Malaria High Throughput Screening on a Global Scale

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Introduction

In the search for new anti-malarial compounds to combat drug resistance, an image based HTS assay was developed by Discovery Biology in late 2007. Evaluation of various *P. falciparum* growth inhibition assay formats indicated that the use of a fluorescent nucleic acid dye to monitor parasite growth would be the most amenable to HTS. The *P. falciparum* growth assay using DAPI was developed using both total fluorescent intensity (TFI) read outs and image based analysis. As image based analysis was preferred to improve data quality, by eliminating potential interferences due to fluorescent or quenching compounds, the original DAPI assay was optimised to significantly improve throughput, whilst maintaining assay precision and the magnitude and linearity of the fluorescent signal. Specifically, this was achieved by altering the assay buffer components, eliminating fixation and centrifugation steps and optimisation of RBC concentration (haematocrit).

To undertake screening of external partner libraries requires the establishment of legal contracts with respect to IP associated with the compounds, and processes to facilitate the importation of these libraries such as shipping, customs and quarantine requirements. Prior to screening taking place all compound information must be entered into the in-house data base and analysis software programmes.

The compound libraries screened were sourced through Medicines for Malaria Venture (Geneva, Switzerland) from various organisations. The external libraries have ranged in size from 150 to 500,000 compounds. They have been received in various 384 well plate types, volumes, concentrations (100nl to 50ul of 1mm to 10mM), plate layouts and different seals. The positioning of empty wells for use as in plate controls has been one of the most variable aspects of the plate layouts.

Throughout all HTS campaigns, the results demonstrate that the response is maintained up to 72 hours after DAPI staining. Stability of the response for reference compounds is consistent both within screening batches and between screening days. Throughout all of the campaigns undertaken to date the Z' has remained consistent between 0.5 - 0.75.

The exact timing and synchronisation of the parasite culture used in the assays also allows for the detection of second generation compounds as the parasite undergoes two replication cycles within the time frame of the assay. See example of compound demonstrating second generation activity at retest.
With full optimisation of the assay protocol and the required equipment we are currently capable of screening half a million compounds in a 6 week screening period.

Equipment

MINITRACK LIQUID handling system

MULTIDROP liquid dispenser

CYTOMAT 240L incubator

BIOMEK FX

EVOTEC OPERA & TWISTER ARM

OPERATOR

The Assay

Diluted compound was added to PerkinElmer 384 well CellCarrier imaging plates and 25µl of complete culture media added to all wells. 20µl of diluted parasite was then added to all the wells and the plates incubated for 72 hours.
After incubation, the plates were stained with DAPI and imaged on the PerkinElmer OPERA HTS imaging system.
Images acquired using the Opera™ were analysed using Acapella for the number of spots based on fluorescence and size.

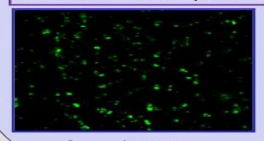
HTS OPERA Assay Image Analysis

"Acapella™ is designed to process complex data in high speed serving the needs for on-line analysis in high content screening. It provides the solution for getting the full spectrum of information out of a collection of primary data." (Quote from PerkinElmer website)

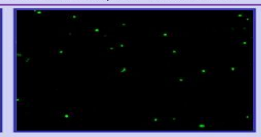
Application of Acapella.

Firstly, local pixel intensity maximums were detected over the entire image of each well, a circle, 4 pixels in radius, was drawn around the intensity maximums and designated as a spot. The spots were then classified as real spots, on the basis of two parameters. Contrast of the maximum fluorescence intensity of the spot with the surrounding pixels in the circle, and Spot-To-Cell Intensity which was used to compare maximum spot intensity to the background intensity found over the whole image. For this assay a contrast of 0.5 and Spot-To-Cell Intensity of 4 was used.

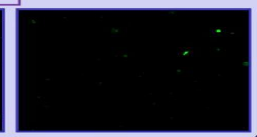
condition	Counts
infected RBC's	1029
infected RBC's + inhibitor	132
non-infected RBC	70



Infected RBCs



Infected RBCs + 2uM Artemisinin



Non-infected RBCs

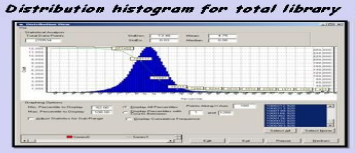
Summary of Libraries Screened

Company	Library (cpd #)	HTS conc	% Hit Rate
DPI	NP: (140,000+)	1.82uM	0.79
	SF1: (34,654)	0.7ug/ml	1.27
MRCT	(42,880)	1.96uM	0.9
Pfizer	Library 1: (150,352)	3.17uM	2.62
	Library 2 : (1,996)	0.784uM 7.84uM	16
	Library 3 : (3,030)	0.784uM 7.84uM	5
	Library 4 (176)	0.784uM 7.84uM	20
	Retest: (15,322)	9pt CRC	
Library X	NP: (141)	6.4,1.28, & 0.25um	**
Chimerix	Lib 1: (10,560)	1.8ug/ml	9
	Lib 2: (3,520)	0.4ug/ml	2.5
Ferrer	(27,035)	3.4uM	4.9
AZ	Validation: (20,000)	1.9uM	3.5
	Library 1 (502,868)	1.6uM	1.88
Evotec*	Library 1 (256,263)	1.92uM	1.25
Library Y	Library 1 (1239)	8 pt CRC	4

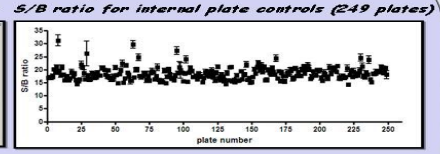
Total samples screened at HTS for these libraries is 1,167,706
Total data points assayed, including empty wells, control wells and plates, retest and IC_{50} determination , plus academic compounds, is greater than 3 million

Example of Screening Data

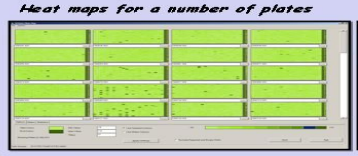
Distribution histogram for total library



S/B ratio for internal plate controls (249 plates)



Heat maps for a number of plates



Summary of Reference compound IC_{50}

	IC50 nM				
	R1	R2	R3	Average	stdev
Pyrimethamine	10.6	7.6	6	8.1	2.4
Mefloquine	11.5	9	7.5	9.3	2
Chloroquine	20.4	14.2	10.1	15.2	5
Artimisinin	6.21	3.9	4.7	4.9	1.2
Puromycin	49.8	36.3	42.7	42.9	6.7
Halofantrine	4.85	NA	2.74	3.8	1.5
Amodiaquin	7.56	3.29	3.3	4.7	2.5

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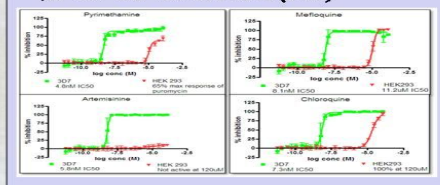
HTS Activity	# of Compounds	# Compounds Not Confirmed	Percentage Confirmed
>50% inhibition	3209	1224	62
>50<80% inhibition	1829	853	53
>80% inhibition	1380	371	73

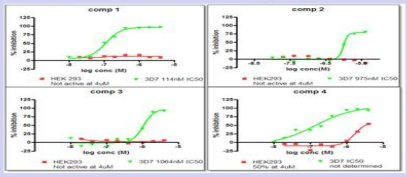
The high non-confirmation rate for the 50-80% is not uncommon in this assay as the Hillslopes of many anti-malarial compounds are extremely steep. In general, retest also involves a 5-9 dose response curve on both the 3D7 parasite and the mammalian cell line HEK 293 for determination of the selectivity index of the actives.

Retest

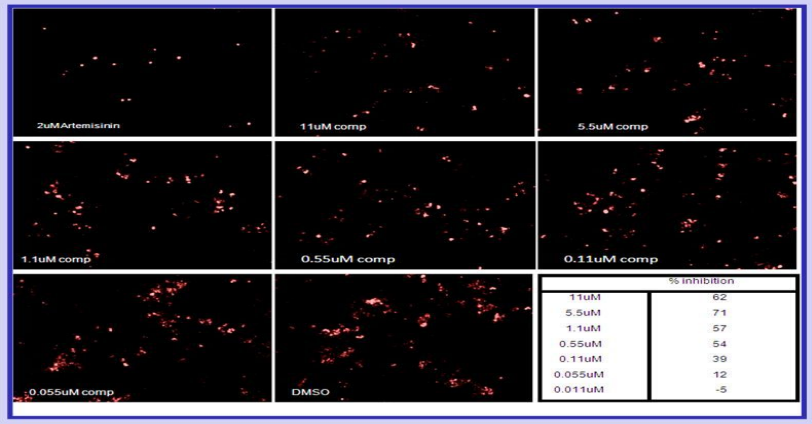
Example of reference compound IC50 values for 3D7 and HEK 293 cells (n=10)



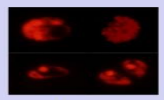
Example of library compound IC50 values for 3D7 and HEK 293 cells (n=1)



Example: Second Generation Active Compound Identified at Retest



Conclusion



The image based anti-malarial screening assay established in Discovery Biology, has proven to be stable, reliable, and sensitive. More than 1 million compounds have been screened, identifying several thousands active compounds with good selectivity, providing novel starting points for drug discovery programmes worldwide.
This assay has recently been demonstrated to be capable of detecting both early and late stage anti-malarial compounds. Thus allowing further biological profiling, and target identification studies to be undertaken on the compounds identified during HTS.

Acknowledgements

The authors gratefully acknowledge financial support to undertake this research from Medicines for Malaria venture (MMV).
We also acknowledge the Australian Red Cross Blood Service for the provision of human erythrocytes.
Sandra Duffy is the recipient of a Travel Award from Perkin Elmer.