingerprint for similarity studies

Isothermal Titration Calorimetry

Solution based assessment of binding interactions, including protein-adjuvant interactions

MMS Detects Structural Changes in RNA

	Pros	Cons
MMS (multifluidic modulation spectroscopy) 1755-1580 cm^{-1}	• Detect population-weighted nucleic acid base pairing/conformation • Can detect conformational changes to as small as 1% of the RNA structure • No labeling requirement • No RNA size limit	• Requires much more RNA than SPR, but <u>much less</u> than NMR or ITC (~0.7 mg) • Requires matched buffer • Due to significant ligand depletion effects, MMS has similar limitations to NMR for accurately measuring KDs (KD > 100 µM) • May be very good for fragment-based drug discovery though!

arrakis THERAPEUTICS

Conclusions

- MMS can detect structural changes in RNA due to changes in formulation and ligand binding
- MMS can detect differences between folded and unfolded RNA
- The individual nucleotides have unique spectra signatures
- We are in the process of assigning spectral regions to different RNA structural elements
- We can determine T_m's for RNA unfolding (data not shown)
- Come to our Symposium on Sept 19th – New RNA data will be presented!