

A High Throughput Small Molecule Binding Assay for Human Trypsin on the Corning Epic® System

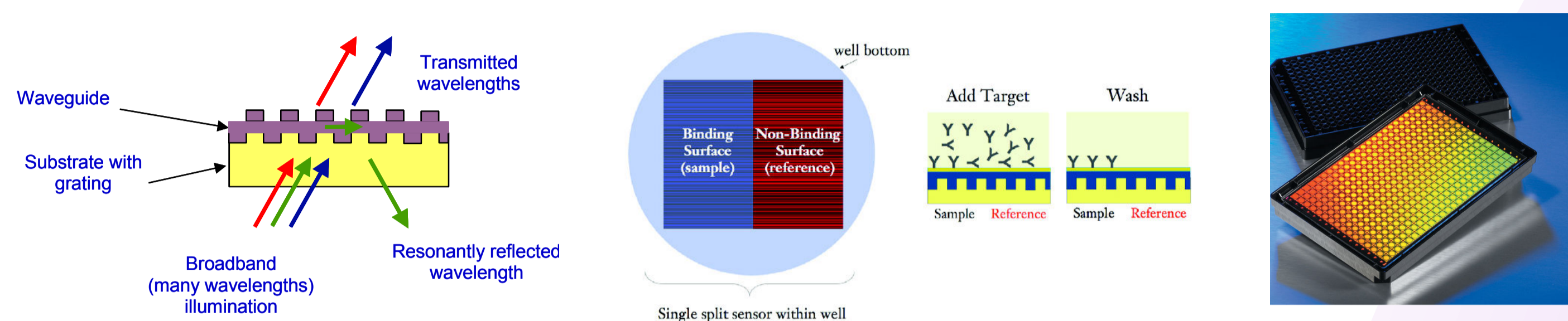
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Introduction

The Epic® System (Corning) is a label-free instrument that incorporates an evanescent waveguide sensor within each well of proprietary 384-well microplates. Direct binding of ligands to target proteins immobilised onto the microplates can be detected by exposing the waveguide to a broadband light source, the resulting resonant wavelength shift is proportional to the mass bound to the surface. The proprietary microplates contain a non-binding intrawell reference area to help determine specific binding (see schematic diagram below). The Epic® System is HTS-compatible with a throughput capability of more than 30,000 compounds/day. The 384-well format enables rapid assay development and/or parallel testing of compounds against other targets (selectivity), species variants (species crossover) and mutant proteins to provide additional information. In addition, it can be envisaged that for some targets (e.g. proteases and kinases), label-free functional assays can be developed.



Due to the availability of known inhibitors and purified protein, human recombinant trypsin (25 KDa) was chosen to develop a proof of principal small molecule direct binding assay on the Epic® System. This trypsin binding assay was validated by determining if various known inhibitors give a binding signal. Subsequently, a diverse set of pharmacologically active compounds were tested three times on separate days to evaluate assay reproducibility and robustness. Finally, a set of approximately 11,400 fragment compounds with formula weight less than 250 Da (figure 8) were spot tested at 100 µM to determine if the Epic® System was sensitive enough for fragment-based screening.

Assay Development & validation

Figure 1. Determination of the optimal pH for immobilisation of recombinant human trypsin (25 µg/well)

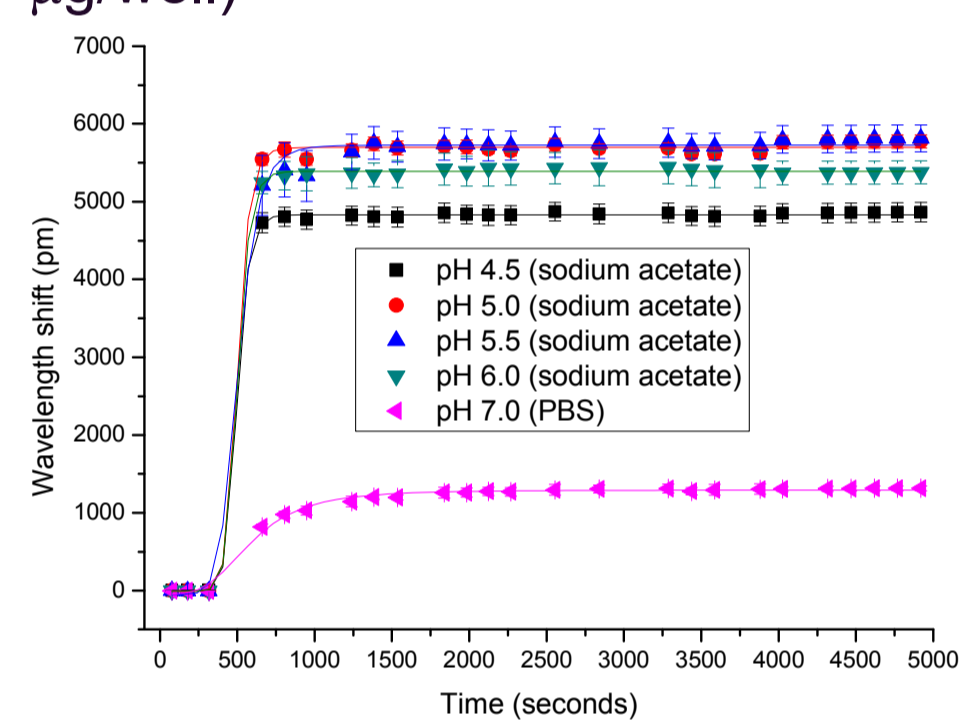


Figure 4. Effect of the concentration of DMSO on the binding of 4-aminobenzamide

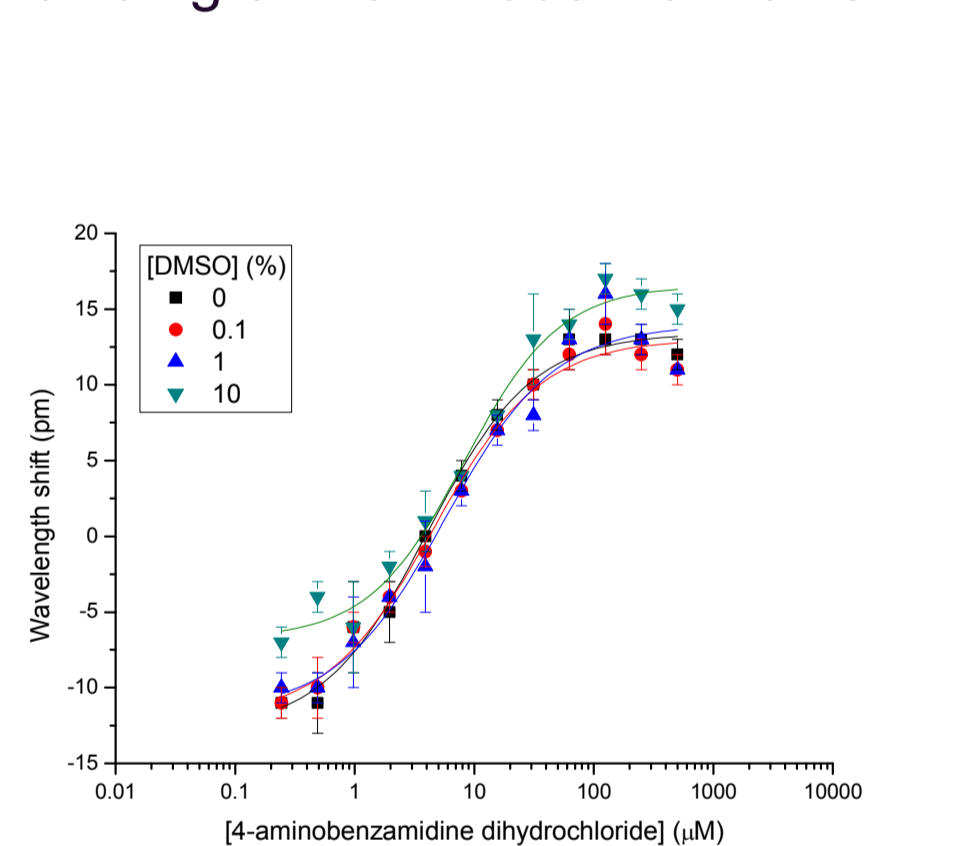


Figure 2. Determination of the optimal immobilisation concentration of human trypsin (pH 5.5)

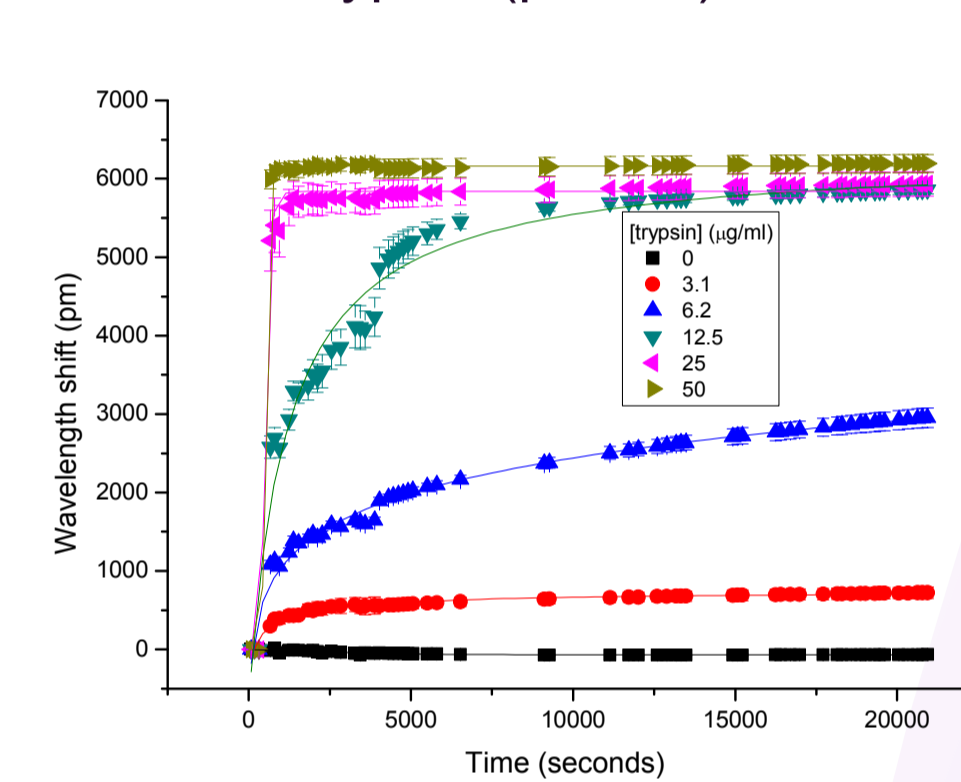


Figure 5. Concentration response curves of standard trypsin inhibitors plotted as % maximum effect.

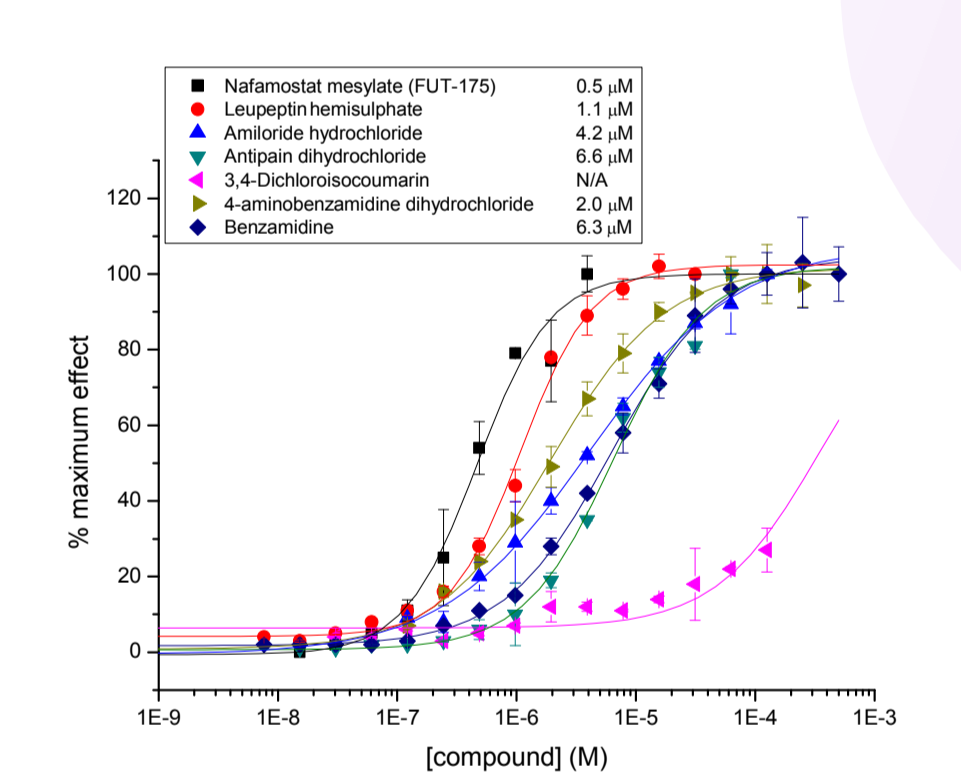


Figure 3. Effect of sodium chloride concentration on the binding of leupeptin hemisulphate

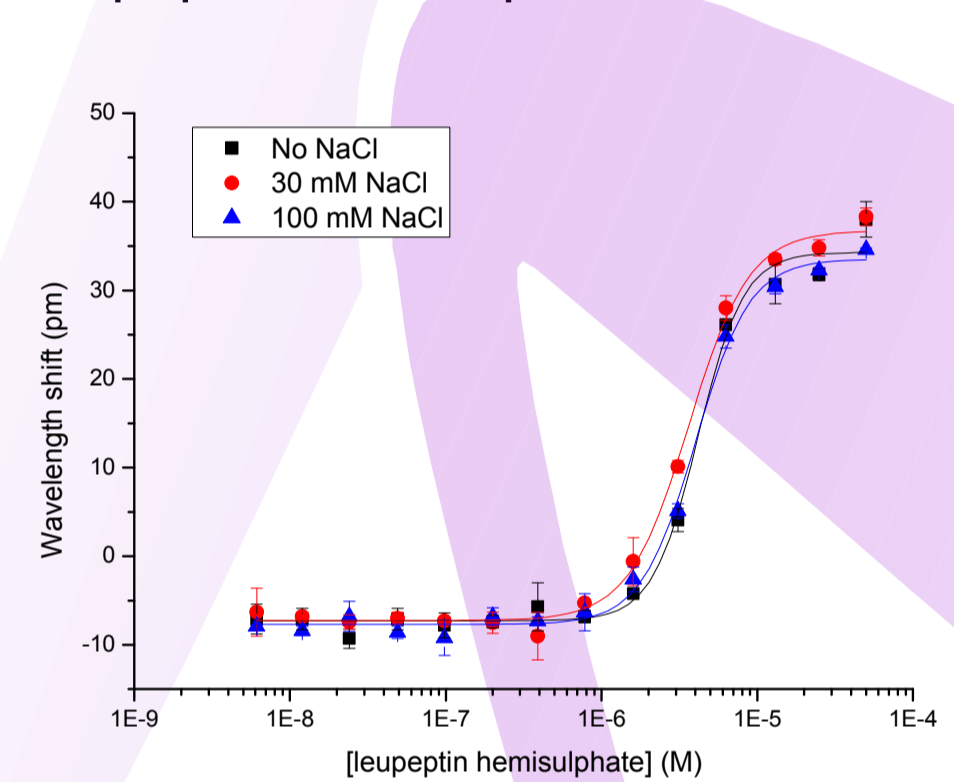


Figure 6. Comparison of interwell versus intrawell controls for the discrimination of specific v non-specific binding of nafamostat

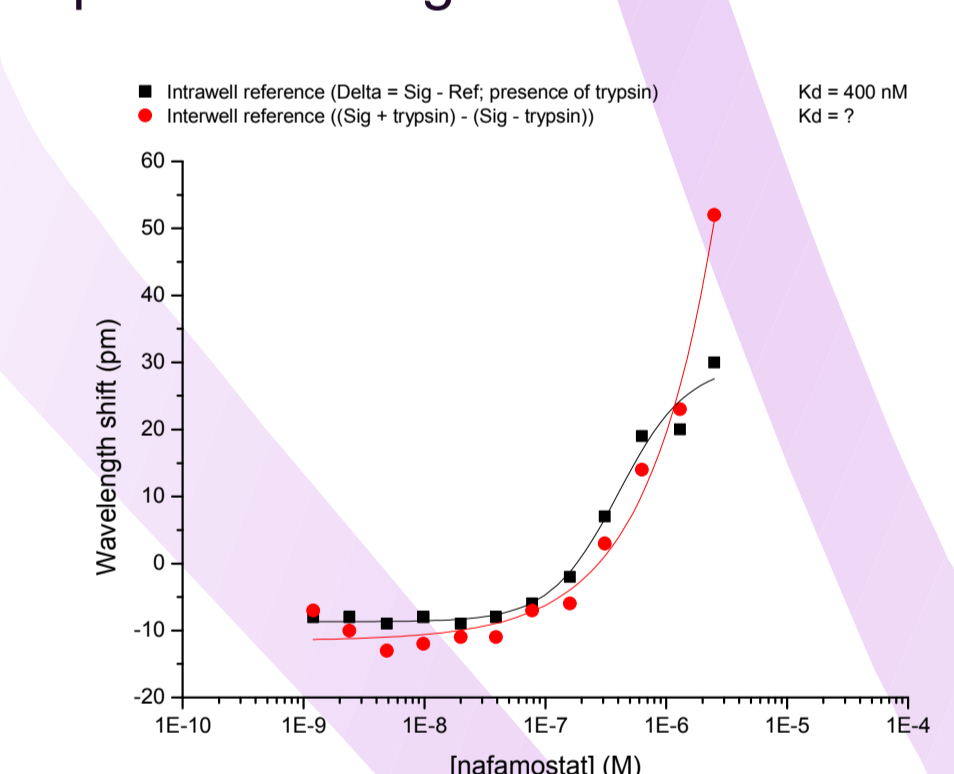
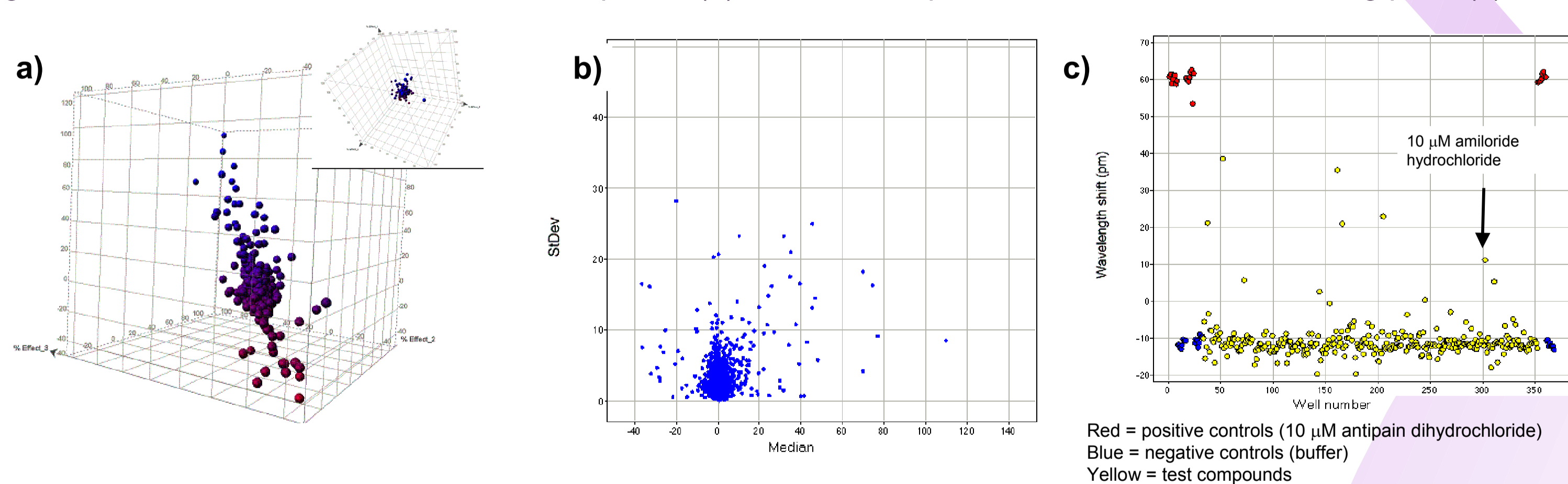


Figure 7. Reproducibility data from a set of 1550 pharmacologically active compounds tested on three separate days. Data is presented as the % effect of the signal obtained with 10 µM antipain dihydrochloride (formula weight 678 Da). 3-dimensional plot of 3 replicates of all compounds tested (a), median of three replicates plotted against the standard deviation for each compound (b) and an example of raw data from a screening plate (c).



The median Z' calculated from the fifteen 384-well plates was 0.85 (range 0.68 to 0.91)

Materials & Methods

Recombinant human trypsin was obtained from PolyMun (catalogue # TRY001-100). Nafamostat (FUT-175) was obtained from Becton Dickinson, all other compounds were obtained from Sigma. Biochemical Epic plates (catalogue # 5046xx1) were activated by adding 15 µl/well of 200 mM EDC (Pierce catalogue # 22981) and 50 mM Sulfo-NHS (Pierce catalogue # 24510), then incubated for thirty minutes at room temperature. Plates were washed with MilliQ water three times and dried by centrifugation. Unless otherwise stated, 25 µg/ml trypsin in immobilisation buffer (20 mM sodium acetate, pH 5.5) was added to an Epic biochemical plate and incubated for one hour at room temperature. Plates were washed three times with binding buffer (50 mM HEPES, 125mM NaCl and 1% (v/v) DMSO), 15 µl/well of binding buffer was added to all wells and the plates were incubated at 26°C for four hours. A baseline read was taken on the Epic reader prior to addition of 15 µl of compound/buffer using the Epic liquid handling accessory (LHA). Assay plates were equilibrated in the Epic for thirty minutes prior to taking a second read. Compounds were prepared by dispensing 60 nl of stock using the Labcyte Echo 555 to a 384-well polypropylene plate. To this, 30 µl of buffer containing 50 mM HEPES, 125 mM NaCl and 0.8% (v/v) DMSO. For the 1500 compound pharmacology subset, 10 µM antipain dihydrochloride was used as the positive control. For the fragment compound subset, 100 µM amiloride hydrochloride was used as the positive control. For data analysis, the signal was expressed as a percentage of the positive control signal. The Flexispense M liquid dispenser (Asys Hitech) was used to dispense binding buffer, trypsin and for wash steps.

For the functional assay, 50 µg/ml of BSA was prepared in 20 mM sodium acetate, pH 5.0 and 15 µl/well added to a biochemical Epic plate (catalogue # 5041) and incubated for one hour at room temperature. Plates were washed in binding buffer (100 mM Tris-HCl, 1 mM CaCl₂, pH 8) and then treated with 50 mM ethanolamine in PBS for five minutes at room temperature prior to washing with binding buffer five times. Plates were equilibrated in the Epic for thirty minutes prior to taking a baseline read and then 15 µl of 0.5 µg/ml trypsin added in the presence or absence of 4-aminobenzamide dihydrochloride.

11K Fragment Compound Screen

Figure 8. Distribution of formula weight in the fragment screening set

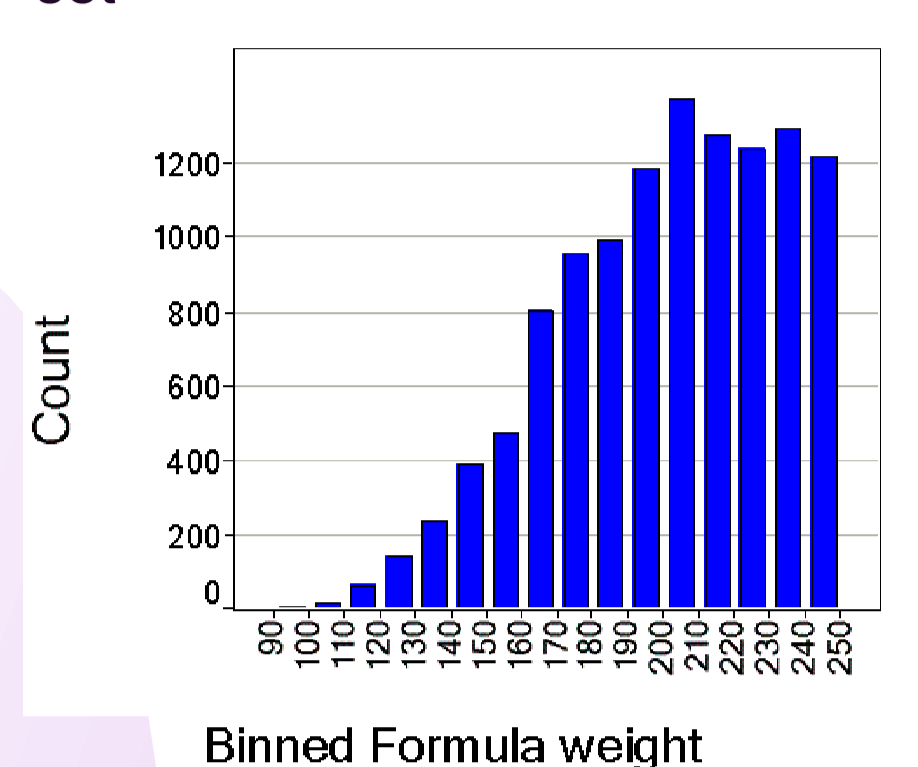


Figure 10. Distribution plot of compound activity (% effect of amiloride hydrochloride signal)

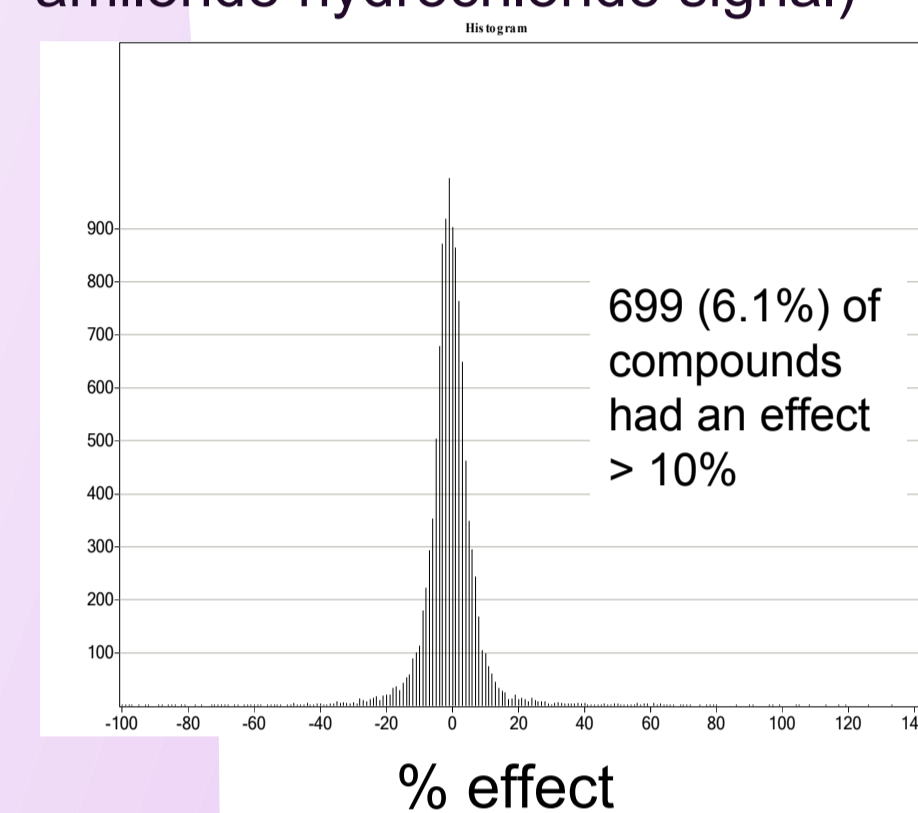


Figure 9. Z factors (a), and positive and negative controls (b) from all fragment screening plates

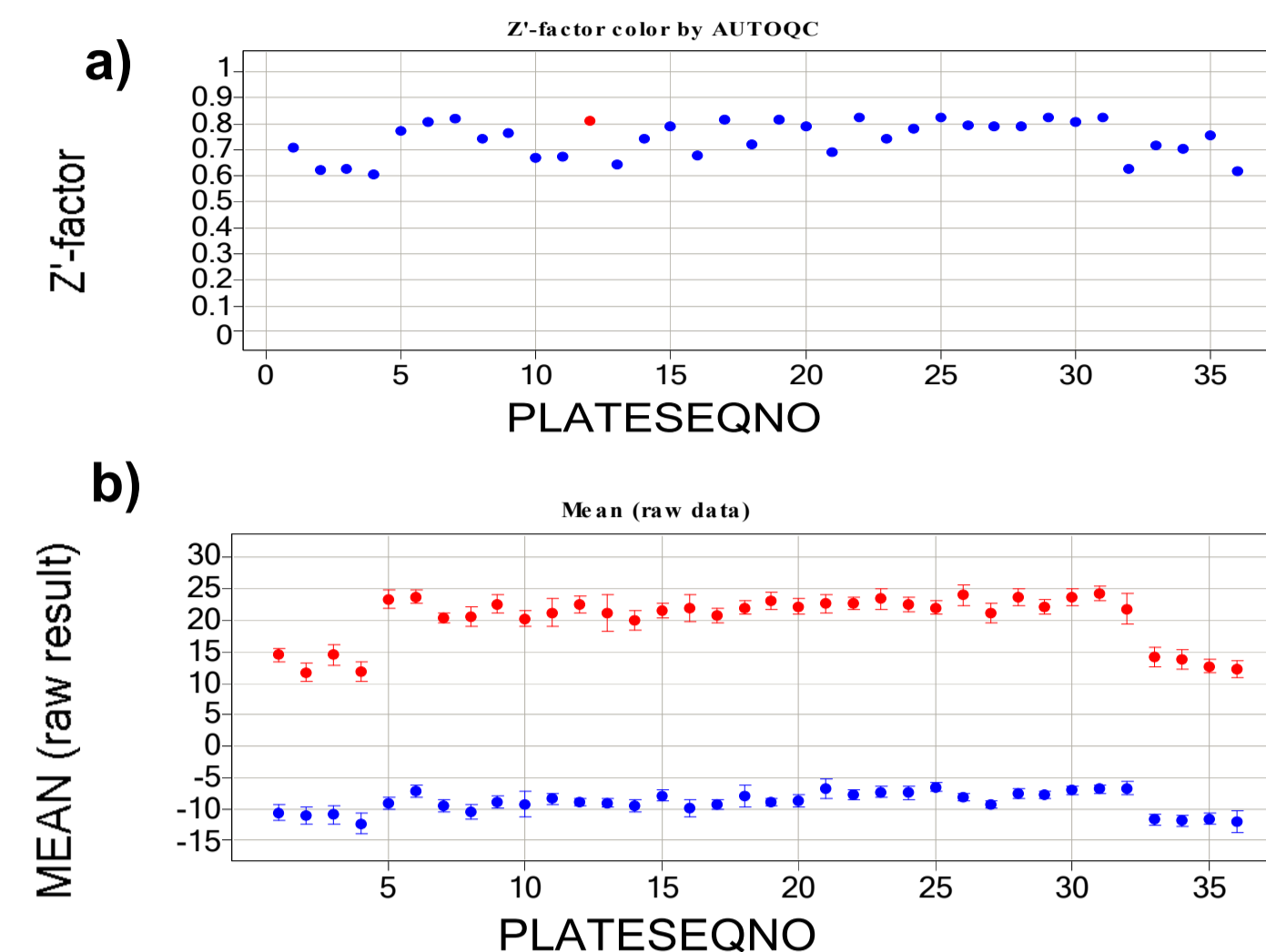


Figure 11. Plot of compound signal versus formula weight

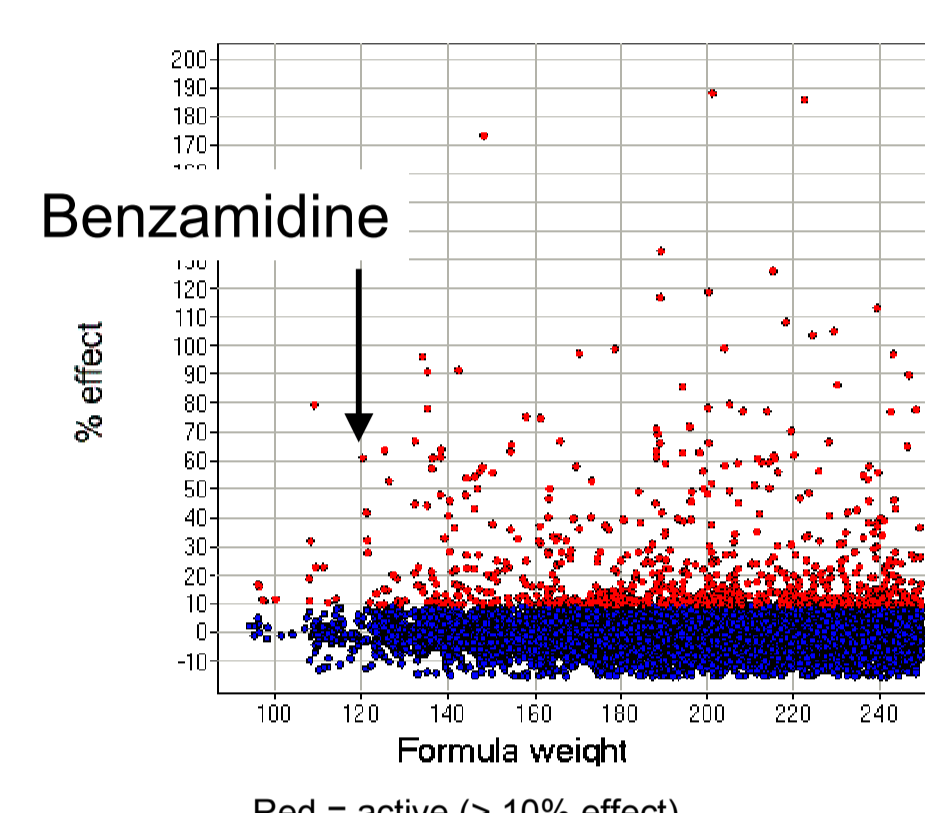
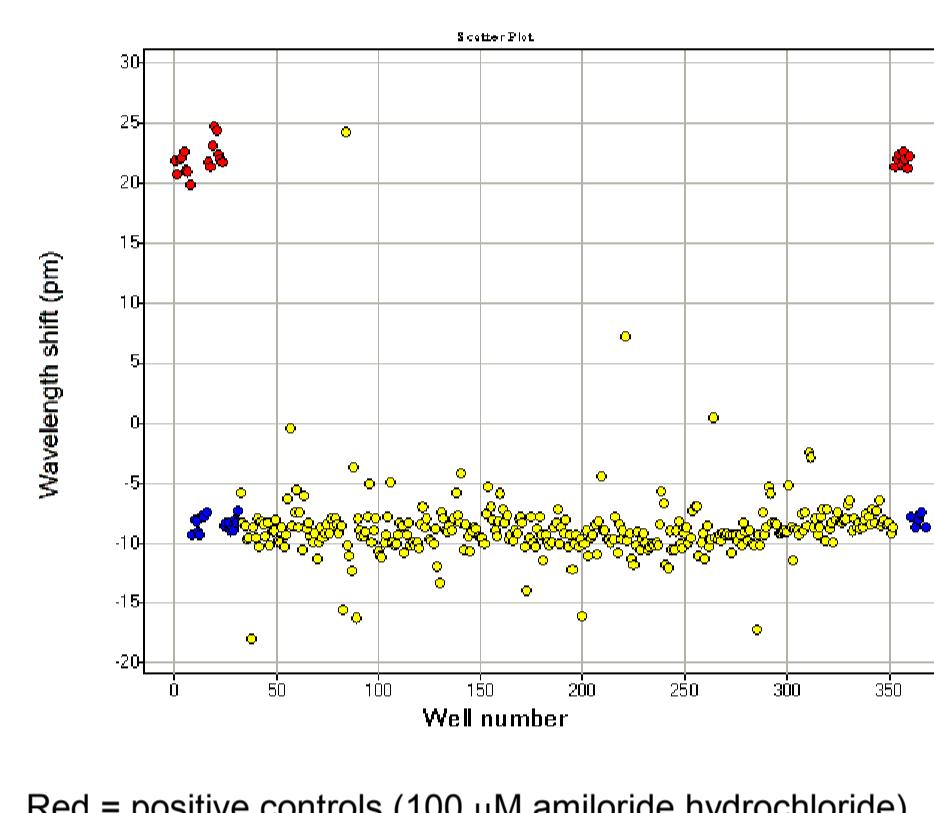


Figure 12. An example plate of raw data



The median Z' calculated from all thirty six 384-well screening plates using 100 µM amiloride hydrochloride as the positive control (formula weight 266 Da) was 0.74 (range 0.61 to 0.82)

Label-Free Functional Trypsin Assay

Figure 13. Cleavage of immobilised bovine serum albumin by recombinant human trypsin

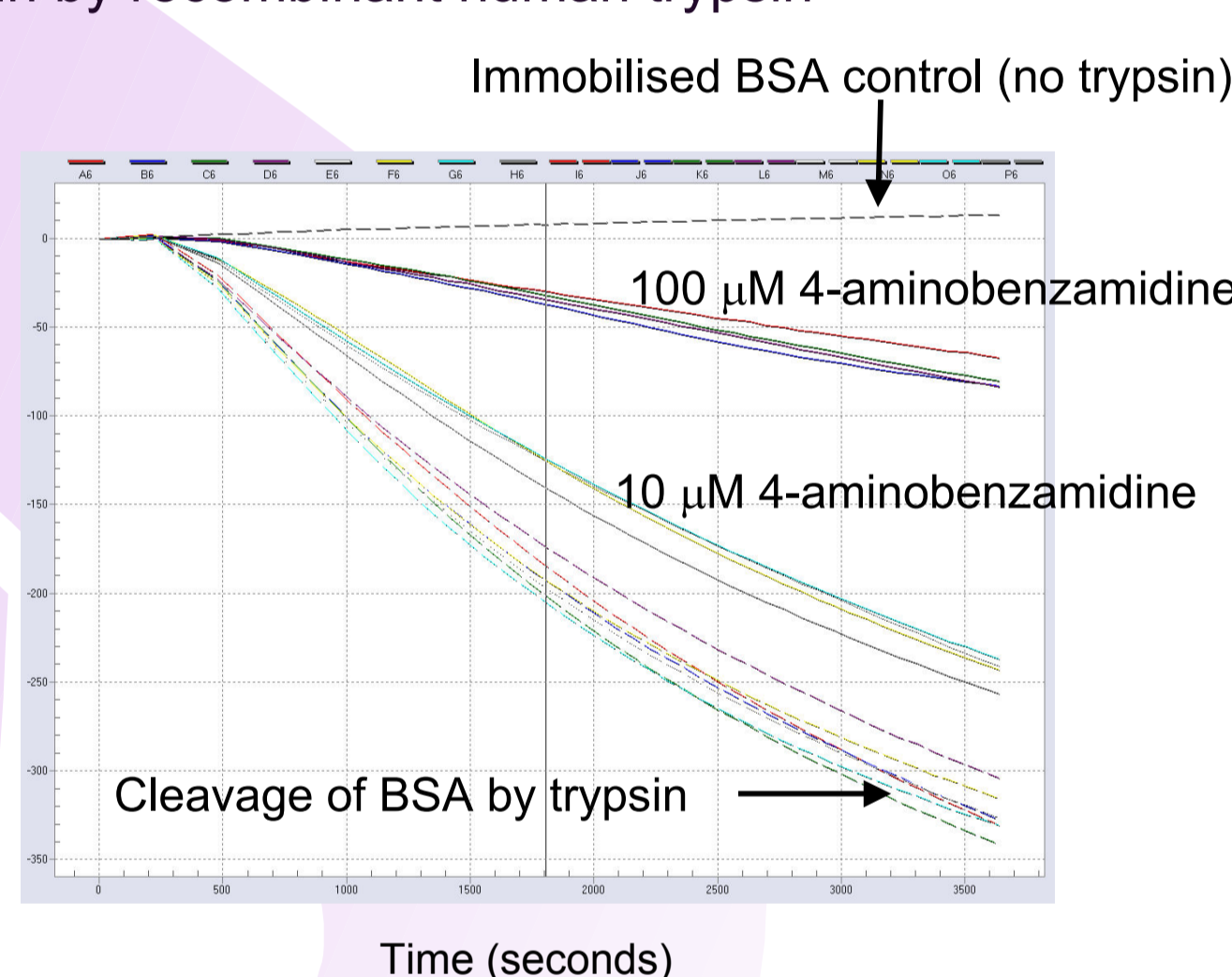
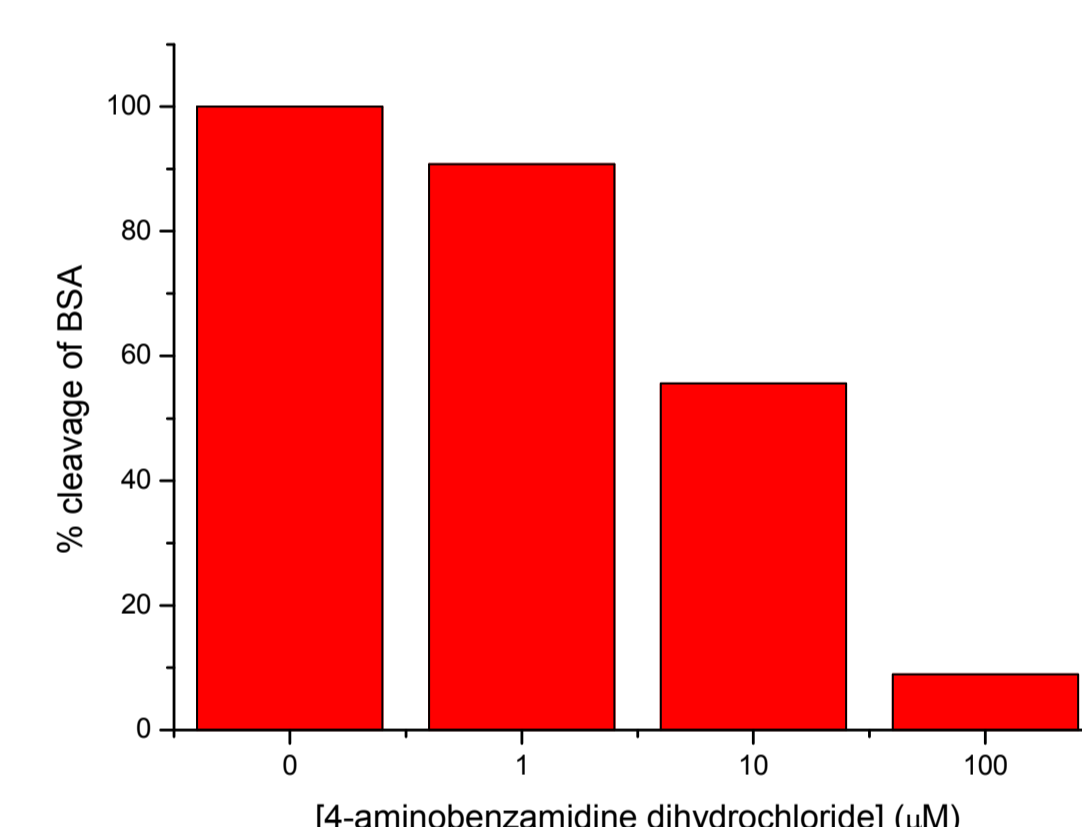


Figure 14. Inhibition of trypsin cleavage by 4-aminobenzamide dihydrochloride at a ten minute time-point (post trypsin addition)



Results

- The immobilization of recombinant human trypsin was optimal at pH 5.0 to 5.5 (figure 1). 12.5, 25 and 50 µg/ml of recombinant human trypsin all gave immobilisation signals of approximately 6000 pm. However, at 12.5 µg/ml of trypsin, a four hour incubation was required to achieve maximum immobilisation. Therefore, 25 µg/ml trypsin in 20 mM sodium acetate pH 5.5 was chosen for future work.
- The concentration of sodium chloride had negligible effect on the binding of leupeptin hemisulphate. A concentration of 125 mM NaCl was chosen for the binding buffer. Similarly, there was no effect on the binding curve of 4-aminobenzamide with DMSO concentrations up to at least 1% (v/v). At 10% DMSO, a small change in the binding curve was observed. 1% DMSO was used in the binding buffer for future experiments.
- Various known trypsin inhibitors were tested in the binding assay. All of the standards demonstrated binding to trypsin. These compounds had a range of formula weights from 120 (benzamide) to 680 (antipain) and had affinities in this assay in the range from approximately 500 nM to 7 µM.
- To test the reproducibility of the assay, a set of 1550 pharmacologically active compounds were tested in the binding assay on three separate days. The median Z' was 0.85 using 10 µM antipain as the positive control. The data was very reproducible, as demonstrated by the 3D plot of the three replicates. 95% of the data had a standard deviation less than 8%. The two known trypsin inhibitors contained within the pharmacology set (amiloride hydrochloride and 4-aminobenzamide dihydrochloride) were both identified as active.
- The median Z' for the thirty six 384-well plates in the fragment screen was 0.74 using 100 µM amiloride hydrochloride as the positive control. The errors in the negative control were very low for the majority of plates (approximately 3%) allowing a cut-off of 10% effect to be used at this stage. 699 compounds had greater than 10% effect in this assay at 100 µM (6.1% hit rate).
- A number of novel fragment compounds were identified from this screen including some compounds closely related to benzamide.
- A compound with a formula weight of 96 Da was detected (17% of the amiloride signal at 100 µM). This and all of the other hits identified need to be confirmed in repeat experiments.
- A functional trypsin assay was also developed using BSA as the substrate. This trypsin activity was inhibited by 4-amino-benzamide.

Conclusions

- This work demonstrates that the Epic System can be used for measuring both binding and functional activity of compounds against trypsin without any requirement for labelling of ligands or protein.
- The trypsin binding assay is extremely sensitive, reproducible and robust and demonstrates that the Epic System can be used for fragment based drug discovery.
- Future work will include confirmation of the identified hits and concentration response testing of the actives to identify compounds that demonstrate saturable binding, 1:1 stoichiometry and high ligand efficiencies.