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Developing a Sterile, Reliable Laboratory Setup for Low-Volume Tuberculosis Antibiotic Discovery Assays

**Pamela Thayalan,
Kakoli Mukherjee
and David Beer**

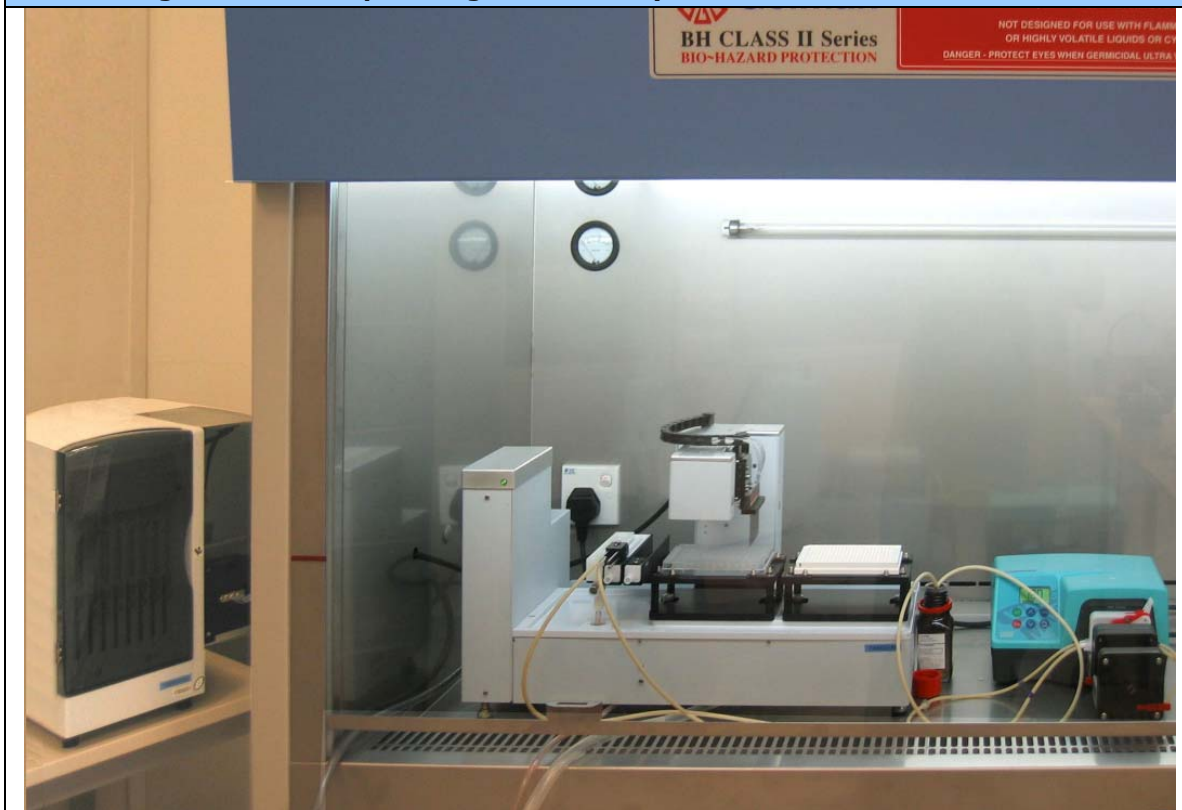
**Novartis Institute for Tropical Diseases
10 Biopolis Road
#05-01 Chromos
Singapore 138670**

One third of the world's population is infected with Tuberculosis and one person dies every 15 seconds from the disease. The existing treatment of Tuberculosis takes 9 months and relies on drugs developed prior to the 1970s. There is an urgent need to develop new drugs to reduce treatment time and also combat the emergence of multi-drug resistant strains of Tuberculosis. One of the goals of the Novartis Institute for Tropical Diseases is to develop new drugs for the treatment of Tuberculosis, particularly multi-drug resistant (MDR) TB and shorten the current treatment times from 6-9 months to 2 months.

Our current approach is to develop a relatively low volume, simple, robust and reproducible assay methodology to test potential antibiotics against proliferating *Bacillus Calmette-Guerin* mycobacteria (the strain currently used in vaccinations against *Mycobacterium tuberculosis*). There are several key challenges in setting up the laboratory to perform such assays reliably, including the need for transfers to occur in sterile, biologically isolated conditions (e.g. within a Biosafety Level II containment cabinet), and the precise and reproducible transfer of stock compounds into the assay plates without cross-contamination. Furthermore, the isolation of the compound transfer device in the containment cabinet necessitated some means for it to perform tip self-cleaning and to operate for relatively long periods of time without maintenance and without loss of precision. The accurate dispensing of low amounts of compound was also seen as desirable, allowing for both conservation of test compounds and the ability to miniaturize the assay.

For the stock compound transfer portion of the assay a Nanodrop™ II (Innovadyne Technologies, Inc.) was evaluated. The small footprint of the Nanodrop II stage, and the separation of the stage and fluidics modules in the design, make possible the inclusion of the stage within the containment cabinet, as shown.

Fig. 1 -- Nanodrop II Stage in Biosafety Level II containment cabinet

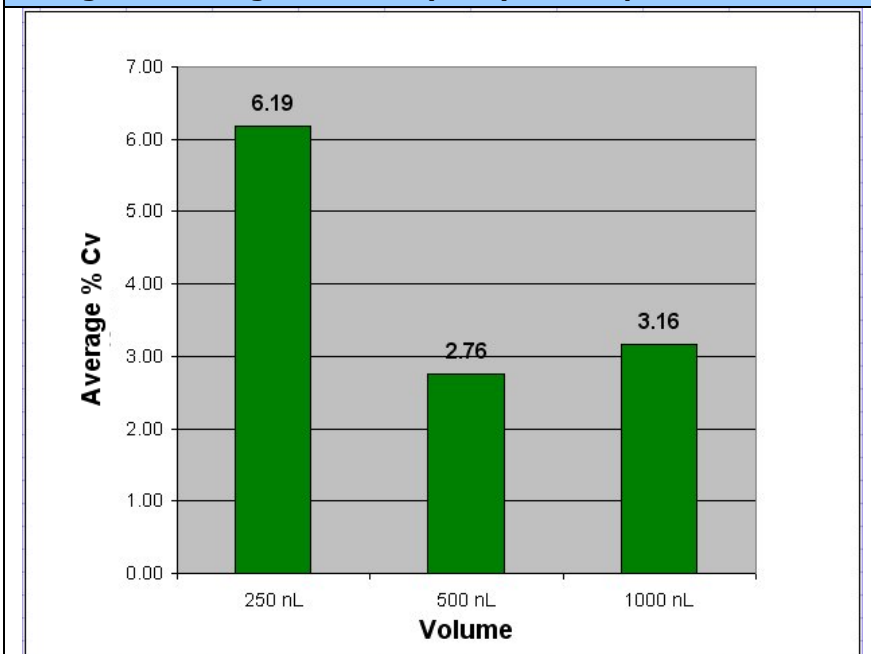


Additionally, the aspirate-dispense architecture of the Nanodrop (non-contact, with valves isolated from the flow path) with customizable tip washing procedures showed promise for meeting our requirements. We set about validating the performance of the Nanodrop II in the areas of dispense precision, accuracy, and cross-contamination suppression.

Validation of Stock Transfer Performance

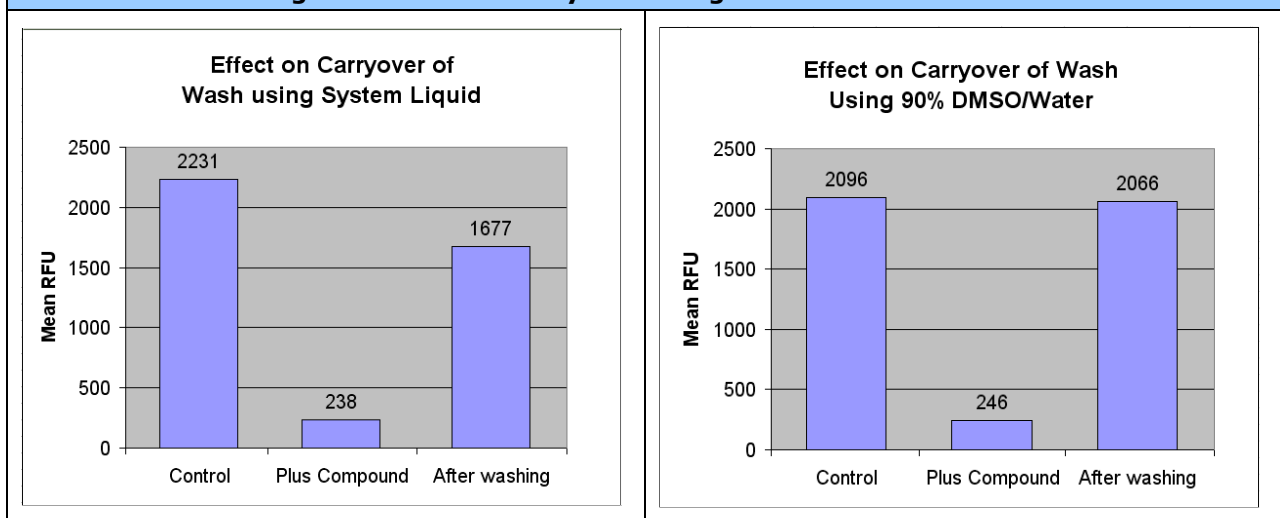
The performance of the Nanodrop II was validated for transferring stock test compound in 90% v/v DMSO/water using the dye Eosin Yellow, which was dissolved in the DMSO solution at a concentration of 100mM. Accuracy and precision were assessed by extrapolating the volume of dye dispensed using the Nanodrop II from an Eosin Yellow calibration curve, with volumes of 250, 500 and 1000nL dispensed in three 96-well plates per volume. The average precision (% CV) was 6.19, 2.76 and 3.16 for 250, 500 and 1000nL volumes respectively and the accuracy for all volumes showed a deviation from expected volume transferred of less than 10%.

Fig. 2 -- Average Precision (%Cv) at 3 Dispense Volumes



Prevention of test compound cross contamination is generally a concern when using fixed tips to transfer test compounds. Several tip wash routines were evaluated for their capacity to eliminate sample carry-over on the Nanodrop II. The diagrams below show the results for two wash procedures. The first bar of each graph refers to control data for cells only (0% of inhibition cell growth). The second bar of each graph includes cells plus test compound (with nM IC50) at 20µM (100% inhibition of cell growth). The third bar shows the efficiency of washing by running the control again after the wash procedure under study. The first wash procedure including only a wash with system liquid after each column of compounds was transferred resulted in 26.4% inhibition of cell growth indicating compound carry-over on the tips. The second procedure using three washes with 90% v/v DMSO/water (from a re-circulating reservoir) and then a wash with system liquid, after each column of compounds was transferred, resulted in no significant inhibition of cell growth, indicating no compound carry-over on the tips.

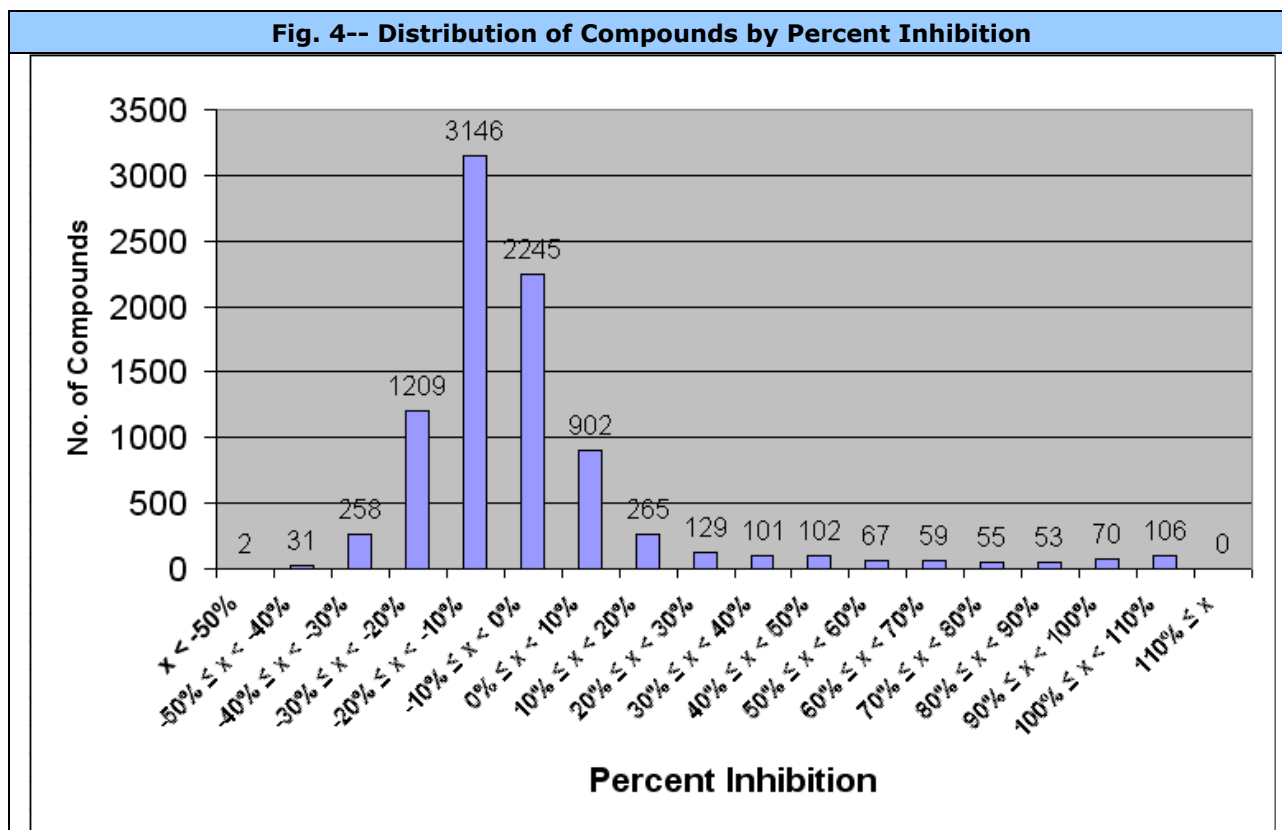
Fig. 3 -- Effect on Carryover Using Two Wash Procedures



exception that the current study was performed at a final volume of 50 μ L (rather than the final volume of 200 μ L used by Collins and Franzblau).

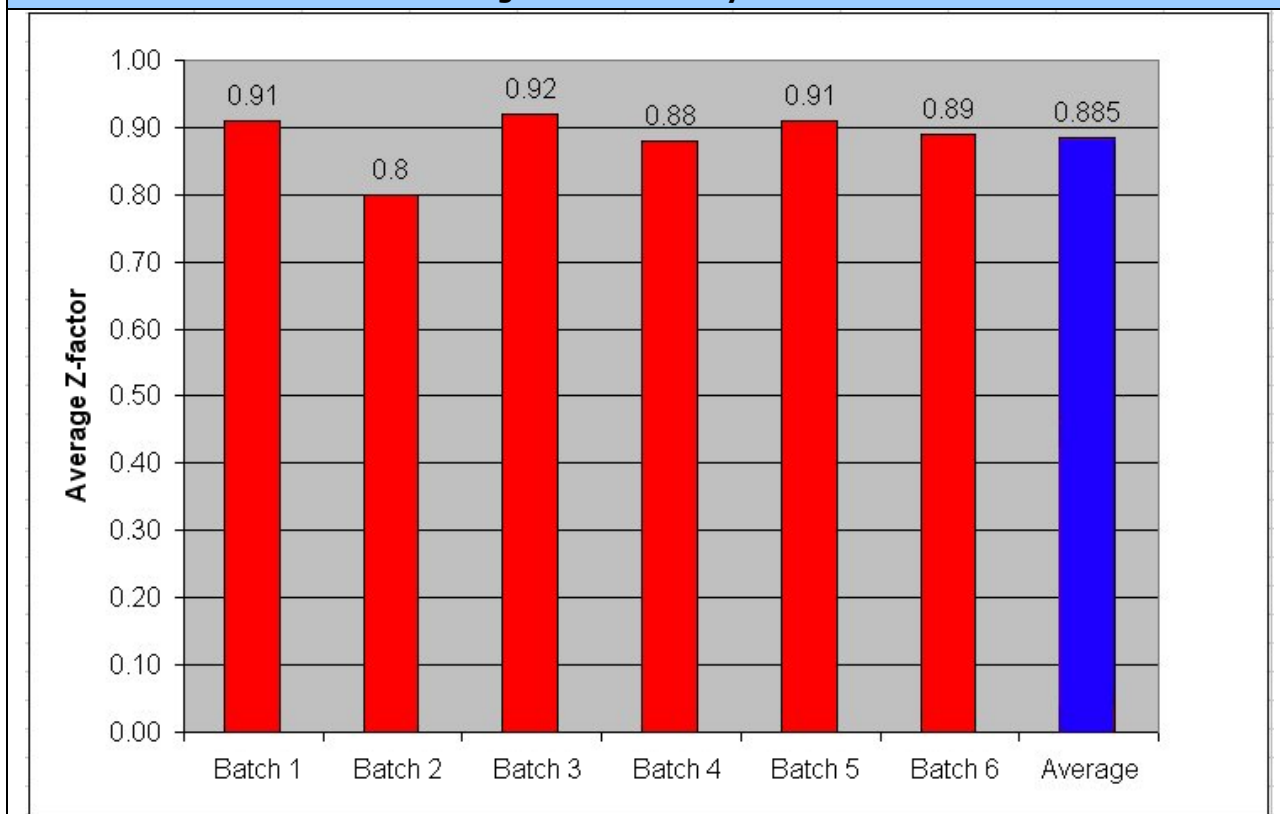
The assay was validated by screening a diverse set of compounds (a total of 8800 compounds in 100 96-well plates) from the Novartis compound archive at a single concentration of 20 μ M. A volume of 500nL of 2mM test compound in 90% v/v DMSO/water was transferred to sterile clear 1/2-well 96-well plates (Corning Costar) using the Nanodrop. 50 μ L Bacillus Calmette-Guerin mycobacterium (diluted to an A650nm of 0.02 from a flask culture) was added to the plates using a Matrix WellMate and the cells were incubated for 4 days at 37 $^{\circ}$ C and 100% humidity. 12 μ L 1:1 Alamar Blue (Serotec):Tween 20 was added to the plates using a Matrix WellMate and the cells were incubated for 24 hours at 37 $^{\circ}$ C and 100% humidity. The resulting fluorescence was detected using an excitation wavelength of 540nm and an emission wavelength of 590nm using a Tecan Saffire II.

The screen was run in 6 batches (1 batch per week) consisting of 10-25 plates with a control plate included in each batch. The screen was run over an extended period to determine if the assay was stable over time. Control plates included a streptomycin dose-response curve (11 concentrations from highest concentration of 20 μ M diluted serially 2-fold in 90% DMSO/water). The minimum inhibitory concentration (equivalent to IC90) for Streptomycin from all control plates was 0.0912 \pm 0.0093 μ M (mean \pm standard error of the mean). Data from the test set of compounds was analyzed as a whole and the histogram below shows the distribution of % inhibition.



The signal to background ratios were 7.8, 8.2, 10.5, 9.4, 12.3 and 12.6 and the z-factors were 0.91, 0.80, 0.92, 0.88, 0.91 and 0.89 for the six runs. The average z-factor for all 100 plates tested was 0.89, as shown:

Fig. 5 -- Z-Factor by Batch



Using a cut-off response of the mean % inhibition + 3 standard deviations from the mean, 270 compounds were picked for re-confirmation by performing dose-response curves (8 concentrations from the highest concentration of 20 μ M diluted serially 2-fold in 90% DMSO/water). Again the Nanodrop was used to transfer the serially diluted compounds to assay plates and a re-confirmation rate (i.e. compounds giving curves with an inhibition of a least 50% at the highest compound concentration) of 80% was achieved using this methodology. The quality of the screen was further confirmed by the identification of the antibiotic, rifampicin, known to be active against *Mycobacterium tuberculosis*, within the compound library. Several novel compound classes were identified from this screen and are being followed up as potentially interesting antibiotics.

The Nanodrop has simplified this assay by allowing the direct pipetting of test compounds into assay plate with high accuracy and precision, allowing for conservation of compound usage and leading to a highly reproducible assay. Positioning of the Nanodrop in a Biosafety cabinet has allowed the ability to preserve the sterility of assay plates. As of this publication over 500 plates have been screened using this assay with no loss in the performance of the assay.

Future work includes moving the assay to 384-well plates, utilizing the same assay format with *Mycobacterium tuberculosis* and further miniaturization.