High-throughput Affinity Selection Mass Spectrometry Using a Novel Two Dimensional RapidFire TOF Platform

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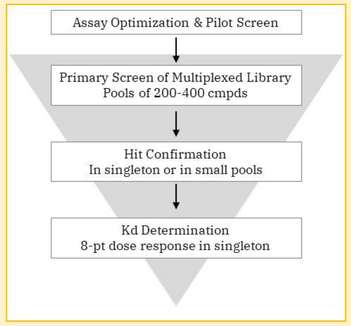
ABSTRACT

Affinity Selection Mass Spectrometry (ASMS) is a solution-phase and label-free technique that enables the binding between small molecules and macro-molecule targets to be determined and measured. In one form of ASMS assay, the target and any bound small molecules are physically separated from unbound small molecules via size exclusion chromatography (SEC) under native conditions. The target fraction from this first dimension is automatically applied to a reverse-phase (RP) second dimension, which separates the binders from the target and delivers them to the mass spectrometer for measurement. This two-dimensional (2D) liquid chromatography (LC) mass spectrometry (MS) method been used extensively to identify and measure small molecule binding to proteins and oligonucleotide targets alike. Recently, ASMS has proven particularly useful in researching molecular glues, PROTAC degraders, and small molecules that can disrupt protein-protein interactions.

In this work, the modification and application of a RapidFire (RF) MS system to execute high-throughput ASMS is described. By introducing a SEC column upstream of the inherent RP cartridge, and leveraging the ability of RapidFire to switch between three solvents instantaneously, the 2D RF MS system was able to execute dissociation constant (K_D) measurements at a sustained cycle time of ~1 minute per sample, representing a significant increase in throughput from what is typically achieved with 2D LC MS. Dissociation constant experiments for multiple established small molecule target pairs were analyzed and results between 2D RF MS and 2D LC MS matched. Two-dimensional RF, therefore, can provide high quality K_D data on hits from primary screens much faster than traditional methods and facilitate hit follow-up and drug discovery, in general.

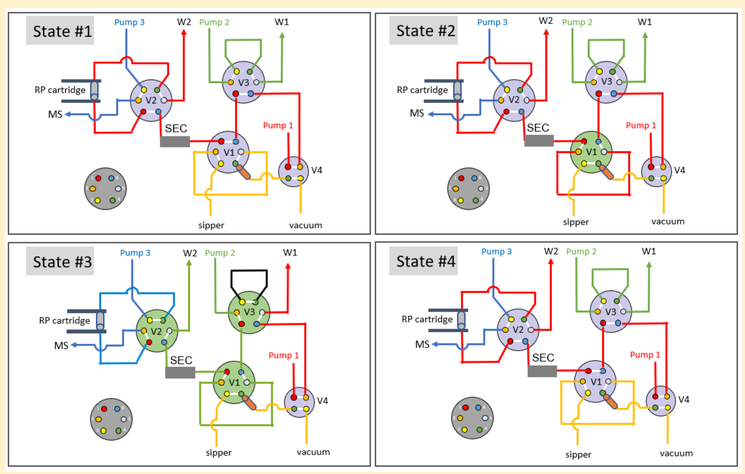
SIGNIFICANCE OF WORK

- K_D experiments on hits found from primary screens are pivotal for informing what compounds get prioritized
- Depending on the size of the screen and the hit rate, following up every hit with a K_D experiment can require significant time.
- This cost commonly limits the number of hits that are followed up - possibly leading to the dismissal of quality candidates.
- Therefore, K_D methods with improved throughput make following up every hit more feasible.



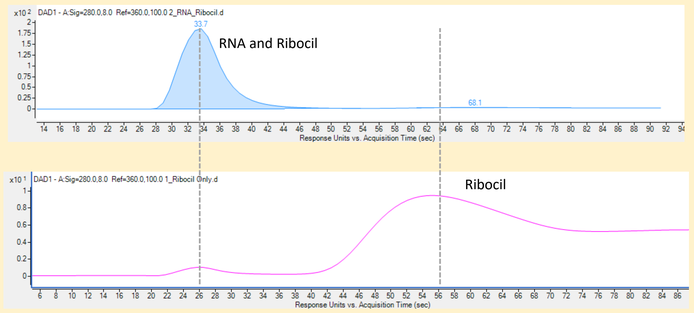
MODIFYING RAPIDFIRE TO BE 2-DIMENSIONAL

- Why start with RapidFire? The RapidFire platform is built for high-throughput operation. The system contains multiple pumps, that each flow their optimized solvent constantly, and multiple valves to instantaneously change solvent conditions within different sections of the fluidic paths.
- How? An SEC column was plumbed in between V1 and V2, just upstream of the inherent RP cartridge of the RapidFire. In this fashion, the RapidFire is able to collect sample (State #1), separate target/binders from non-binders and trap the fraction of interest on the RP cartridge (State #2), elute the binders (hits) from the RP cartridge into the MS for measurement (State #3), and re-equilibrate (State #4) quickly.

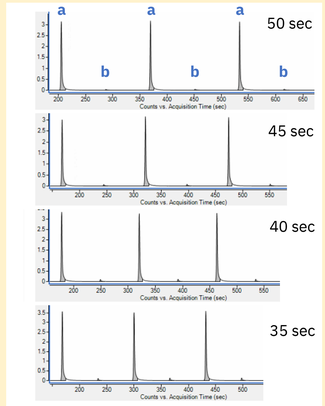


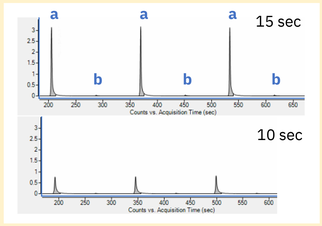
OPTIMIZATION OF SEC TIME

Separation of 10 μM Ribocil +/- 2.5 μM target FMN riboswitch (RNA) using a custom SEC column and 700 mM ammonium acetate (weak solvent) at 0.5 ml/min. Under these SEC conditions, the target fraction is separated from unbound compounds in 12 seconds.



OPTIMIZATION OF STRONG SOLVENT AND RE-EQUILIBRATION TIMES



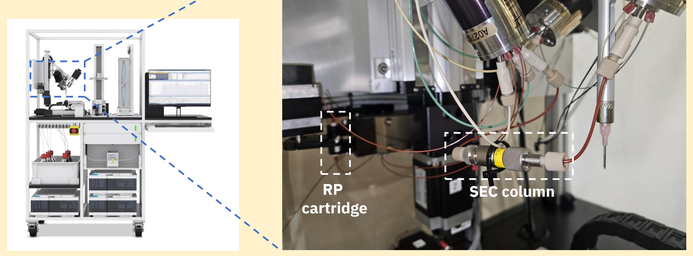


Re-equilibrating the SEC column back to initial conditions before the next injection is critical for column performance. To evaluate the effect of different re-equilibration times, 10 μM Ribocil with (a) and without (b) 2.5 μM RNA were injected in triplicate with different settings. In this experiment, 15 seconds provided the best balance of method throughput and performance.

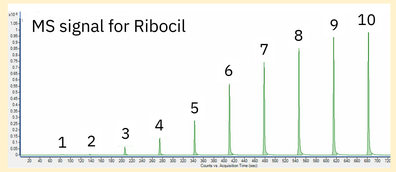
After isolating the target and any bound molecules on the RP cartridge, strong solvents are applied to the RP cartridge to elute the binders into the MS for measurement and the SEC cartridge for washout before the next injection. If strong solvent conditions are not sufficient, carry over can occur. To evaluate the effect of different strong solvent times, 10 μM Ribocil with (a) and without (b) 2.5 μM RNA were injected in triplicate with different settings. In this experiment, there was minimal impact to decreasing the strong solvent time down to 35 seconds. Times less than 35 seconds showed unacceptable carry over (not shown)

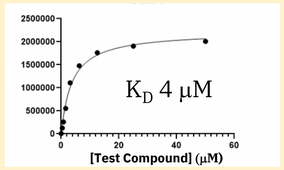
OPTIMIZED 2-DIMENSIONAL RAPIDFIRE METHOD

- Pump 1 (weak solvent), 700 mM ammonium acetate in water at 0.5 ml/min
- Pump 2 (strong solvent for SEC), 70% acetonitrile in water at 1.5 ml/min
- Pump 3 (strong solvent for RP), 80% acetonitrile in water + 0.09% formic acid + 0.01% trifluoroacetic acid at 1 ml/min
- Cartridge type, C18
- Weak solvent time, 12 sec
- Strong solvent time, 35 sec
- Re-equilibration time, 15 sec



10-POINT K_D IN 10 MINUTES



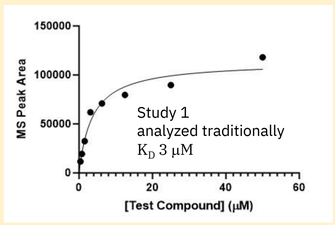


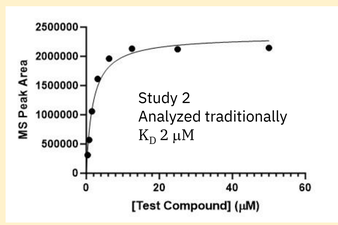
10 injections in ~600 seconds

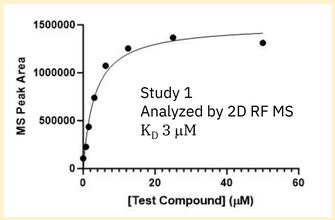
To test the optimized 2D RF TOF method to provide meaningful K_D data, 10 concentrations of Ribocil (2-fold dilutions from 50 μM top) were incubated with 2.5 μM RNA. The data for the 10 samples were acquired in about 600 seconds, illustrating the method sustains roughly a 60 second cycle time. Raw data for Ribocil showed a concentration response in the range tested and plotting the peak areas against their concentrations allowed a K_D of 4 μM to be extrapolated. This value is consistent with what is observed by the traditional 2D LC MS method, which is 6-fold slower than the 2D RF MS method presented here.

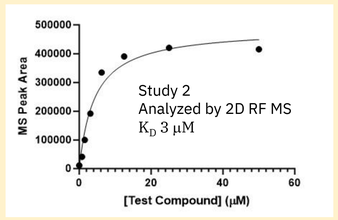
ADDITIONAL TOOL COMPOUNDS

K_D data on multiple RNA targets/tool compounds were acquired using the high-throughput and traditional 2D platforms for comparison.









CONCLUSIONS

- High-throughput ASMS K_D measurements were achieved on multiple RNA targets using a modified 2-Dimensional RapidFire MS platform.
- The novel platform acquired data at sustained rate of ~60 seconds per sample, more than 6-fold faster than a traditional method.
- K_D results from 2D RF MS match those from 2D LC MS.
- This high-throughput platform, therefore, provides a practical way to determine the K_D of 60 compounds in an 8-hour shift, label-free and in-solution.

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