

Development of a 384-well small molecule binding assay for trypsin on the Epic[®] label-free reader

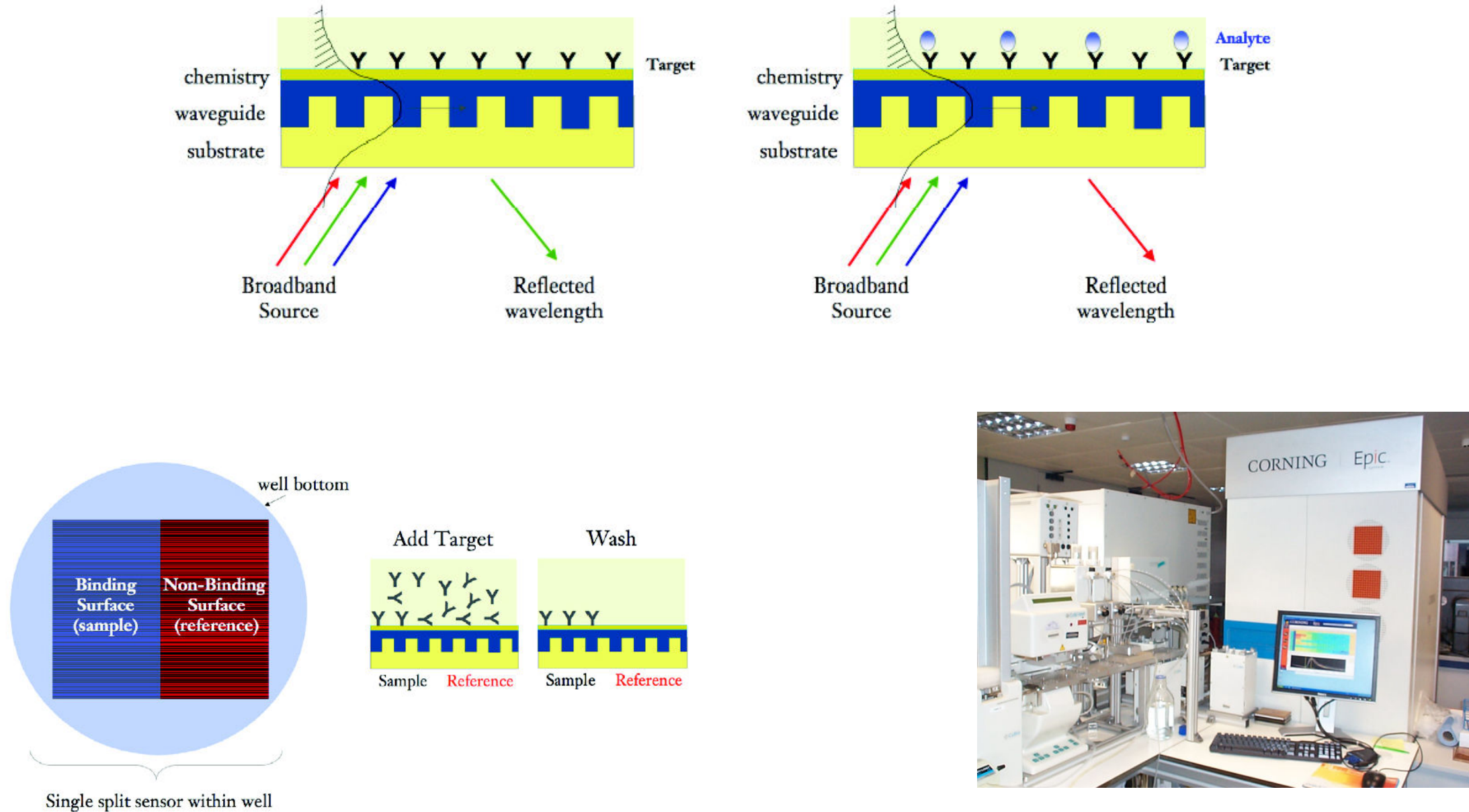
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Introduction

Biochemical assays are routinely developed for high throughput screening using fluorescence, luminescence or absorbance readouts to measure product formation or substrate depletion but these assays can be prone to compounds interfering with the assay detection non-specifically. Following an HTS, surface plasmon resonance (SPR) can be used to measure the direct binding of compounds to target proteins but these approaches have limited throughput. The Epic[®] label-free detection system (Corning), is a novel optics-based instrument that combines the 384-well plate-based approach of standard biochemical assays with some of the label-free advantages of SPR systems to enable the measurement of compounds binding directly to an immobilized protein. The Epic[®] system combines an evanescent waveguide sensor within each well of the 384-well microtitre plate and a reader that measures changes in resonant wavelength obtained from binding events. Proteins are immobilized on the surface of the waveguide grating by standard amine coupling. Direct binding of ligands is detected by exposure to a broadband light source, the resulting resonant wavelength shift is proportional to the mass bound to the surface. Epic[®] plates supplied by Corning contain a self-referencing area that is used to determine specific binding by the Epic[®] software. The Epic[®] system has been integrated at AstraZeneca with a Cybi-well liquid dispenser and a Cytomat incubator. The CyBio Composer software co-ordinates plate handling and compound additions to enable screening of over 100x 384-well plates/day.



Materials & methods

Recombinant human and porcine trypsins were obtained from PolyMun (catalogue # TRY001-100) and Promega (catalogue # V5111) respectively. Nafamostat was obtained from Toronto Research Chemicals, all other compounds were obtained from Sigma. Unless otherwise stated, 50 µg/ml trypsin was immobilized in 20 mM sodium acetate, pH 5.5 for one hour at room temperature (RT) in an Epic biochemical plate (catalogue # 5041). Plates were then washed three times with PBS and the plate was incubated overnight at 26°C. Buffer was exchanged for 15 µl/well binding buffer (PBS containing 125 mM sodium chloride and 3% (v/v) DMSO). Plates were incubated in the Epic carousel at 26°C for one hour before starting the assay. A baseline read was taken of the plate on the Epic reader prior to addition of 15 µl of compound/buffer using the Cybi-well liquid dispenser. The plates were then equilibrated in the Epic for twenty five minutes prior to taking a second read. The difference between the baseline read and second read was taken as the binding signal. Where the immobilization signal was taken, a baseline read was taken in 20 mM sodium acetate prior to the addition of trypsin or streptavidin. A second read was taken after incubation at RT for one hour in 20 mM sodium acetate at various pH. For screening assays, trypsin was immobilized using a Flexispense M liquid dispenser (Asys Hitech), plates were incubated for an hour at room temperature followed by washing three times with PBS on a Tecan Powerwasher and incubated overnight at 26°C. The assays were scheduled with CyBio Composer software which scheduled the buffer exchange, Cybi-well compound additions and Epic reads.

Trypsin immobilization

Figure 1. Determination of the optimal pH for the immobilization of porcine trypsin

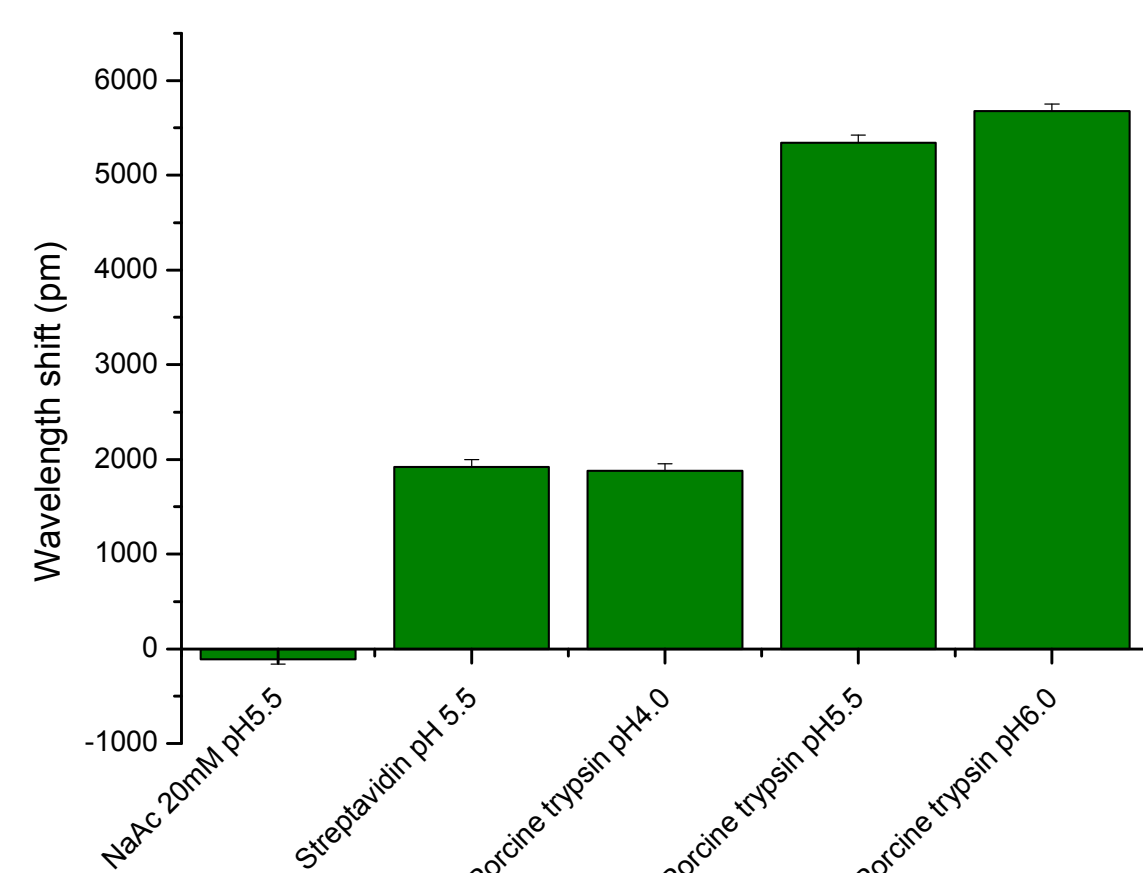
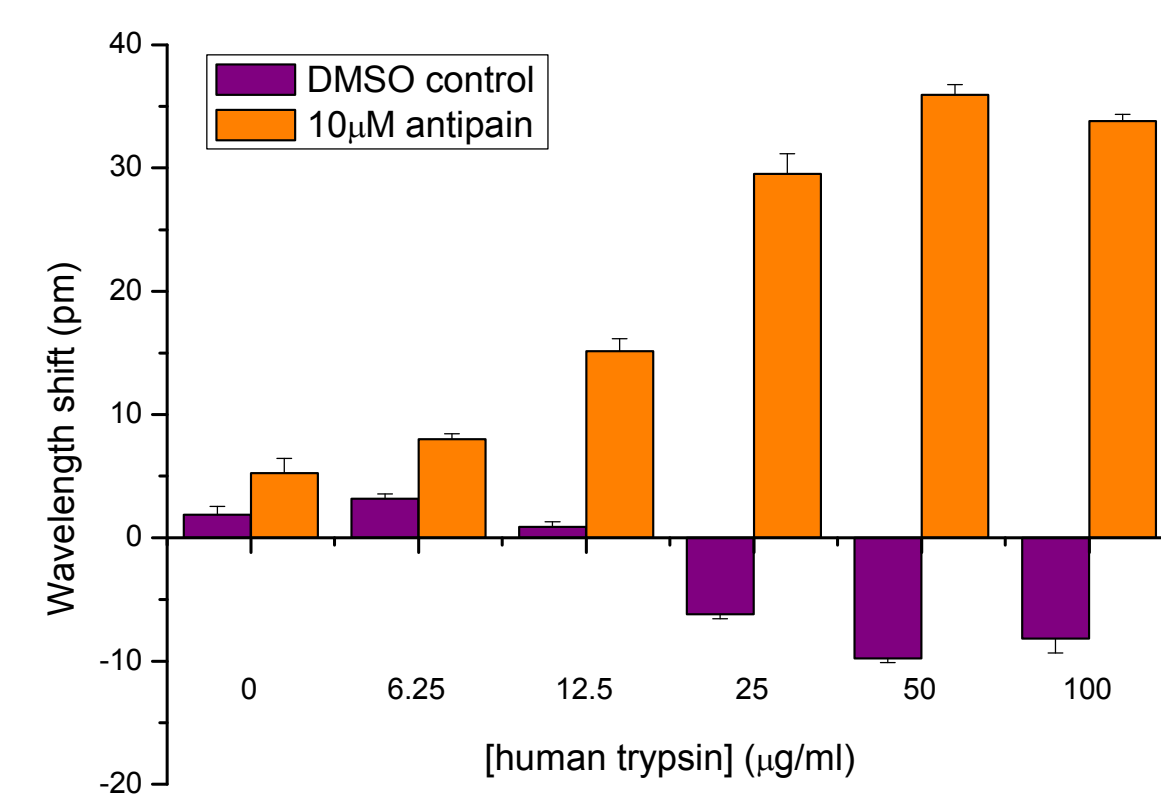


Figure 2. Determination of the optimal concentration of human trypsin



Assay optimisation

Figure 3. Effect of varying sodium chloride concentration on antipain binding to human trypsin

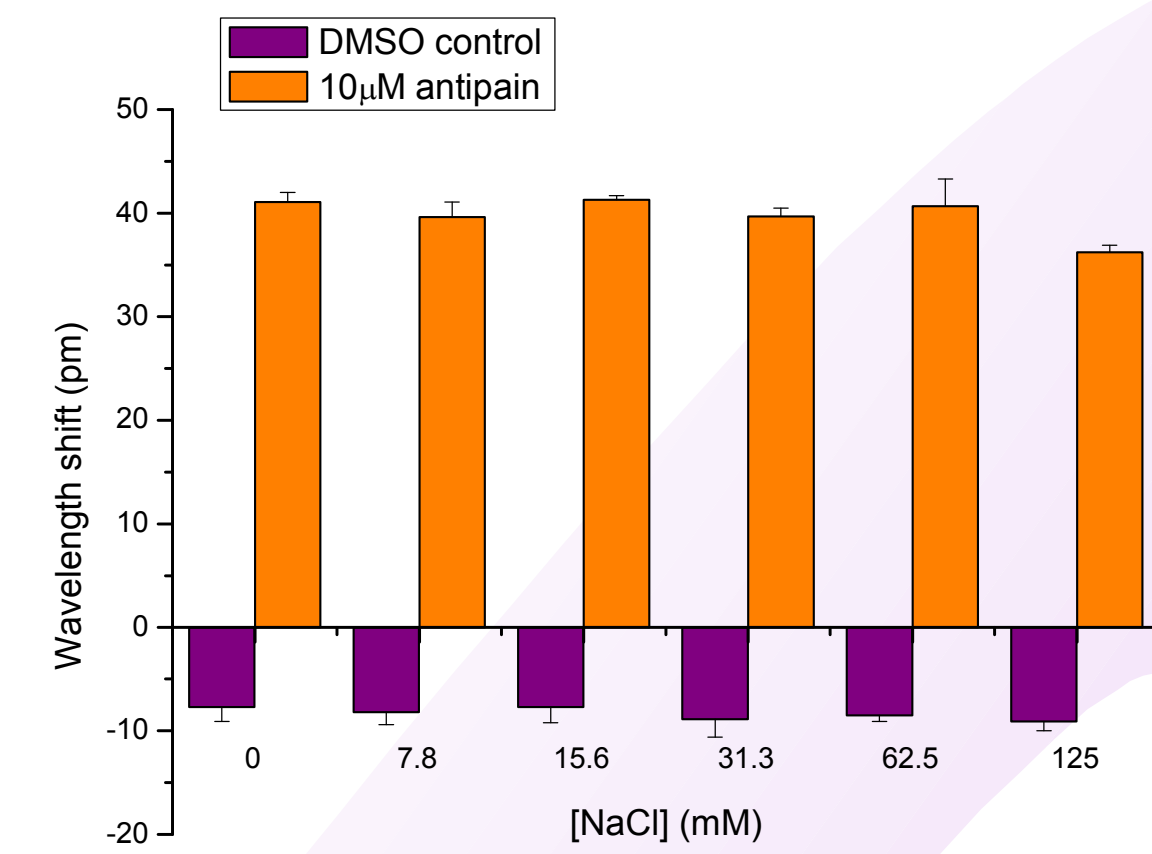
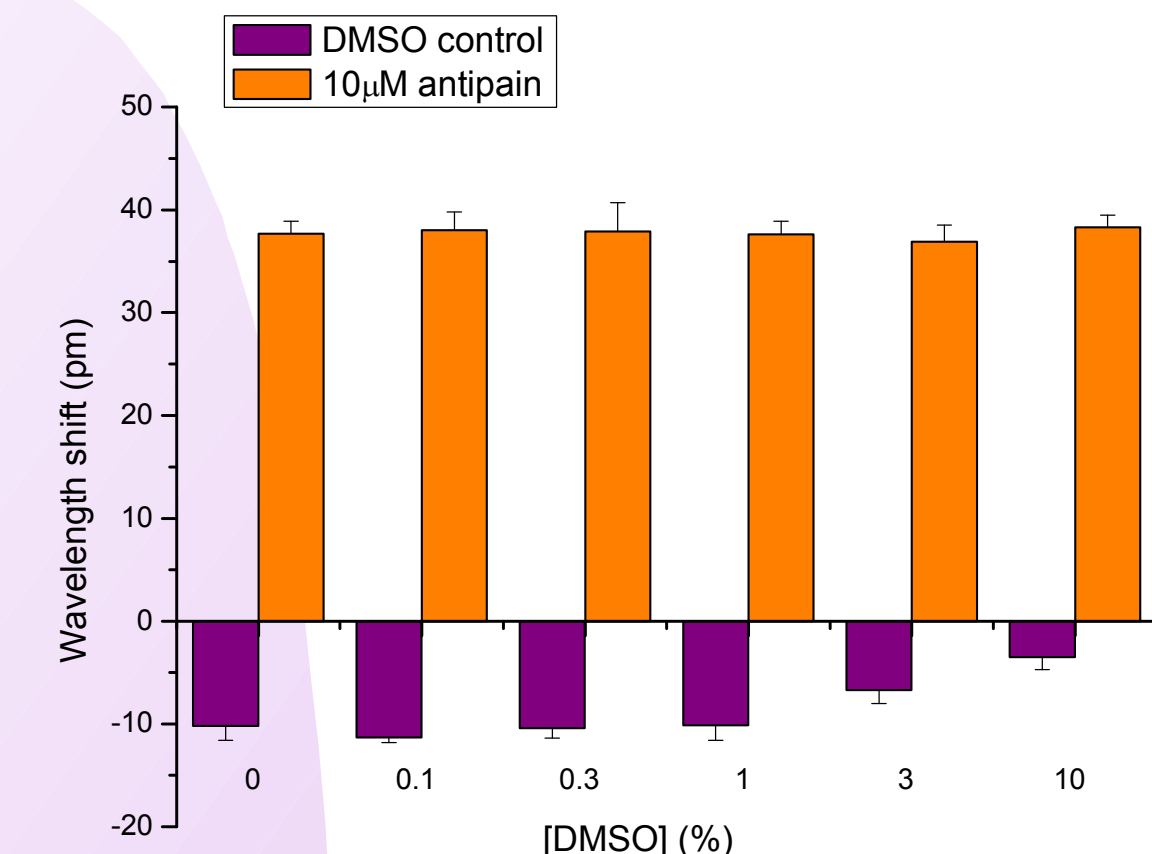


Figure 4. Effect of varying DMSO concentration on antipain binding to human trypsin



Assay validation

Figure 5. Determination of the binding signal of known trypsin inhibitors at a single concentration

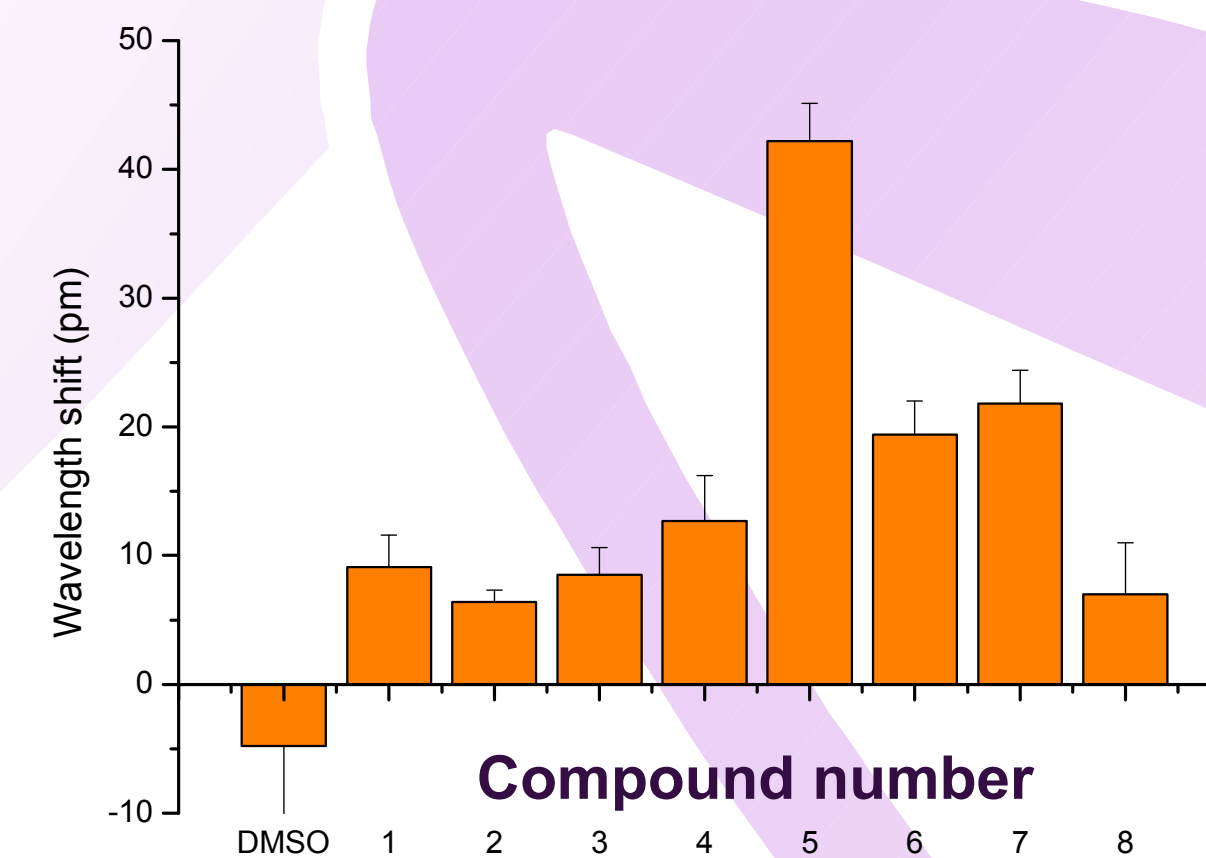


Table 1. Summary of the single concentration testing of known trypsin inhibitors

Compound number	Compound Name	Conc. (µM)	Mwt.	Mean Signal	SD
-	DMSO control	-	-	-4.8	6.3
1	benzamidin	1000	120	9.1	2.5
2	benzamidin hydrochloride	100	157	6.4	0.9
3	3-aminobenzamidin hydrochloride	100	208	8.5	2.1
4	4-aminobenzamidin hydrochloride	100	208	12.7	3.5
5	antipain	10	678	42.2	2.9
6	nafamostat	1	540	19.4	2.6
7	leupeptin	10	476	21.8	2.6
8	amiloride hydrochloride	10	266	7.0	4.0

Mwt = molecular weight

Figure 6. Determination of the K_d (apparent) of antipain (molecular weight = 678)

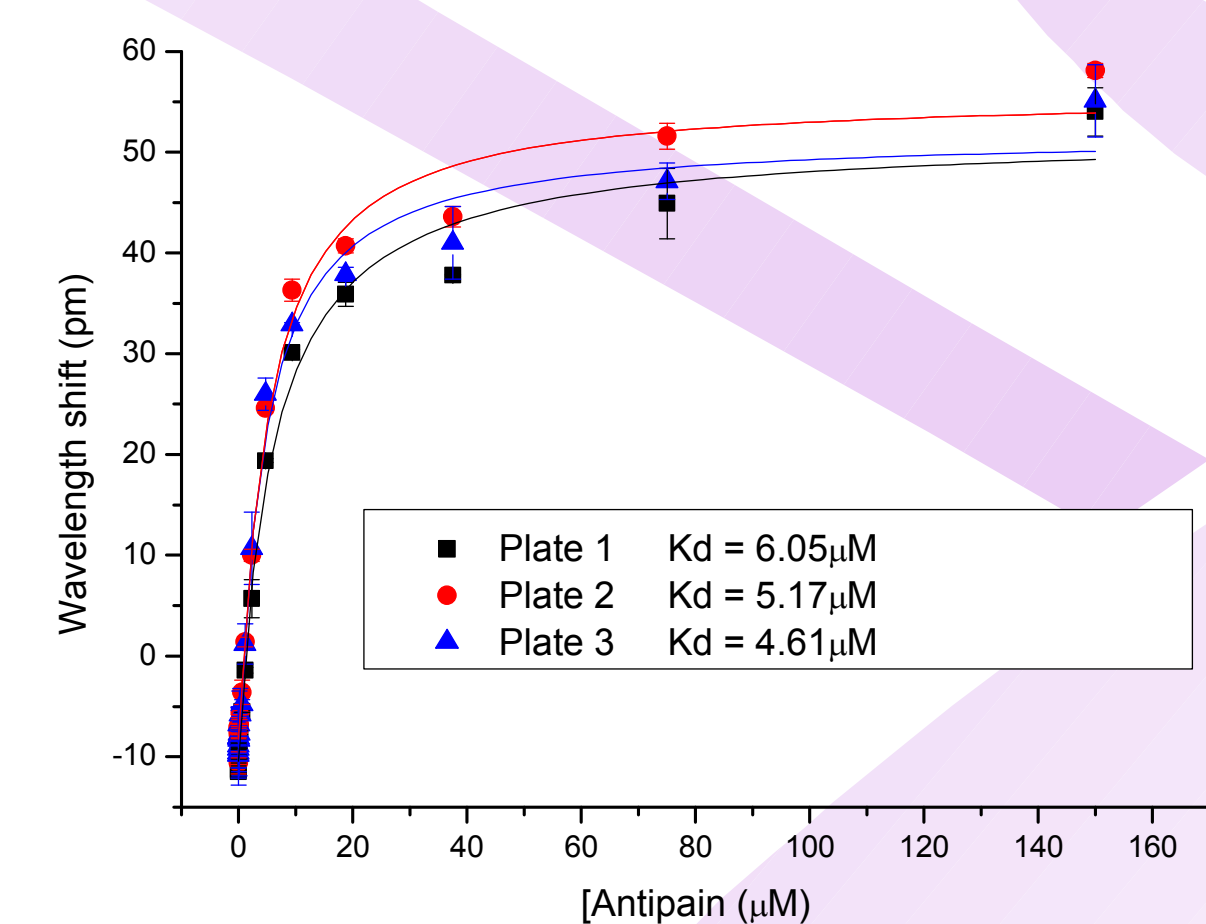


Figure 7. Determination of the K_d (apparent) of leupeptin (molecular weight = 476)

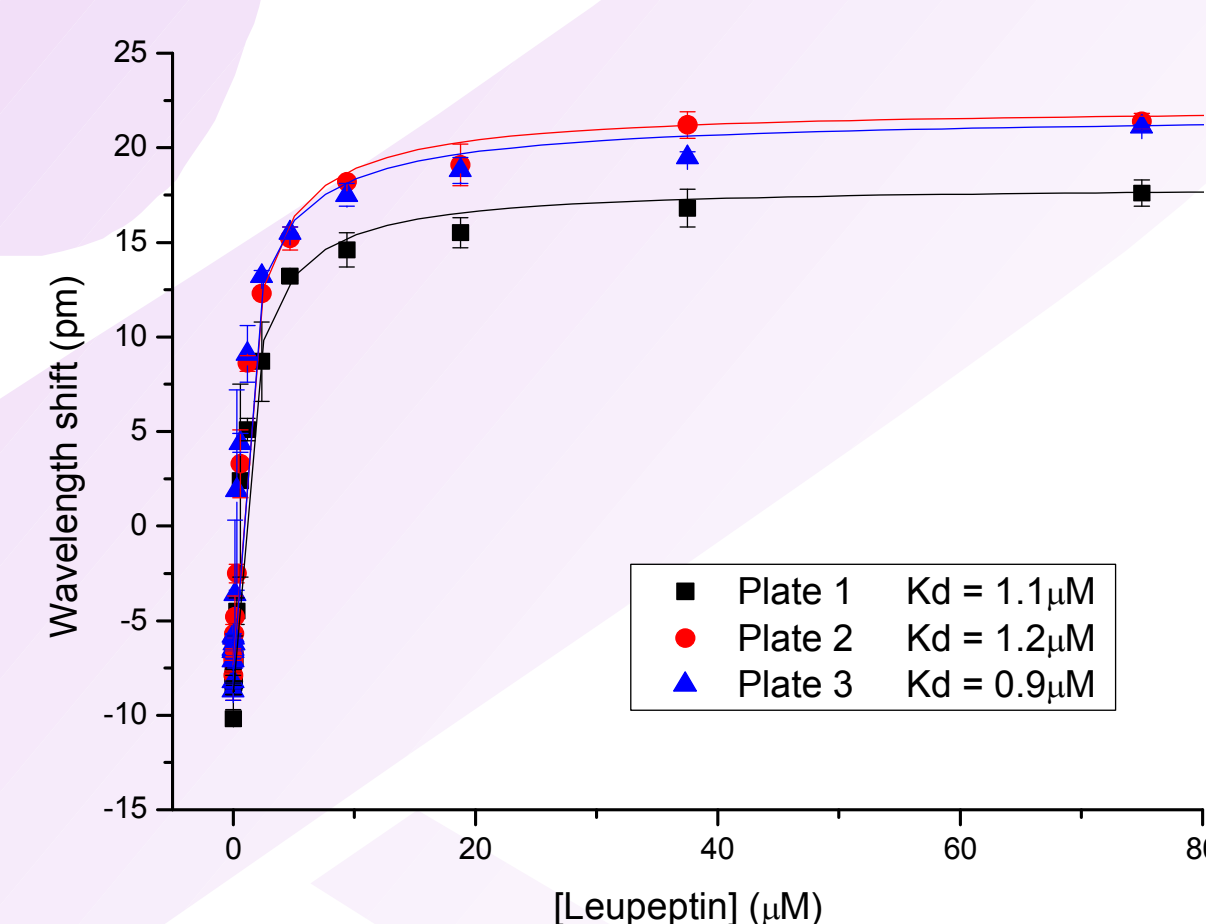


Figure 8. Determination of the K_d (apparent) of 4-aminobenzamidin (molecular weight = 208)

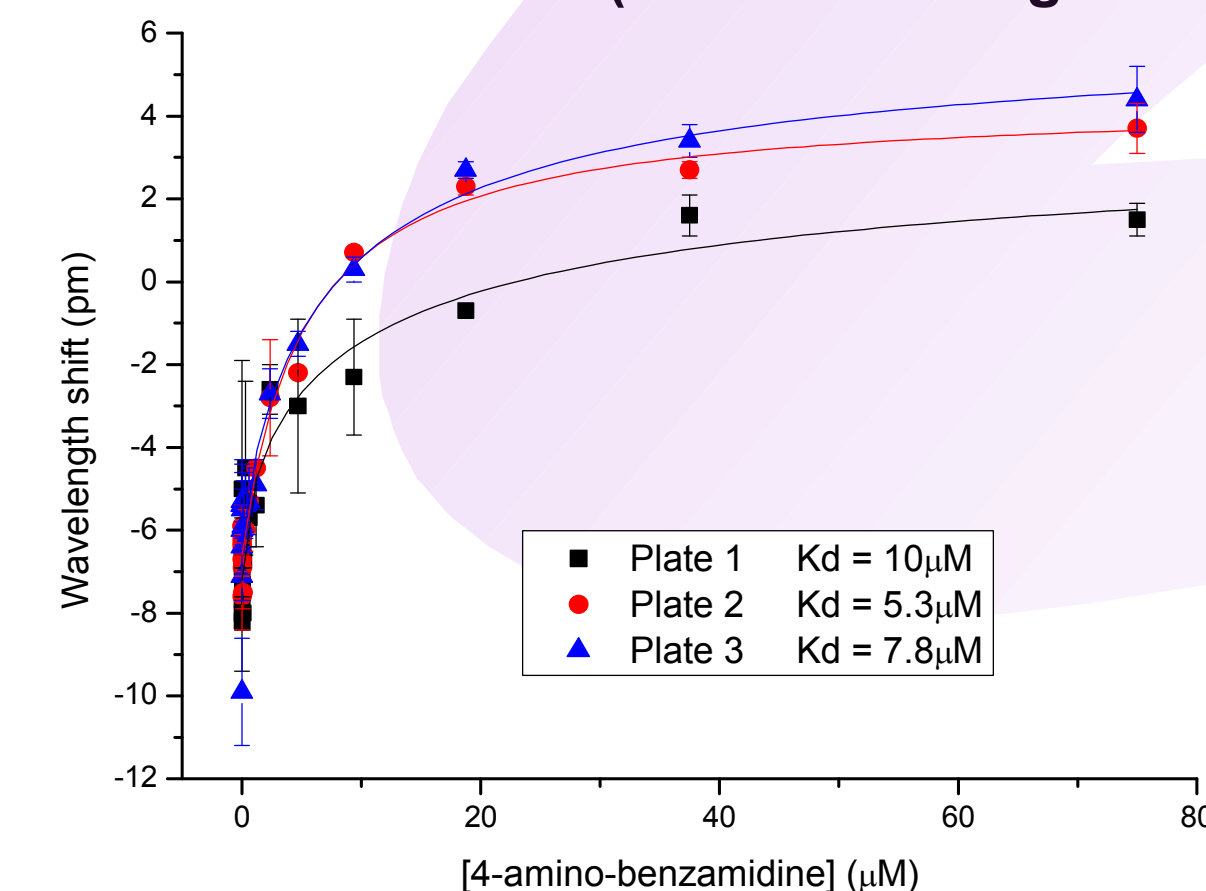


Table 2. Summary of the determination of K_d (apparent) of standard compounds (n=3)

Compound Name	Mwt.	K _d (µM)	
		Mean	SD
Antipain	678	5.3	0.7
Leupeptin	476	1.1	0.1
Benzamidin	120	21.8	3.6
Benzamidin hydrochloride	157	13.8	6.0
3-aminobenzamidin	208	11.8	0.7
4-aminobenzamidin	208	7.7	2.4
Nafamostat	540	0.3	0.1

Screening of a library of pharmacologically active compounds

Figure 9. Screening histogram of the activity of a library of pharmacologically active compounds in the trypsin binding assay

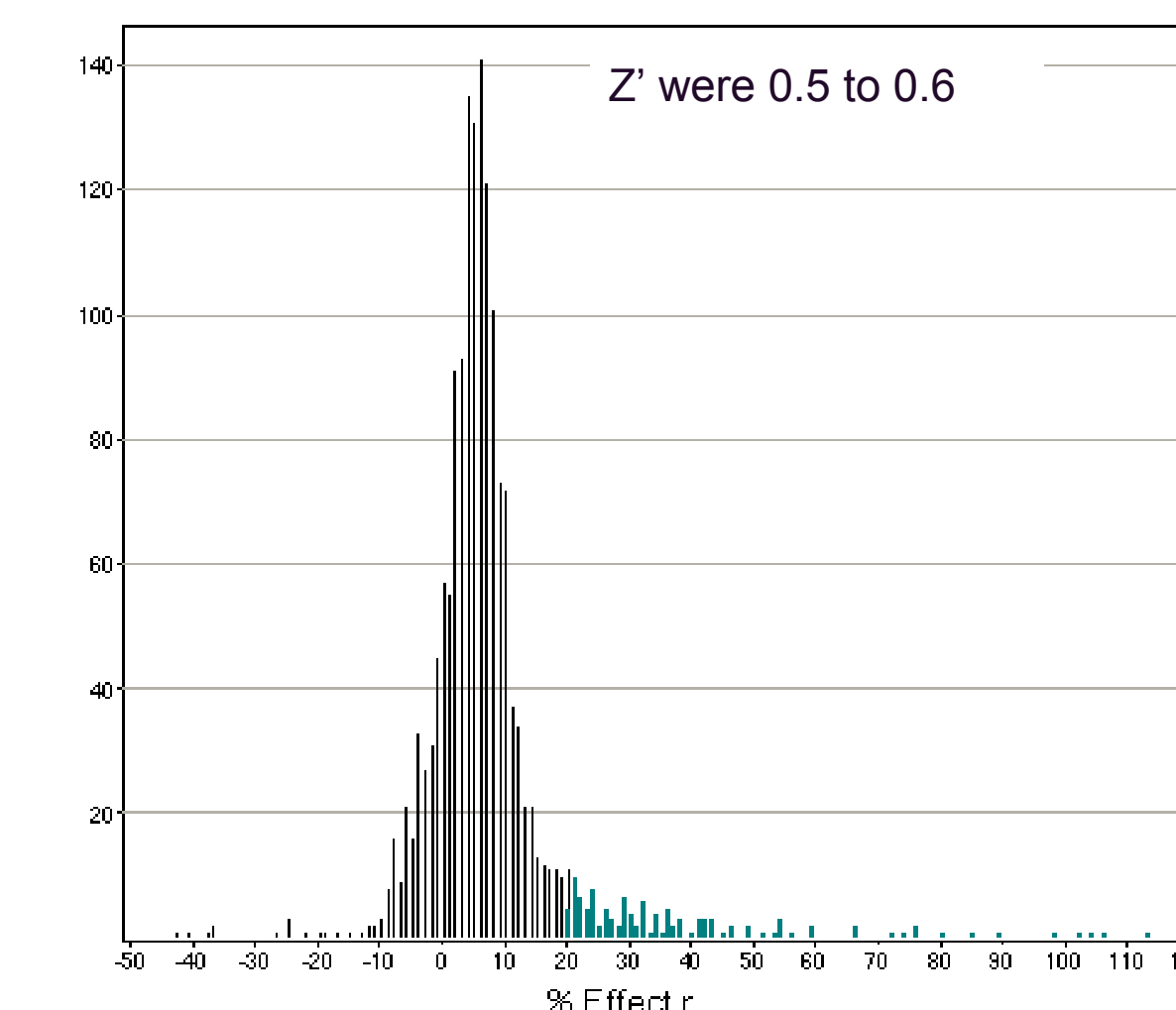


Table 3. Examples of some hits obtained from screening the library of pharmacologically active compounds

Compound Name	Formula weight	Raw signal (pm shift)	% effect
4-aminobenzamidin dihydrochloride	135	6.6	26
Amiloride hydrochloride	230	9.5	32
5-(N,N-Dimethyl)amiloride hydrochloride	258	12.2	38
Sperminine NONOate	262	18.1	54
Pentamidine isethionate	340	24.4	66
DIPPA hydrochloride	448	34.6	89
Ruthenium red	560	45.4	113
WIN 64338	712	38.3	98

The percentage effect was defined as the percentage difference between DMSO control and the signal obtained from 10 µM antipain binding (n=32/plate for controls)

Compounds were tested once at 10 µM, 124/1540 compounds had a % effect greater than 20%

Results

- The immobilization of porcine trypsin was optimal at pH 5.5 (figure 1). This pH was also used for the immobilization of human trypsin.
- The immobilization concentration of trypsin that gave the greatest assay signal with 10 µM antipain was 50 µg/ml for both human (figure 2) and porcine trypsin (data not shown). There was some negative drift in the signal observed for buffer controls. However, this was constant for all wells and may be due to enzyme autodigestion.
- The concentration of sodium chloride had negligible effect on the binding of antipain to human trypsin (figure 3). A high (125 mM) concentration was used in the binding buffer to obtain a physiological buffer concentration.
- Up to 1% (v/v) DMSO had no effect on antipain binding to human trypsin (figure 4). Higher concentrations of DMSO could also be used with only a marginal decrease in binding signal.
- Eight standard protease inhibitors were spot tested in the trypsin binding assay, binding of all eight compounds could be measured above DMSO controls (figure 5 and table 1).
- The K_d (apparent) of seven of these compounds was determined on three separate plates. The K_ds from the three plates were within half a log of each other for each compound and the rank order of affinities were as expected (table 2). The maximum wavelength shift obtained at saturating concentrations of compound was dependent on the mass of the compound (figures 6 to 8).
- The Z' obtained from automation testing were good (0.5 to 0.6 from eight plates tested; data not shown), only one well was removed from the analysis of three plates of alternating positive/negative controls (1152 wells).
- 124/1540 compounds from a set of pharmacologically active compounds demonstrated greater than 20% effect in the trypsin binding assay. A number of compounds known to bind to trypsin were identified including 4-aminobenzamidin dihydrochloride and amiloride analogues.

Conclusions

- This work demonstrates that the Epic[®] system can be used to screen for compounds binding directly to human trypsin without the need for labelling of the ligand or protein.
- This assay can be used to determine the affinity of small molecules binding to human trypsin.
- Compounds with molecular weights as low as 120 Daltons could be detected with this assay, suggesting that this approach could be used for fragment-based screening.
- The signal measured at a given compound concentration is a combination of its mass and the affinity of the compound for the target protein.