pivotpark screeningcentre

Target Engagement Assessment of Drug-like and Pan-assay Interfering Compounds using Affinity Selection Mass Spectrometry

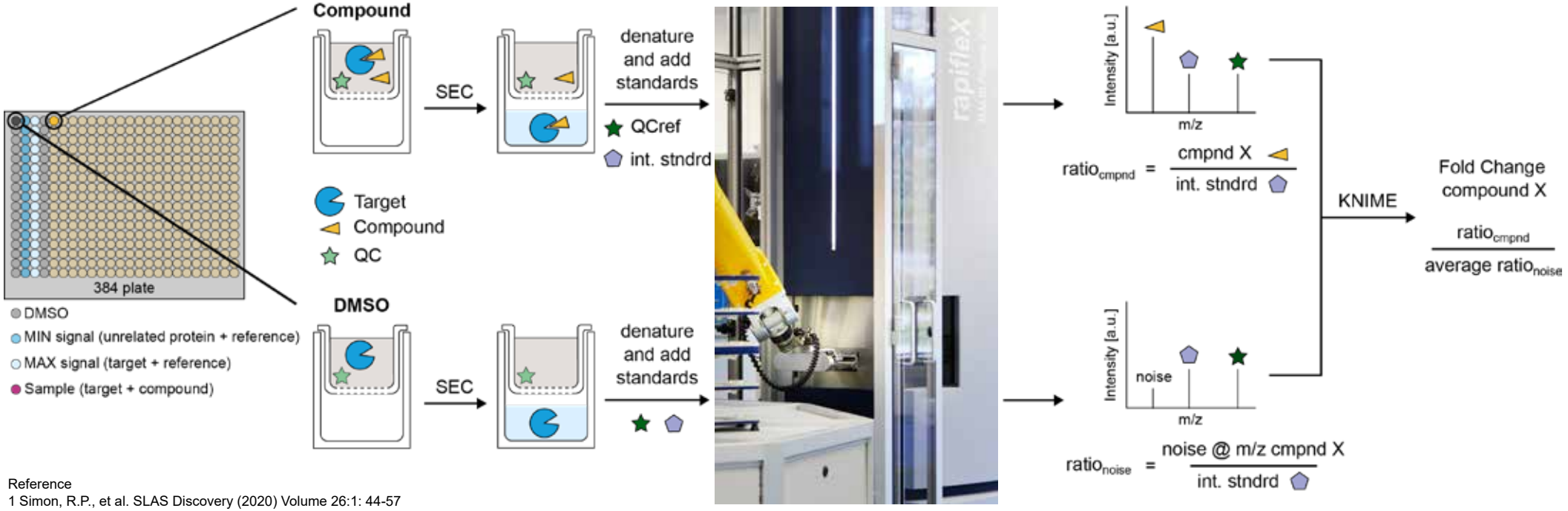
Pivot Park Screening Centre (PPSC) has adopted Affinity Selection Mass Spectrometry (ASMS) workflow into its daily high-throughput screening (HTS) operations. ASMS is a biophysical method to study protein-ligand interactions using mass spectrometry (MS) in microtiter plates. High-throughput ASMS is based on separation of protein-ligand bound complexes from unbound compounds achieved by in-plate size exclusion chromatography (SEC), followed by identifying the ligands using MS. In contrast to other conventional biophysical assays, ASMS is label-free, does not require mobilization or extensive assay development, and is HTS-amenable, allowing the analysis of large compound libraries.

Thrombin, a well-studied protease was used to establish an ASMS-based screening pipeline. To this end, we setup a label-based and a label-free functional biochemical, as well as a biophysical ASMS binding assay to screen the ‘Robustness Set’ compound collection against thrombin. The Robustness set collection is comprised of various classes of compounds with assay interfering properties and non-drug-like mode-of-action such as autofluorescence, aggregation, chelation, chemical reactivity, and redox activity, as well as a focused chemically diverse drug-like subset for which no obvious interferences are expected. In the primary ASMS assay, a total 14 compounds were identified as potential binders to thrombin, of which only two were identified as inhibitors of the functional enzymatic activity. We examined the specificity of the 14 binders by comparing their binding to bovine γ-globulin (BGG) using a confirmation ASMS setup. This resulted in confirming target engagement of two compounds with inhibitory activity in the biochemical enzymatic setup and identifying one compound that selectively binds to the target, yet didn’t inhibit the functional activity of thrombin.

The results from testing robustness set compound collection show that the ASMS setup, coupled with unspecific binders counter-screen confirmation setup is complementary to the biochemical workflows for early assessment of target engagement. Although the throughput is around 5,000 samples/wells per day, compound pooling can drastically increase the throughput of ASMS-based screening for use in primary screening of large compound libraries.

Affinity based selection mass spectrometry at Pivot Park Screening Centre

A published ASMS HTS workflow (Simon et al. SLAS Discovery, 2020)[1] was used for the pilot study. For the ASMS protocol, we made use of our general in-house plate layout, with control samples in columns 1-4 (DMSO, minimum and maximum effect) and compounds samples in columns 5-24. The target proteins were then incubated with either DMSO, reference (maximum signal, MAX), or compound, and an unrelated protein was incubated with reference to estimate the minimum signal (MIN). Additionally, a quality control (QC) peptide was added to the sample reaction as a technical control. Subsequently, compounds bound to the protein were separated from the non-bound ligands and the QC peptide using in-plate size-exclusion chromatography (SEC). Since buffer components will not elute during the SEC process, the assay buffer can be optimized for the target protein. The protein-ligand complex was then denatured by adding acetonitrile to the eluate, including two peptide standards: (1) an internal standard (int. stndrd) to normalize the compound intensity (2) an isotope of the quality control peptide (QCref) to quantify possible SEC leakage. After preparing the samples using the CyBio Well vario system (Analytik Jena AG), the mass of each compound was traced by the rapifleX® MALDI PharmaPulse® (Bruker AG). Samples with a SEC leakage of more than 1 %, as well as samples missing one out of two peptide standards were invalidated. Relative quantification of the compound was achieved by calculating the fold change based on dividing the signal of the compound to the noise in the DMSO well. Compounds with equal or above 2.5 fold signal compared to the background (FC ≥ 2.5) were considered to be binding to the target. In addition, compounds were also identified as binders if the compound mass was detected (intensity > 0) and lacked the noise signal (intensity of noise is 0).



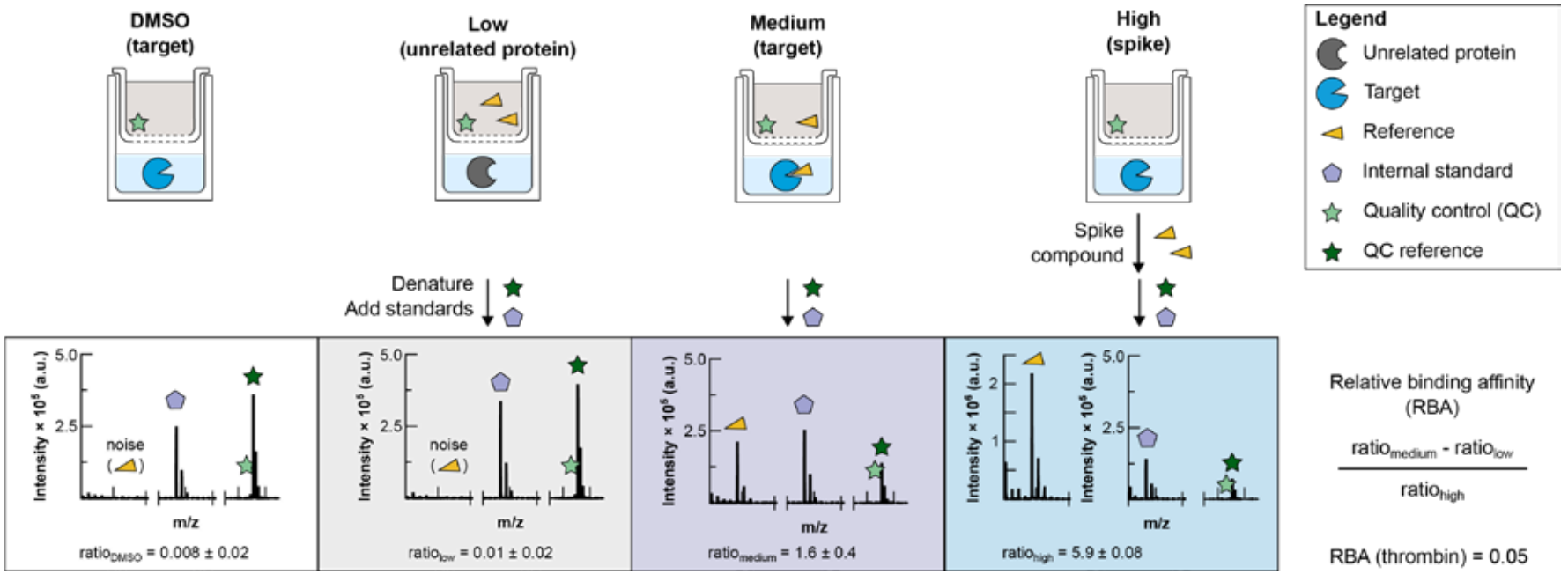
Reference
1 Simon, R.P., et al. SLAS Discovery (2020) Volume 26:1: 44-57

Specific binding of thrombin reference in ASMS

To establish the biophysical ASMS workflow in our screening lab, we setup an assay for thrombin, a well-studied serine protease. Thrombin catalyses many coagulation-related processes with high specificity, e.g. converting fibrinogen into insoluble strands of fibrin.[3] The activity of this enzyme is crucial for 'coagulation' and thrombin is implicated in the blood clotting process acting as a potent vasoconstrictor.[4] Many anticoagulation medicines have been developed over the last 50 years to prevent certain cardiovascular and thromboembolic diseases.[5,6]

To select an appropriate reference molecule, we tested multiple known inhibitors in the primary ASMS setup and selected the monovalent inhibitor argatroban6 as a reference tool compound during the ASMS screen. This reference ionized well and showed binding to thrombin. Since the intensity of the MS cannot be used for absolute quantification and ASMS is prone to aspecific binding, it is recommended to validate the primary ASMS screen results by analysing the specificity of the compounds using an active confirmation setup.

In the active confirmation assay , three sets of control samples per compound were required indicated by low, medium, and high. The medium sample was similar to the primary assay, compound incubated with the target protein (thrombin). In the low sample, an unrelated protein (BGG) was incubated with the compound, and the compound is spiked in the eluate of the high sample containing thrombin (1:1 ratio). Calculating the relative binding affinity (RBA) indicates whether the compound is specifically binding to the target and estimates the amount of compound that is binding. The specificity for the reference argatroban was tested in this setup, showing binding to thrombin with an RBA between 0.034-0.043 , indicating specific compound binding.

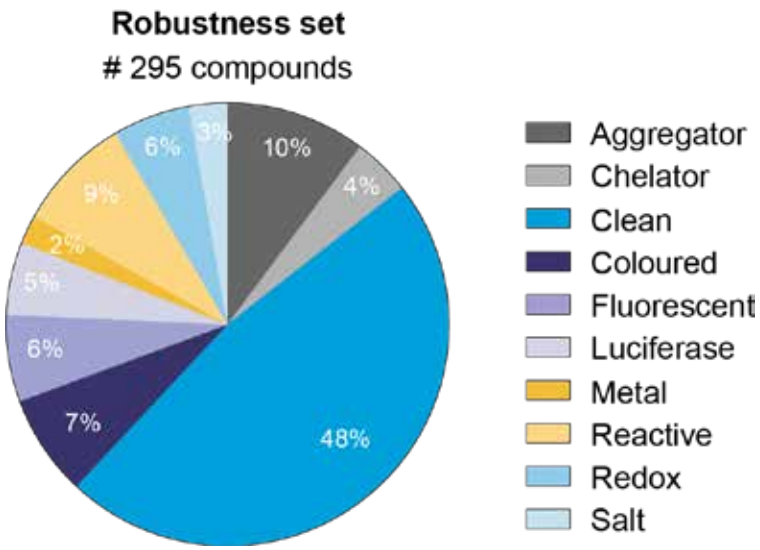


References
3 Hulshof, A. et al. Int J Mol Sci 2021, 22 (5): 2590; 4 Vanhoutt, P.M. et al. Acta Physiol (Oxf) 2017, 219 (1): 22-96; 5a Lee, C.J. and Ansell, J.E. Br J Clin Pharmacol 2011, 72(4): 581-592; 5b Grossmann, K. Biomedicines 2022, 10(8):1890; 6 Garcia, D.A., et al. Chest 2022, 141(2): 11-2291

The robustness set: a focused drug-like and pan-assay interference compound collection

The ASMS protocol for thrombin was screened using the robustness set collection. This compound set was specifically designed to dissect primary assay liabilities and inform on requirements for triaging assay cascade prior to performing (ultra-) HTS campaigns.[2] This collection comprises of selected molecules with well-defined assay interfering properties, like aggregation, metal ion chelation, redox activity, autofluorescence, absorbance, luciferase inhibition, and chemical reactivity, as well as so-called 'clean' diverse drug-like compounds for which no obvious assay interfering properties are expected. The compounds are present in quadruplicates and scattered throughout a 1536-well plate. Perturbing an (ultra-)HTS assay with the robustness set compound collection gives insight on susceptibility of the assay to technology interference and non-drug-like mode-of-action, along with an indication of active rate prior to progressing full-deck HTS.

[2] Honarnejad, S. et al. SLAS Discovery (2020) 26(2):192-204



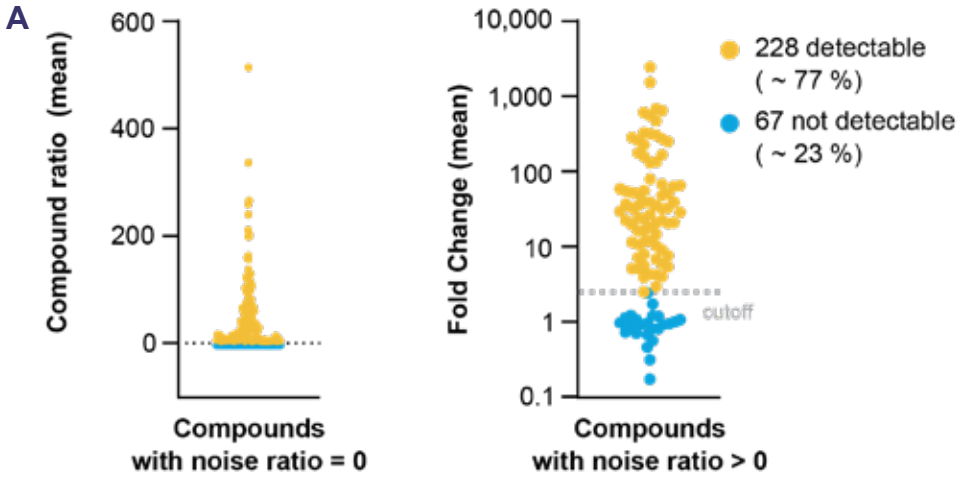
Detectability of the robustness set by MALDI-TOF MS

As a first step towards ASMS, we assessed the detectability (ionization) of each compound present in the robustness set by the rapifleX.* In total 228 compounds out of 295 (~ 77 %) were detectable based on the peak ratio and FC ≥ 2.5 in n = 4. We compared the detectability for each single measurement to the detectability of the average in four replicates. The false positive rate and false negative rate per single measured were 3 % and 2 %, respectively. This means that from 295 compounds, on average 222 compounds were detectable in a single measurement, including 2 false positives. In addition, on average 73 out of 295 compounds were not detectable based on single point data, whereof 69 compounds were true negatives and 4 false negatives.

Furthermore, evaluating the datapoints based on p-value calculated with a t-test (two tailed distribution, unequal variance) resulted in 220 compounds (~ 75 %) with significantly higher signal relative to the DMSO background (p-value ≤ 0.05), all of which had a FC ≥ 2.5. The eight compounds showing no statistically significant difference relative to background according to the t-test generally had a very large standard deviation; either for the compound intensity or the noise intensity ranging from 60 – 121 %.

Therefore, only those compounds showing FC ≥ 2.5 and a significant p-value were considered to be detectable, highlighting the importance of running replicates in ASMS.

* Remark: The samples were prepared using two crystallization methods and analyzed in positive ionization mode. A minor subset of the compounds were detectable in negative ionization mode and therefore not subject to further analysis (< 1 %).

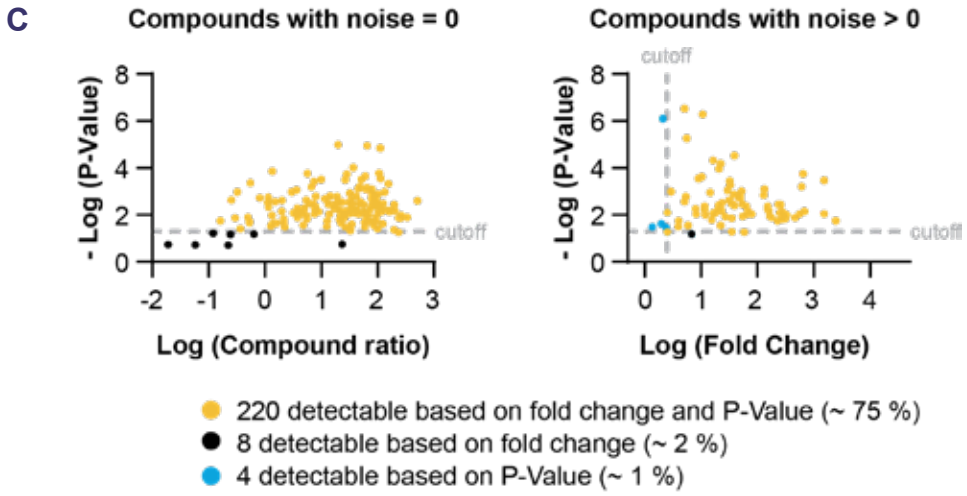


B **Detectable based on fold change**

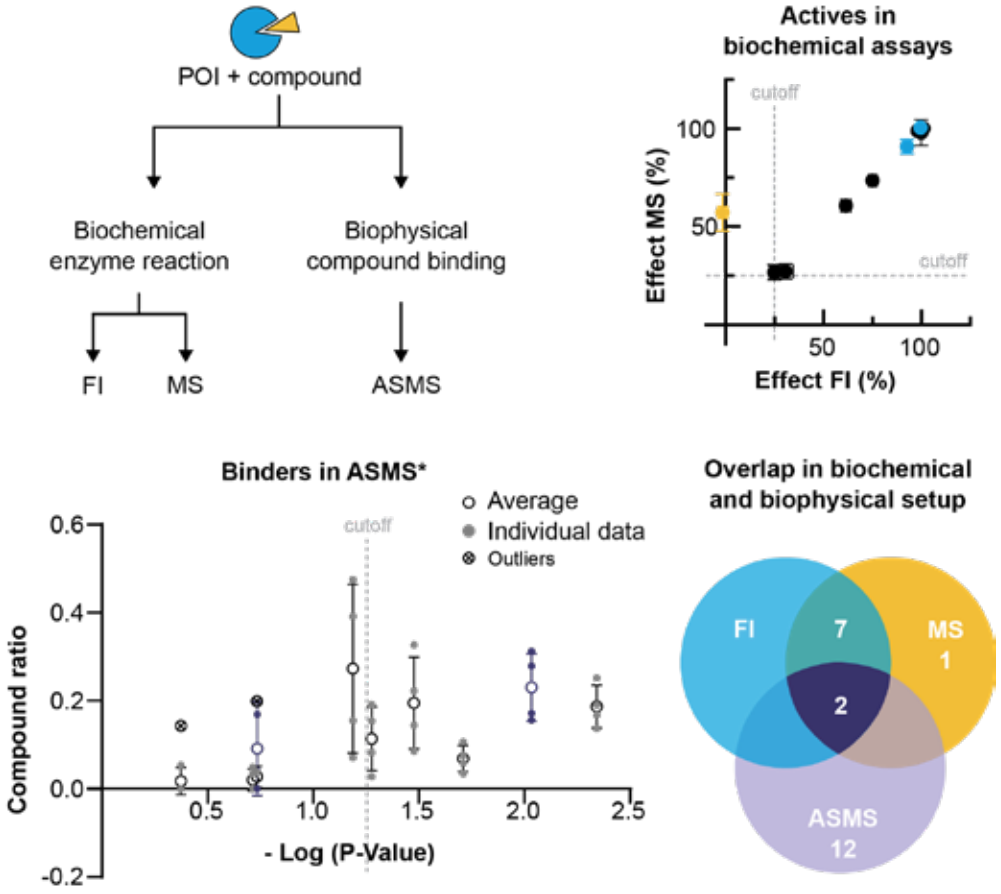
Mean n = 4 \ Single n = 1	True	False
True	220	4
False	2	69

Statistics

False positive rate (FPR)	0.031
False negative rate (FNR)	0.017
Sensitivity	0.98
Specificity	0.95
Accuracy	0.98



Primary ASMS screen: target engagement of thrombin binders



The binding of compounds from the robustness set collection to thrombin was evaluated in three different setups: functional protease activity was followed in a biochemical setup by fluorescence intensity (FI) and mass spectrometry (MS), and biophysical binding was analysed in the primary ASMS setup.

From the 295 robustness compounds, we detected 14 unique compounds binding to thrombin based on FC ≥ 2.5 (n = 4) in the biophysical ASMS setup using two different crystallization methods. Identifying binders based on individual peak ratios (n=1) resulted in 11 primary compounds, whereof 3 were falsely annotated as binder compared to the observed binders in n = 4 (~27 %). From the total 14 unique compounds, t-test showed that the signal of 5 compounds was significantly increased compared to the DMSO control (p-value ≤ 0.05).

In total, 10 compounds in the robustness set collection were inhibiting the enzymatic activity. There was an overlap of 2 compounds between the three different setups, while 8 hits were only observed in the biochemical setup. From the 8 functionally actives, 4 compounds were not ionising in the rapifleX and the other 4 were not detected in the primary assay by ASMS. Although these compounds were not considered as binders, they are not per definition non-binders as the detection limit of the MS can be of influence. This means that the ASMS setup is biased towards ionization and detection limitations by MS and performing an ionization check of the compound library before running an ASMS screen is recommended.

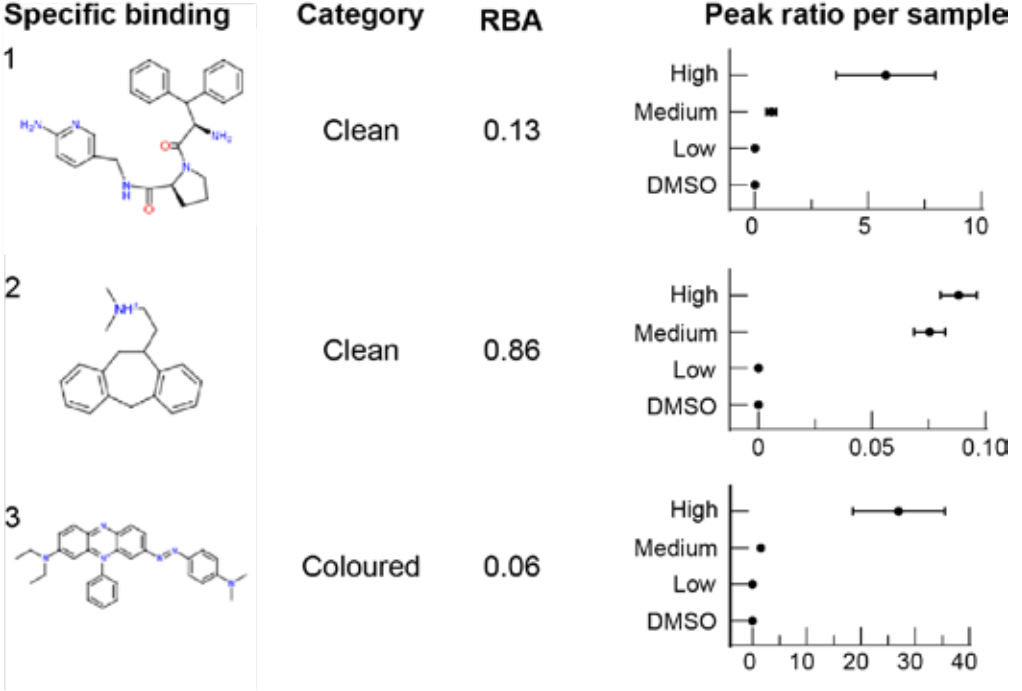
Evaluating ASMS actives for specificity in active confirmation

The 14 binders observed in the primary ASMS screen were subsequently analysed in the confirmation ASMS setup, including DMSO, low, medium, and high samples.

3 out of 14 binders identified in the primary ASMS screen were confirmed to bind specifically to thrombin, whereas no binding was observed to BGG (n=4). The remaining 11 compounds were neither detected in the medium or low control sample (4 compounds) nor were ionized as observed in the high sample (7 compounds). The crystallization and ionization in active confirmation is influenced by the higher amount of DMSO in the samples, because of spiking in the eluate of the high sample. Since the binding assessment is semi-quantitative, including the high sample is not recommended such that interference of the DMSO in the crystallization process during active confirmation phase is avoided.

Compound 1 and compound 2 are part of the drug-like subset and showed specific binding in ASMS, whereof an inhibitory effect (90%) was observed also for compound 1 in the biochemical assays. Although compound 3 is a redox cycling dye and is highly aromatic, specific binding to thrombin and enzyme inhibition was observed. Further validation of (un)specific binders and orthogonal confirmation through other secondary assays is recommended.

With identifying two specific ASMS binders having inhibitory activity, we show successful target engagement of novel hits identified in a biochemical setup and confirmed their binding specificity by ASMS. Our data shows that the ASMS setup is complementary to the biochemical workflows and can be applied as a standalone screening method.



Pivot Park Screening Centre has

- imlemented ASMS assay for an automated setting (384-well format)
- throughput 5K compounds per day (without pooling)
- developed a biophysical label-free HTS assay
- performed a primary ASMS screening campaign on the robustness set compound collection
- profiled target-specific engagement by ASMS confirmation assay
- identified three specific ASMS binders, whereof two were functional catalytic inhibitors
- ASMS is complementary to biochemical workflows

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