Identification and Characterization of Non-Covalent Small Molecule Binders of mRNA using Affinity Selection-Mass Spectrometry (ASMS)

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ABSTRACT

Recent publications[1] demonstrating inhibition of downstream gene products through the selective binding of compounds to DNA or RNA targets have led to a renewed interest in Affinity Selection-Mass Spectrometry (ASMS) based high-throughput screening approaches. Here we describe an approach using the Automated Ligand Identification System (ALIS) to identify test compounds that bind the target of interest from pools of hundreds of small molecule test compounds. The ALIS approach uses 2D size-exclusion/reversed-phase chromatography coupled to a high-resolution time-of-flight mass spectrometer to identify unique binders in pools of up to 600 test compounds in 6.5 minutes. Data analysis and hit identification is performed using a combination of Agilent Mass Hunter and proprietary custom software. Putative primary screening hits are confirmed in singleton or small pools of up to 5 compounds at 3 different concentrations. Validated hits can be followed up as full dose-titrations to determine binding characteristics and affinity. With this approach, high-throughput screens with 125K test compounds can be completed in 36 hours and require only a few mg of target RNA.

INTRODUCTION

The target in ASMS is a macromolecule that can be separated from small molecule test compounds by size exclusion chromatography (SEC). The target can be RNA, protein or a protein/RNA complex. Depending on the compression ratio of the pools, greater than 100,000 compounds can be analyzed in 36 hours. The ASMS screening platform is highly versatile, allowing for affinity assessment against a range of traditionally intractable targets to identify binders. The approach can be used on targets where the biology may not be well understood and a conventional functional assay is challenging to develop.

ADVANTAGES OF ASMS

- High throughput: Over 100K test compound in pools of 320 can be screened in 36 hours
- Low protein usage: Primary screen of 100K+ test compounds requires ~1 mg of a 50 KDa RNA or protein target
- Solution-based binding with native target: target or test compound does not need to be modified or immobilized on a surface
- Ability to detect small molecule binding on very large targets including protein-protein and protein-oligonucleotide complexes

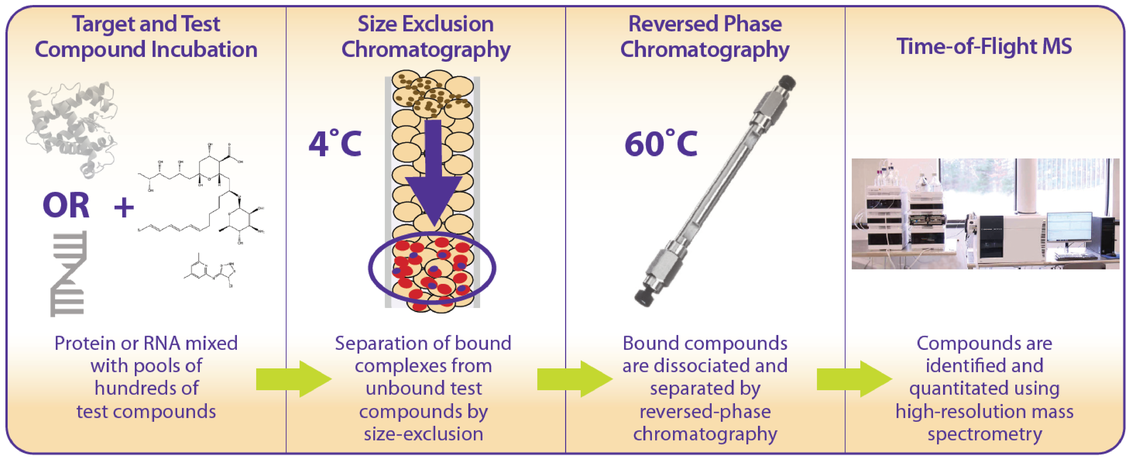
METHODS

The ALIS instrumentation consists of an Agilent 1260 HPLC pump for the size-exclusion chromatography coupled to an Agilent 1290 UHPLC pump for the reversed phase chromatography with a high-pressure switching valve interfaced to an Agilent 6230 Time-of-Flight Mass Spectrometer. Data analysis is performed with a combination of Agilent Mass Hunter and proprietary custom software

SEC conditions: Buffer A: 700 mM Ammonium Acetate
Buffer B: 70% Acetonitrile

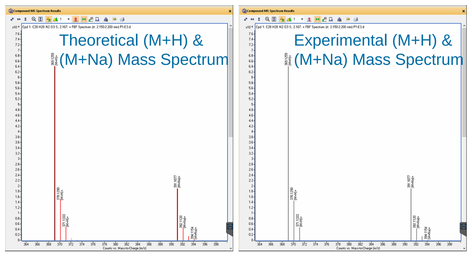
RP conditions: Buffer A: 0.1% Formic Acid in Water
Buffer B: 0.1% Formic Acid in 90% Acetonitrile

ALIS-ASMS WORKFLOW



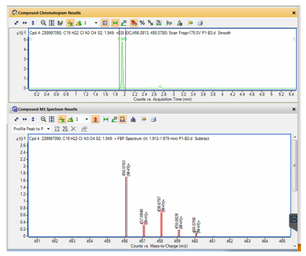
DATA ANALYSIS & HIT ID

The Find-by-Formula algorithm in Agilent MassHunter SW is used to search for all test compounds in each pool. Every potential feature identified is given a score from 1 to 100 based on the mass accuracy of the main peak together with the ratio and spacing of naturally occurring isotopes

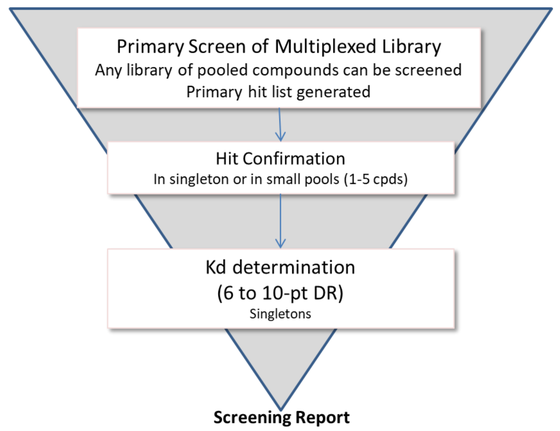


Features identified by MassHunter SW are determined to be unique to the correct test compound pool by running a proprietary two-step cross-hit filter. In step one, features are compared to a series of DMSO-only blank injections. In step two, those unique compounds are compared against all other test compound pools. Only features that are unique to the correct test compound pool are selected for confirmation and further characterization.

Example of an ASMS Hit: $C_{19}H_{22}ClN_3O_4S_2$



ASMS SCREENING CAMPAIGN FUNNEL



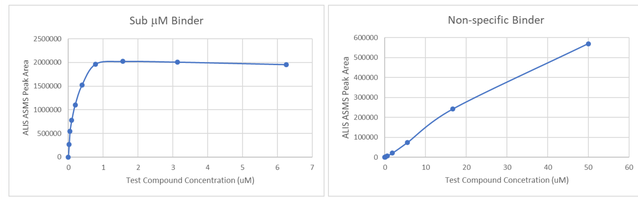
HIT CONFIRMATION

Test compounds identified as hits in the primary screen are confirmed as binders in singleton or small pools of 2-5 compounds run at 1 uM and 10 uM test compound in the presence of target as well as 10 uM test compound without any target present to ensure that the signal is target-dependent and not an artifact or breakthrough in the SEC column

LEAD CHARACTERIZATION & BINDING AFFINITY DETERMIMATION

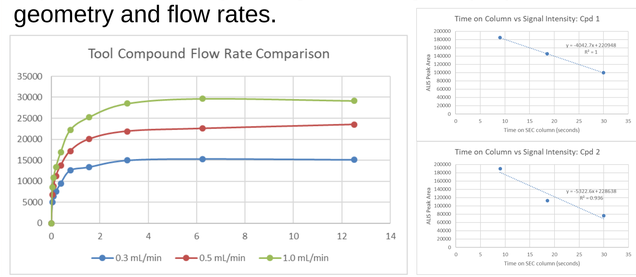
Confirmed hits may be further characterized and the Kd determined by running test compound dose-response curves. Typically 8- to 12-point titrations ideally straddling the Kd are performed. Selective and specific high-affinity binders have binding curves that asymptote at the target concentration while non-specific or non-stoichiometric binders will have linear dose-response binding curves.

SPECIFIC AND NON-SPECIFIC BINDING CURVES



ASSAY OPTIMIZATION

Bound compounds start dissociating in the SEC column as the equilibrium between on and off rates is disturbed due to the physical separation of the target and test compounds. It is important to minimize the time spent on the SEC column, which can be done by maximizing the separation efficiency through selecting the proper column geometry and flow rates.



CONCLUSION

A version of an Affinity Selection Mass Spectrometry binding assay based on an approach originally described as the Affinity Ligand Identification System (ALIS)[2] has been implemented in our laboratory. The platform has been successfully used to screen dozens of RNA and protein targets and to further confirm and characterize putative hits. The approach can be further used to support SAR and medicinal chemistry programs to improve the desired characteristics of lead compounds.

ASMS enables high throughput with greater than 100,000 test compounds screened per day and has low target consumption requiring only a few mg of target for most screens. Additionally, binding assays with very large targets are compatible with ASMS. Traditional technologies such as Surface Plasmon Resonance (SPR) or interferometry are incompatible with many RNA targets due to the difficulty of detecting the small mass change upon test compound binding a large target. Because ASMS directly measures the dissociated test compound, there are no limitations on the size of the target.

References:

1) Flusberg, DA *et al*, (2019) SLAS Discovery: Advancing Life Science R&D **24**(2), 1-16
2) Annis DA et al, (2004) International Journal of Mass Spectrometry **128**(2), 77-83

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