Development of an HTS FabD inhibition assay using a new 384-well filter plate to screen novel antibiotics

Several enzymes are involved in the fatty acid biosynthesis (Fab) system of bacterial organisms. Unlike the mammalian FAS enzyme system in which all the active sites are present in a single, multifunctional protein with several domains[1], the multi-enzyme system prevalent among bacteria[2] makes these proteins attractive targets for novel antibiotics with little or no cross reactivity to the mammalian enzyme.

by Dr Gaëtan Touyer, Dr Christine Miossec, and Dr Irvin Winkler

Several Fab enzymes have been previously targeted for HTS screening. Researchers at AstraZeneca screened 200,000 compounds using FabI as the target from which they identified triclosan as the lead candidate[3]. A scintillation proximity assay (SPA) developed for FabH by He et al is a candidate for use as a high throughput screening (HTS) assay[4]. In 2002, Warne et al screened 600,000 compounds using an assay in which three Fab reactions (FabD, FabG and FabH) were coupled with a luminescence detection system[5].

One enzyme in this system, malonyl-Coenzyme A: acyl carrier protein (ACP) transacylase, also known as FabD, also plays an essential role in the elongation of the fatty acid chain. In 2003, Molnos et al reported the development of a medium throughput, non-radioactive FabD inhibition assay based on monitoring a continuous coupled enzyme reaction for NAD^+ reduction[6]. Until recently, however, screening of candidate libraries for activity using the FabD enzyme as the target had not resulted in the identification of any FabD inhibitors.

In early 2004, Miossec et al at Aventis identified a biphenyl pyrrole acid as a moderate inhibitor of *E. faecium* FabD activity using radiolabelled malonyl-CoA as the substrate in a high throughput screening inhibition assay using 96-well filtration plates[7]. The same assay was used by Michel et al to find a series of 4-hydroxyquinolines FabD inhibitors[8]. These initial reports suggest FabD as a promising target for antibiotic screening which has not been fully explored. To increase the probability of selecting compounds with a broader microbiological spectrum of inhibitors than in these studies, a higher throughput method was required.

In this communication, we report an HTS assay using FabD as the target configured on a new 384-well glass-fibre filter plate to screen more than 280,000 novel antibiotic candidates. The current

study converts the 96-well assay used previously to a miniaturised 384-well filtration format. Modifications to the assay have resulted in a robust, sensitive, robotics-friendly, high throughput method with a Z' factor consistently >0.5. In addition, screening time and radioactive waste have been reduced eight-fold.

Background

The FabD radioactive inhibition assay was originally described by Joshi et al[9]. This enzymatic assay measures the amount of ^{14}C-malonyl-ACP produced in the condensation step of Fab (see **Figure 1**). The ^{14}C-labelled malonyl group is transferred from coenzyme A to ACP and the reaction is stopped by the addition of trichloroacetic acid. ^{14}C-malonyl-ACP is precipitated and separated by filtration from the unconverted ^{14}C-malonyl-CoA which remains in solution. Scintillant is added to the precipitate and radioactivity trapped on the filter plate is measured. The assay was adapted to a 96-well plate format where incubations are performed in a standard 96-well plate, and then precipitated protein is transferred to a 96-well glass-fibre filter plate for separating protein bound radioactivity from soluble ^{14}C-malonyl-CoA[5,6].

The 96-well assay was an adequate high throughput screen during the primary screening of a 240,000 compound library in which eight compounds were evaluated per well. Each compound was evaluated in two different wells accompanied by seven unique compounds in each of those wells. This screen yielded a 'hit' series with a narrow microbiologic spectrum. In order to obtain broader selectivity, including gram-positive and gram-negative pathogens, a new library (made up of historic in-house libraries and additional purchased libraries for a total of more than 280,000 compounds) was screened. The availability of a new 384-well glass-fibre filter plate (Millipore Corporation, Billerica, MA) led to the development of a faster, higher throughput method, in which both the enzymatic reaction and the separation steps are carried out in one filtration plate, eliminating the need for an additional reaction plate.

Materials and methods

Assay validation

Validation of the 384-well filtration assay was determined by evaluating the Z' factor and establishing equivalence to the 96-well version in terms of IC_{50} and signal-to-noise ratio. The Z' factor was calculated from assay results in three plates. IC_{50} value of a reference compound, A000326719[7], was measured using both the 96-well and 384-well assays and the one-plate and two-plate 384-well assays. A000326719 is a weak inhibitor of FabD with an IC_{50} of approximately 50µM. Signal-to-noise in the one-plate and two-plate systems was compared for both plates as well.

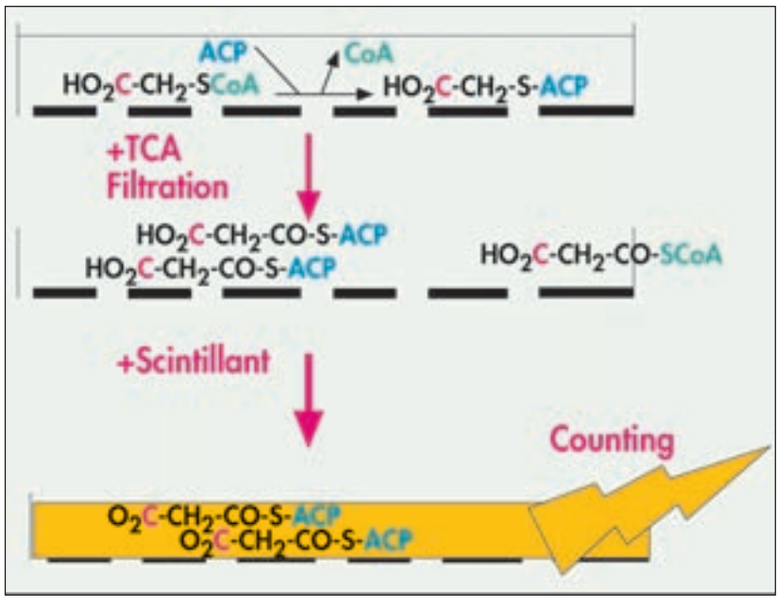

Figure 1: Scheme of FabD assay

Preparation of assay components

Genes encoding FabD and ACP were PCR amplified from *Enterococcus faecium* (EF) genomic DNA and cloned in *E. coli* plasmid expression vectors. Fermentation and protein purification were achieved using standard procedures. Addition of phosphopantetheine to purified apo-ACP was enzymatically performed with purified *E. coli* ACP synthase to produce holo-ACP. Co-substrate working solution consisted of malonyl-CoA (Sigma) and [2-^{14}C]-malonyl-CoA (Amersham/GE Healthcare).

Working solutions for enzyme inhibition assay were prepared as follows. Assay buffer contained 110mM sodium phosphate, pH7, 1.5mM dithiothreitol (DTT) and 0.1% bovine serum albumin. Holo ACP substrate stock solution was 874µM in assay buffer. The stock solution is freshly diluted in assay buffer and incubated for 30 minutes at room temperature prior to addition to assay wells in order to recover Cys 35 free SH (malonyl reactive site). Final concentration was 58.3µM. Co-substrate consisted of 220µM cold malonyl-CoA (Sigma) and 2-^{14}C malonyl-CoA (Amersham/GE Healthcare). One volume of a 54mCi/mmol solution at 20µCi/ml (370µM) ^{14}C malonyl-CoA and

Table 1: Determination of assay quality. Z' values were determined by the formula $Z' = 1-((3\sigma_u+3\sigma_i)/(\mu_u-\mu_i))^{\times}$ where σ = standard deviation, μ = mean, u and i = negative control and positive control respectively

	Plate 1		Plate 2		Plate 3	
	Negative Control	Positive Control	Negative Control	Positive Control	Negative Control	Positive Control
n	192	192	192	192	192	192
Mean cpm	3738.68	307.88	3434.39	293.63	3616.12	275.20
SD cpm	254.36	43.95	290.30	47.44	293.88	44.08
CV %	6.80	14.28	8.45	16.16	8.13	16.02
z-prime	0.74		0.68		0.70	

four volumes of 220µM cold malonyl CoA were combined to yield a 250µM substrate working solution. Enzyme solution was 3.9ng/mL of FabD.

FabD enzyme inhibition assay

Final assay conditions for validation experiments were as follows. The filter in each well of the 384-well plate with glass-fibre filter was wet with 100µL of water and then filtered on the MultiScreen vacuum manifold. Five microliters of assay buffer were added to each well of the plate. Test compounds were prepared to a concentration of 100µM in DMSO and 2.5µL were added per well to the filter plate. Positive controls contained 2.5µL of DMSO per well. Thirty-five microliters of Holo ACP substrate (58.3µM), 5µL of co-substrate solution and 5µL of enzyme solution were added consecutively to each well. The plates were covered with the lid and incubated for 90 minutes at 37°C. Reaction was stopped with 50µL of 10% trichloroacetic acid (TCA) and plates were incubated for 15 minutes at room temperature. Wells were washed twice with 100µL water, placed on MultiScreen vacuum manifold (Millipore Corporation) and filtered. Filters were dried overnight at room temperature. The plate bottom was covered with MultiScreen clear tape (Millipore Corporation) and 10µL of Microscint 40 (PerkinElmer) scintillation fluid were added per well. The plate was covered with clear tape and incubated for >15 minutes at room temperature before counting on either a Perkin Elmer Microbeta®Trilux or Topcount® scintillation counter.

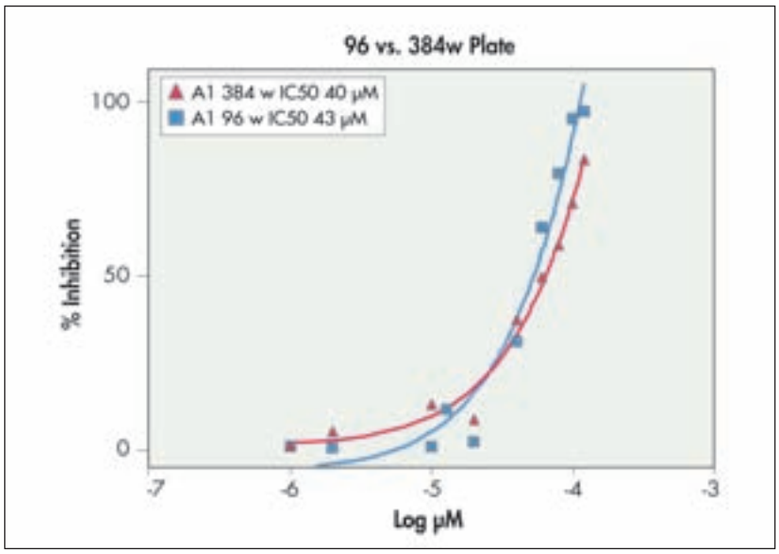

Figure 2: Comparison of the inhibition of FabD by reference compound A000326719 in the 96-well assay vs the 384-well assay

Inhibition assays were run in two different modes depending on whether the assay was performed directly in the filter plate or not. In the first mode, indicated as the 'Two-Plate' assay, reactions were initiated, incubated and terminated in a reaction vessel or solid bottom multiwell plate. The stopped reaction is then transferred to and captured on a filter plate for several rounds of washing followed by signal quantification by radioactive counting. In contrast, all of the reaction steps in the second assay mode, referred to as the 'One-Plate' assay, were performed in the filter plate without any additional reaction plate or transfer.

HTS experimental procedures

Preparation of assay components

FabD, ACP, holo-ACP, and co-substrate working solution were all produced as in assay validation.

Working solutions were prepared as in assay validation with the following exceptions. Holo ACP

stock solution is prepared to a concentration of 966µM and diluted in assay buffer and incubated 30 minutes at room temperature to recover Cys 35 free SH (malonyl reactive site). Final working solution concentration was 205µM. FabD working concentration was 1.95ng/mL.

Instrumentation

Beckman-Coulter's Sagian ORCA robot with SAMI-NT3 Software was used for plate handling and scheduling, and LabSystems Multidrop® dispenser was used to pipette reagents. Plate washing after the precipitation step was accomplished using the Biotek EL405 plate washer and radioactivity was measured on a PerkinElmer Microbeta® Trilux Scintillation counter. The filtration step was performed off-line on a Polyfiltronics UniVac 3 vacuum filtration unit. The decision was made not to integrate the filtration step into the robotics system in order to avoid contamination of the filtration block. (Integration of the filtration step using non-radioactive labelled substrate, eg Europium, is a future possibility.)

FabD enzyme inhibition assay

The order of addition of assay components, the volume of each component and incubation times were modified from the validation assay conditions above to accommodate the automated method and improve throughput and ease-of-handling. Materials and reagents are the same as in the validation assay unless otherwise noted. Final volume and concentration of assay components were the same for both assays. Five microliters of assay buffer were added to each well to block the filter and reduce non-specific binding. Addition of 10µL of Holo ACP was made to each well, followed by 5µL of test compound or 50% DMSO (positive control) and 10µL of enzyme solution or assay buffer (negative control). Next, 20µL of co-substrate working solution were added and the plates were incubated, precipitated, filtered and washed as in the assay validation method above. Filters were dried overnight at room temperature. The plate bottom was sealed with clear tape and 10µL scintillation cocktail was added to each well. The top of the plate was sealed with clear tape and the plate was incubated for a minimum of two hours at room temperature. Radioactivity was counted with a ^{14}C standard setting for 60sec/well.

Single point measurements were made for primary screening of the 282,158 test compounds. Each plate in this study included 352 compounds, 16 negative controls (all reagents except test compound) and 16 positive controls (all reagents except DMSO for test compound and no enzyme). The controls were used to determine the Z' factor for each plate, and data from plates with a Z' factor <0.5 were recalculated after elimination of the outliers. Results were calculated from the quench corrected raw data of each plate using the controls.

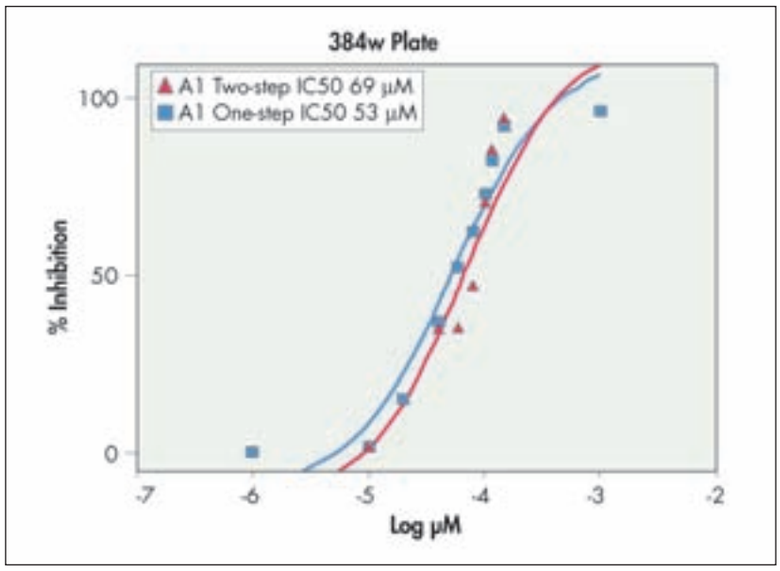

Figure 3: Inhibition of Fab D by reference compound A000326719 in the two-plate and one-plate methods

$$\text{Inhibition of ligand binding (\%)} = 100^{*}\left(1 - \frac{(sample - positivecontrol)}{(negativecontrol - positivecontrol)}\right)$$

Confirmation was performed in triplicate.

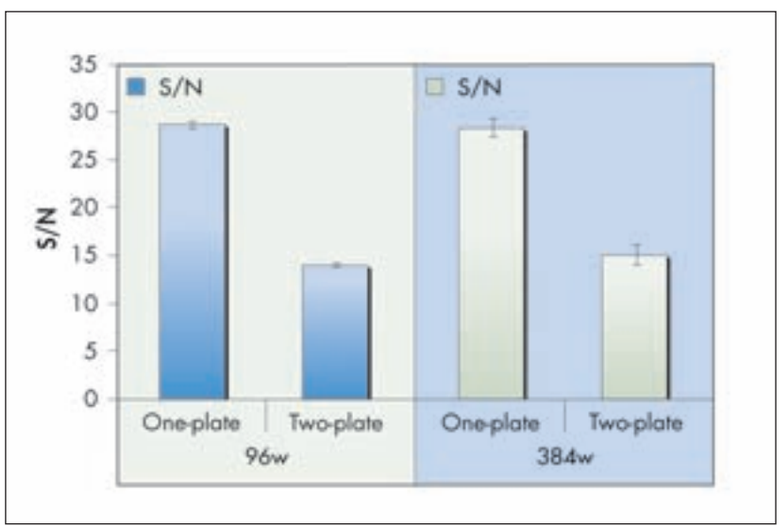

Figure 4: Comparison of signal to noise (S/N) in 96-well and 384-well MultiScreen glass-fibre filter plates using the two-plate vs one-plate methods (see Methods)

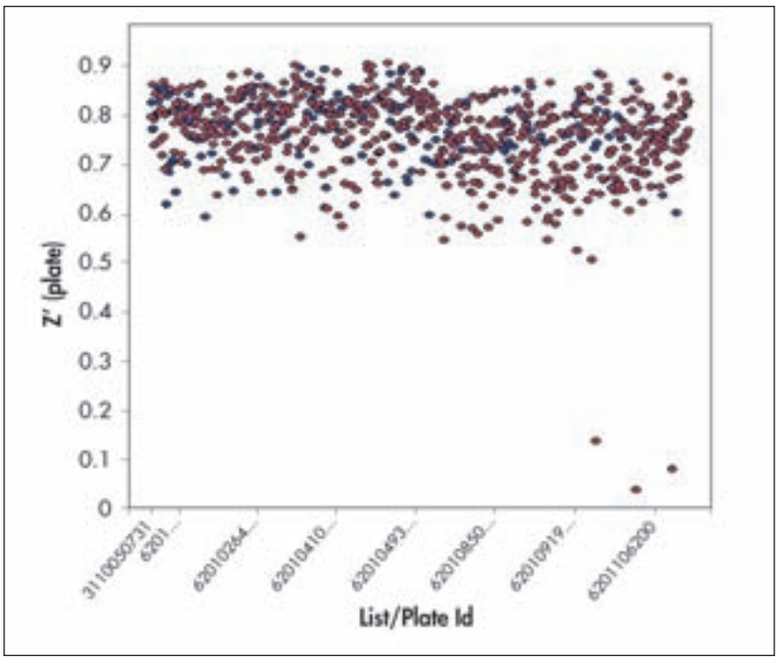

Figure 5: Distribution of the Z' values of all plates tested (n = 813)

Results

Assay validation

In order to optimise the FabD inhibition assay, we used Z' values to validate assay quality[10]. Z' values were calculated for the three 384-well plates run in the assay validation phase and results are presented in **Table 1**. A Z' limit of 0.5 or above was set to indicate an assay procedure with high reproducibility and good separation between negative and positive controls suitable for later screening experiments. As can be seen in **Table 1**, values were significantly above the 0.5 minimum target set for establishing the assay as useful for high throughput screen.

During assay validation, assay performance in the 96-and 384-well filter plate formats, as well as the 'two-plate' and the 'one-plate' assay formats (see Methods for details) were compared. IC_{50} results are presented in **Figures 2** and **3**. When the 'one-plate' 96-well assay was compared to the 'one-plate' 384 well-assay, the IC_{50s} for A000326719 were 40µM and 43µM respectively (see **Figure 2**). When the 'one-plate' assay was compared to the 'two-plate' assay in a 384-well plate the IC_{50s} for A000326719 were 53µM and 69µM respectively (see **Figure 3**) while the IC_{50} obtained by an HPLC method was 60µM. Results indicate no significant difference between the plate formats and methods.

We did observe a significant performance advantage in the one-plate assay method. In both the 96-well and 384-well filter plates, signal-to-noise ratio for the one-plate method was approximately double the two-plate method, due to a lower specific signal and a higher background in the 2-plate method (See **Figure 4**). The lower signal is probably due to a slight loss of material during transfer from plate to plate. Signal-to-noise was equivalent for both plate formats; therefore, sensitivity of the assay was not compromised.

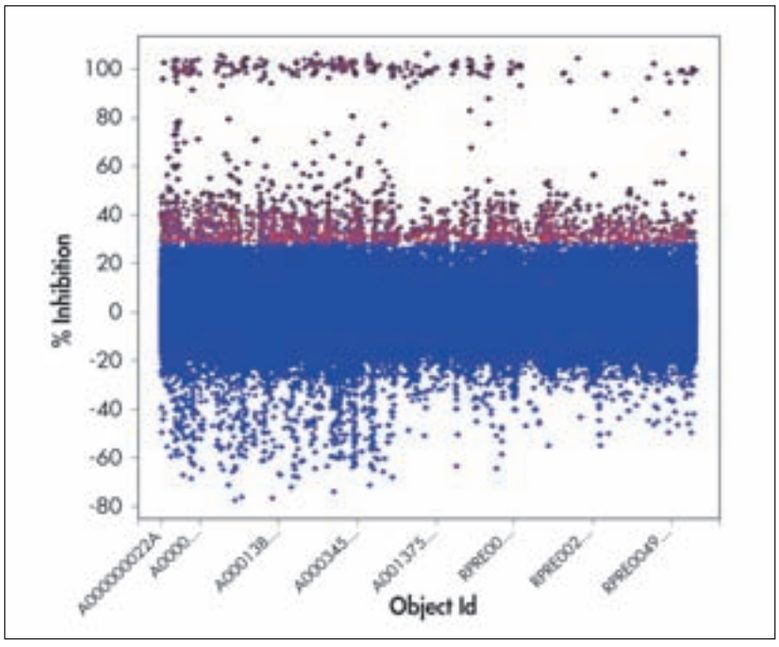

Figure 6: Distribution of the Primary Data according to Compound Codes (Positives in pink) (n = 282158)

HTS of test compounds

Z' value for all except three out of the 813 plates used in the screening of 282,158 compounds was calculated to be 0.77 ± 0.08 (see **Figure 5**). The Z' factor of <0.5 for the three plates were due to single outliers. Data from these plates were included in the analysis after the single outlier points were identified and eliminated. Results for all compounds in the screening are presented in **Figure 6**. The mean inhibition value for this screening study was 0.12 ± 9.61%. Using a calculated threshold value of 28.9%, a total of 2,087 compounds were identified for retesting in triplicate. Approximately 100 additional false positives were also included in the retesting in order to confirm them as negatives. Additional compounds were added for confirmation to fill up partially filled test plates in order to avoid empty wells, as only completely filled plates can be vacuum filtered.

Discussion

As fatty acid synthesis is an essential process for cell growth, the enzymes involved in this biosynthetic pathway represent attractive targets for novel antibiotics. An essential enzyme in this system, FabD, plays a major role in the elongation of the fatty acid chain. Antibiotic development by inhibition of this well conserved bacterial enzyme may result in a new class of drug with good selectively and broad spectrum of action.

The filter-based radiometric FabD inhibition assay was originally described by Joshi et al[9]. The assay was adapted to a 96-well plate format where incubations are performed in a standard 96-well plate, and then precipitated protein is transferred to a 96-well glass fibre plate for separating protein bound radioactivity from soluble ^{14}C-malonyl-CoA. The current study takes the initial 96-well assay and miniaturises it to a 384-well filtration format.

To reduce radioactive waste and streamline the procedure, the assay was converted from the original 'two-plate' to a 'one plate' system. In the original 96-well format, the reaction mixture was transferred from a standard 96-well plate to a 96-well FC glass-fibre filter plate after precipitation for the filtration step. This assay was compared to a 'one-plate' system using a 96-well then a 384-well MultiScreen plates to perform the reaction, incubation and filtration steps. In both the 96-well and 384-well plates, signal over noise ratio was increased for the one-plate method (see **Figure 4**). Thus, the 384-well assay plate provided a platform in which the reaction, incubation and separation steps could all be achieved in one plate.

The miniaturisation of enzyme inhibition assay for FabD resulted in a high-throughput, robotics-friendly assay that can process 12,000 samples per day. Here we document the utility of this assay for large scale screen of more than 280,000 compounds for their ability to inhibit FabD activity.

Analysis of the data identified 100 wells as false positive (see **Figure 6**). These results could be due to several possibilities – the most likely of which is failure of the robot to add radioactive substrate (or less likely, enzyme) to those wells, either due to a bubble in the pipette tip or other pipetting errors. If <0.04% (100 out of 282,158 wells) is the false positive rate for this assay, future use of the assay could include data acceptance criteria whereby wells with less than a certain level of the starting radioactivity count rate would be flagged for additional verification.

The initial screen showed great reproducibility and separation between positive and negative control as measured by Z' values greater than 0.5 and in many plates as high as 0.8. However, even with excellent assay performance, only 24 hits were confirmed from the 2,715 compounds retested in triplicate (**Figure 7**) as active for at least two data points. Together with these positives, eight inactive compounds were randomly selected for dose-response determination as negative controls. From these 24 reconfirmed samples, 23 compounds exhibited a dose-dependent activity with IC_{50} values below 40µM. The randomly selected samples did not exhibit a significant dose dependent activity. Although the hit rate was extremely low, the identified hits represented valuable structures for the project and have considerably increased the number of FabD inhibitors identified to date from six to 29.

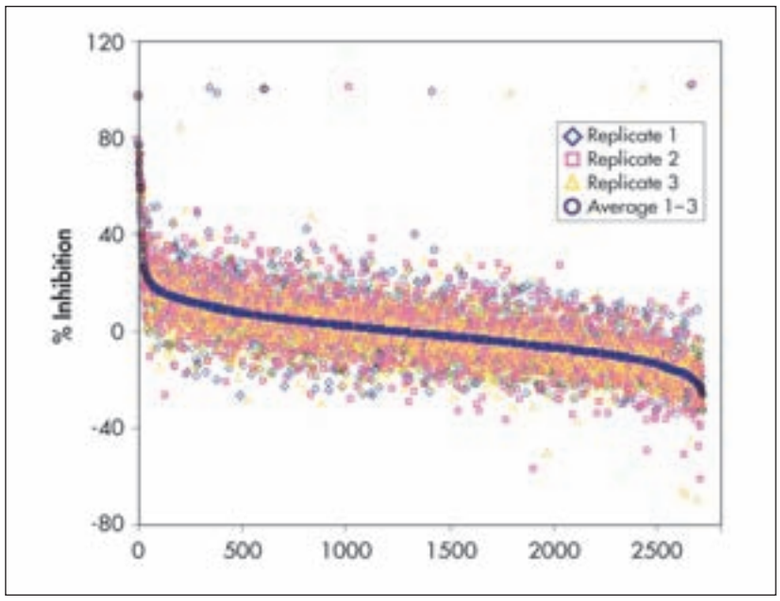

Figure 7: Results of confirmation screening in ranked order (n = 2715)

Conclusion

A 384-well enzyme inhibition filtration assay has proven to be well suited to HTS and is capable of handling 12,000 samples per day. Reformatting the 96-well assay made it possible to screen additional chemical collections in a robot-friendly format, to find new inhibitors, and to confirm what has been theorised for many years – that FabD is an appropriate target for novel antibiotic identification. In the search for a compound with a broad antibiotic spectrum, primary screening using the *E. coli* FabD inhibition assay prior to microbiological evaluation is a valid and valuable tool.

The drive towards further miniaturisation of

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assays for drug discovery improves throughput, conserves compound libraries and reduces radioactive waste. Future uses of the new filter plate format for novel chemical entity screening may include improvement of classically homogeneous assays (ie Scintillation Proximity Assays; SPA) by decreasing background through washing of unincorporated label or other assay formats using non-radiolabelled substrates or ligands (eg Europium-label) and ligand binding assays with animal tissues (membranes). DDW

Dr Gaëtan Touyer *was appointed Head of the MTS screening group for the Sanofi-Aventis, Vitry Research Centre (Paris, France) and manages five engineers. Previously he served as the Assay Development Manager, where he was responsible for managing three engineers in the development and validation of high throughput screening (HTS) assays. Previously, Dr Touyer led the Radioiodination and Immunochemistry laboratory for Hoechst-Marion-Roussel (Paris, France). In this position, Dr Touyer synthesised I125-labelled compounds, bioconjugates and immunochemical reagents for DMPK and HTS. He also proposed the 'broad spectrum immunoassay' concept for throughput improvement in early ADME studies. Prior to that, Dr Touyer developed enzymatic tools to synthesize C14 uniformly labelled nucleotides for the Centre of Nuclear Studies (Saclay, France). Dr Touyer holds engineering degrees in biochemistry and business administration from Paris University. He has published numerous papers on the topics of radioimmunoassay, high throughput screening and monoclonal antibodies. Additionally, Dr Touyer has obtained several European patents for his work.*

Dr Irvin Winkler *is the Deputy Head of the HTS screening group for the Sanofi-Aventis Frankfurt Research Centre (Frankfurt, Germany). He has been engaged in high throughput screening for nine years. Previously, Dr Winkler was group leader in the chemotherapy department at Hoechst AG (Frankfurt, Germany). In this position, he was responsible for antiviral research, including HIV. Dr Winkler holds scientific degrees in biology from the University of Wuerzburg and Hannover (Germany). He has published several papers on antibacterial and antiviral chemotherapy. Additionally, Dr Winkler has obtained several European patents for his work.*

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