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High Throughput Screen for Inhibitors of Rac1 GTPase by Flow Cytometry

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Additional information is available at the end of the chapter

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Abstract

High throughput (HT) screening is at the starting point for most drug discovery programs. As the range of targets being pursued widens new technologies have to be deployed to enable assays built to measure the activity of proteins previously deemed challenging. Flow cytometry is a technology providing multi-parametric analysis of single cells or other particles in suspension, such as beads. High throughput (HT) flow cytometry has become a very attractive screening platform for drug discovery. In this chapter we describe a 1536 well format high throughput screen of 500,000 compounds to find inhibitors of Rac1 GTPase to prevent allergic airway hyper-responsiveness in asthma. We discuss the assay development, miniaturization and validation carried out prior to the full screening campaign. We then describe how we have automated our iQue® HD screener instruments and how we proceed with the data analysis and explain why we chose to run this screen on a flow cytometer and how it enabled us to reduce cost and timelines for the project.

Keywords: HTS, drug discovery, bead-based assay, GTPase

1. Introduction

Asthma is a heterogeneous inflammatory disorder of the airways characterized by chronic deregulated inflammation, bronchial hyperreactivity, and symptoms of recurrent wheezing, coughing, and shortness of breath. Its prevalence has increased considerably over the past three decades, particularly in Western countries. Asthma is a major public health problem that affects 300 million people worldwide [1, 2].

Airway hyperresponsiveness (AHR), one of the hallmarks of asthma, directly results from excessive contraction of airway smooth muscle cells (aSMC). The degree of AHR always correlates with asthma severity and the need for therapy [3, 4]. Regular treatment of chronic asthma consists of a combination of inhaled anti-inflammatory corticosteroids and long-acting beta2-adrenergic receptor agonists for bronchodilation. However, severe asthma escapes to usual treatments or frequently requires higher doses. In acute asthma, short-acting beta2 agonists or anticholinergics are used as bronchodilators. These drugs are rapidly effective but can be insufficient in some cases of severe acute asthma attack. Despite available therapies, many patients with severe asthma remain uncontrolled and the number of asthma deaths is still elevated [5]. There is thus an obvious need for new drugs acting through other pathways to prevent or reverse AHR and decrease severe asthma attacks, hospitalizations and deaths.

The molecular mechanisms regulating aSMC contraction and proliferation involved in AHR are still largely unknown. Understanding the intracellular signaling pathways responsible for AHR is thus essential to identify new targets and design new treatments. In this way, the Rho protein Rac1 has been identified as a promising candidate.

The Rho/Rac proteins constitute a family of small GTPases of more than 25 members in mammals. So far, the best characterized members of this family include the members of the Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2, RhoG), and Cdc42 (Cdc42) subfamilies. Rho/Rac proteins share a common structure (40–95% identity) composed of a six-stranded mixed β sheet flanked by five α helices. The core G domain is made up of five polypeptide loops (G1–G5) involved in nucleotide, regulators and effectors interactions. Like the majority of Ras superfamily GTPases, most members of the Rho/Rac family behave as molecular switches that cycle between inactive (GDP-bound) and active (GTP-bound) states. In basal conditions, these proteins are inactive and sequestered in the cytosol due to their binding to Rho GDP dissociation inhibitors (RhoGDIs). Upon cell stimulation, Rho proteins became GTP-bound, translocate to the plasma membrane after release from RhoGDI, and interact with their primary effector molecules to trigger different signal transduction pathways. In addition to RhoGDIs, the activation/inactivation cycle of Rho/Rac proteins is regulated by a complex set of regulatory proteins that include GDP/GTP exchange factors (GEFs) and GTPase activating protein (GAPs) (**Figure 1**). Interaction of Rho/Rac protein with a GEF promotes the exchange of GDP for GTP molecules, thus leading to the rapid activation of Rho/Rac proteins during cell stimulation events. By contrast, GAPs promote the hydrolysis of the bound GTP molecules to GDP, thus allowing the transfer of the GTPases back to the inactive state at the end of the stimulation cycle [6].

During the last decade, Rho/Rac protein signaling pathways have been recognized as major regulators of essential cellular functions. Rac1 is a key regulator of cytoskeletal structure and dynamics, leading to lamellipodia and ruffle formations [6, 7]. Thanks to this last function, Rac1 controls cellular migration and adhesion. Recently, we demonstrated that Rac1 regulates vascular SMC contraction, and consequently modulates arterial pressure [8]. Accordingly, we hypothesized that Rac1 could also be involved in aSMC contraction. We demonstrated that the specific SMC deletion of Rac1 (SM-Rac1-KO) in mice prevents bronchoconstriction *ex* and *in vivo*. Our results showed that the decreased expression or activity of Rac1 in aSMC impairs

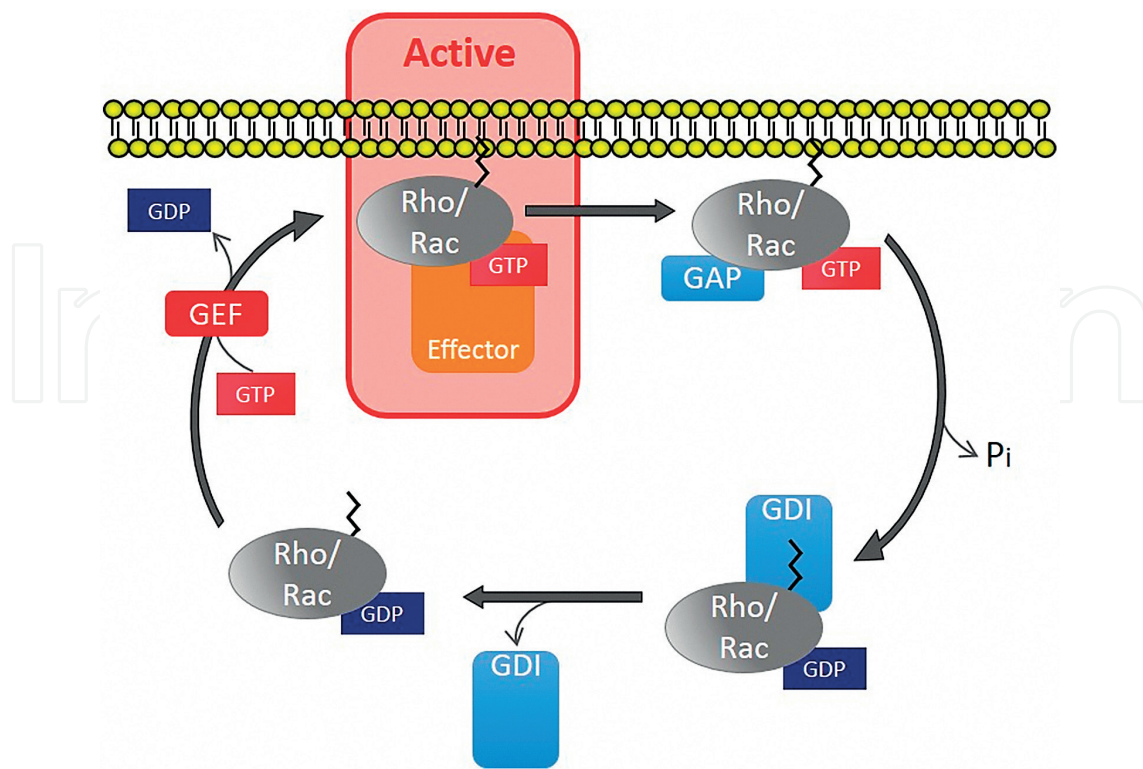


Figure 1. Activation cycle of Rho/Rac proteins and their regulatory proteins. Activation of Rho/Rac proteins is mediated by a guanine nucleotide exchange factor (GEF) leading to GTP loading and to the translocation of Rho/Rac proteins to the plasmatic membrane. This active configuration of Rho/Rac proteins promotes effector interactions. To “turn off” the cycle, a GTPase-activating protein (GAP) accelerates the intrinsic GTPase activity of Rac, allowing Rac to return to its inactive state in the cytosol. The guanine nucleotide-dissociation inhibitor (GDI) binds specifically to GDP-bound Rho/Rac proteins, prolonging the inactive state and sequestering the GTPase in the cytosol.

agonist-induced rise in intracellular Ca^{2+} concentration through a mechanism involving Rac1-dependant control of PLC activity. Interestingly, Rac1 deficiency has no impact on the respiratory system in basal, physiological condition. However, deletion of Rac1 expression in aSMC or nebulization of the Rac inhibitor NSC23766 prevented AHR in an allergic asthma model in mouse. These data indicate that (1) Rac1 is a critical component in aSMC contraction and (2) inhibition of Rac1 activity or expression may represent a novel therapeutic approach for patients with airway AHR such as asthma.

Unfortunately, the currently available drugs that inhibit Rac1 (EHT 1864 and NSC23766) have low affinity and induce critical off-target effects, thus highlighting the obvious need to discover new Rac inhibitors [9, 10].

In this chapter we describe how a published flow cytometry assay was taken and converted to a 1536 well format suitable for high throughput screening (HTS) to enable the screening of 500,000 compounds in the AstraZeneca Global HTS Centre to attempt to find new Rac1 inhibitors. We describe the whole protocol from receiving assay ready plates through to the data analysis and discuss the criteria that are important for an assay to be suitable for a high throughput screen by flow cytometry and describe the validation process we go through prior to starting a high throughput screening campaign.

2. Assay development

Several papers have demonstrated the utility of a bead-based flow cytometric GTP-binding assay for screening GTPase targets for small molecule inhibitors in both singleplex and multiplex formats [11–13]. As part of AstraZeneca’s Open Innovation initiative, we collaborated with researchers at l’Institut du thorax to identify small molecule inhibitors of Rac1 GTPase. We therefore set out to develop a bead-based assay for screening Rac1 in a singleplex fashion (**Figure 2**) for a screening campaign in 1536 well plates on an iQue® Screener HD. The Intellicyt iQue® Screener HD is a flow cytometer with an autosampler able to sample from 96, 384 and 1536 well plates. Using proprietary technology the instrument separates well samples with an air bubble allowing the software to determine what sample comes from what well making the instrument a true high throughput instrument.

The assay is based on glutathione-coated beads used to capture GST-tagged Rac1. Bodipy-labeled GTP (FL-GTP, ThermoFisher Scientific), is then used as a tracer for the detection of compounds that compete with its binding to the GTPase resulting in a decreased fluorescence signal.

We purchased commercially-available glutathione coated particles (GSH beads, size 4.0–4.9 μm , Spherotech) that have glutathione covalently coupled to their surface and glutathione S-transferase tagged Rac1 protein (human wild type) (GST-Rac1, ThermoFisher Scientific) and bound the GST-Rac1 onto the GSH beads overnight. Assay development was performed in 384 well plates before miniaturizing the assay for 1536 well plates. We optimized the concentration of FL-GTP to use in the assay to achieve the largest signal to background window and also confirmed that FL-GTP did not bind to unconjugated beads to demonstrate Rac1/GTP binding specificity. Based on the data shown in **Figure 3** and associated table, 100 nM FL-GTP was selected.

To validate that the beads were coated with GST-Rac1 and that they would bind bodipy-labeled GTP the prepared beads were mixed with serial dilutions of unlabeled GTP (ThermoFisher

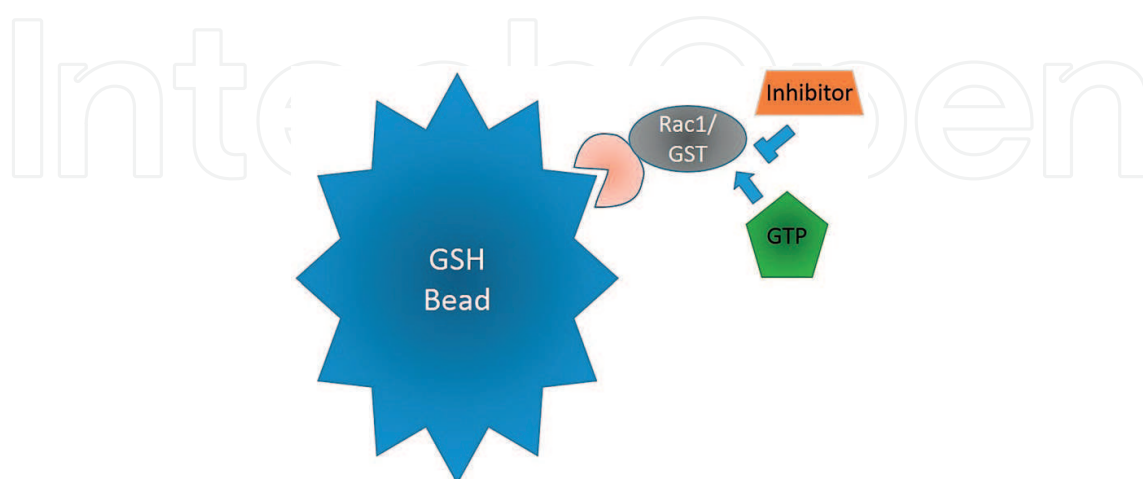


Figure 2. Pictorial representation of GST-Rac1 bound to GSH beads. Inhibitors of fluorescent GTP binding result in decreased FL1-H signal.

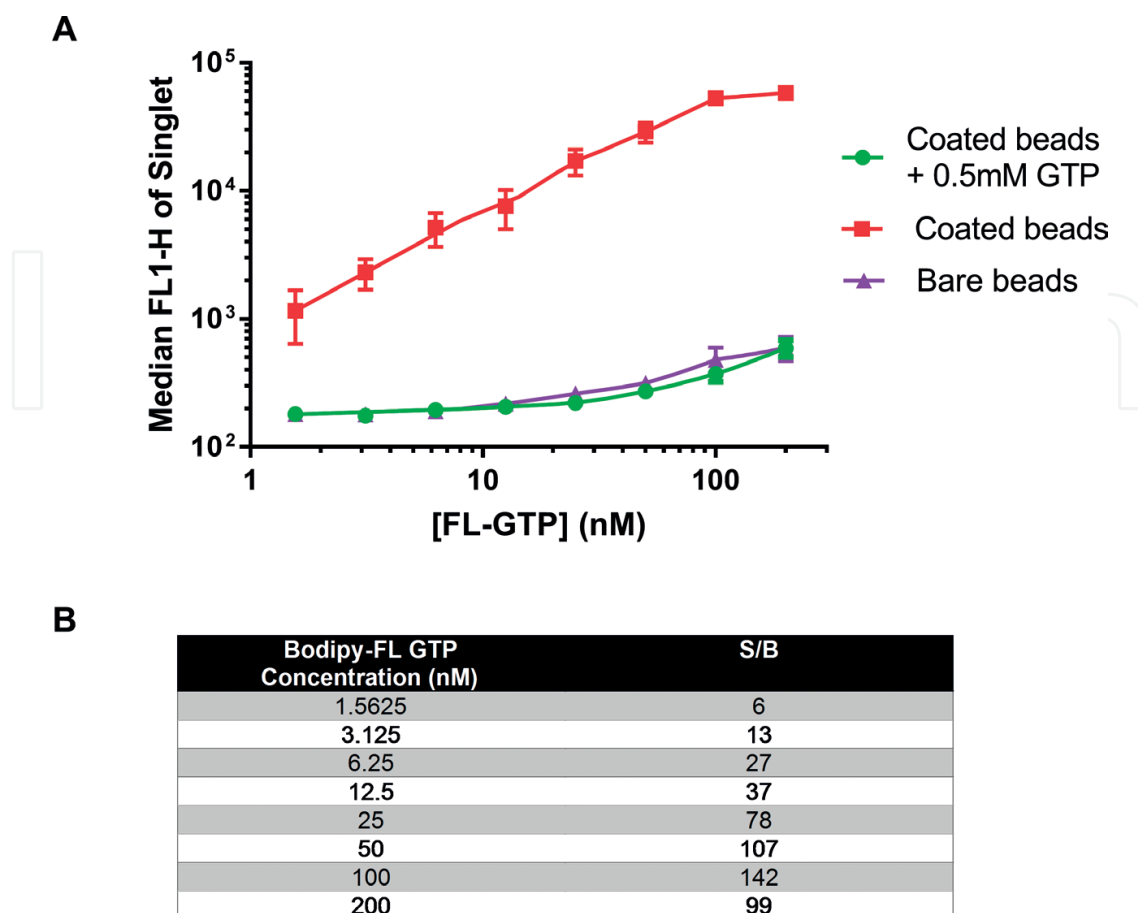


Figure 3. Optimization of FL-GTP concentration and confirmation of binding specificity. (A) Unconjugated or Rac1-coated beads were incubated with varying amounts of FL-GTP in the presence or absence of 0.5 mM unlabeled GTP and bead fluorescence measured on the iQue[®]. ■: Rac1 coated beads in the presence of 0.5 mM unlabeled GTP, ▲: bare beads, and ●: Rac1 coated beads. (B) Signal to background table for each concentration of FL-GTP.

Scientific) as a competitor followed by the addition of FL-GTP. To prepare the assay, 5 μ L of coated beads were dispensed into wells on a 384 well plate followed by 5 μ L of buffer containing unlabeled GTP in serial dilutions in triplicate, then 5 μ L of 100 nM FL-GTP was added for a final volume of 15 μ L and incubated for 30 min. The methods described in Refs. [11–13] included a final incubation for up to 60 min at 4°C. We wanted to be able to prepare and incubate the plates at room temperature (RT) for large scale screening, so we incubated plates both at 4°C or RT and then read the plates on the iQue Screener HD immediately after incubation or after 2 h at RT to determine if the signal window was still adequate for screening at that time. The beads are not fluorescently labeled, therefore exhibit low fluorescence intensity in the FL-1 channel (excitation 488 nm; emission filter 530/30 nm). Beads with bound FL-GTP exhibit a marked increase in fluorescence (approximately 200–500 fold). Inhibitors of GTP binding decrease the FL-1 fluorescence which is the measured signal for the samples read on the iQue[®] Screener HD. For analysis, beads were gated on forward scatter height (FSC-H) vs. side scatter height (SSC-H), then doublets were excluded by gating on the singlet population on forward scatter height vs. forward scatter area (FSC-A). **Figure 4** shows representative data for the gating scheme and the decrease in bead fluorescence with three concentration response curves (0–333.3 μ M unlabeled GTP).

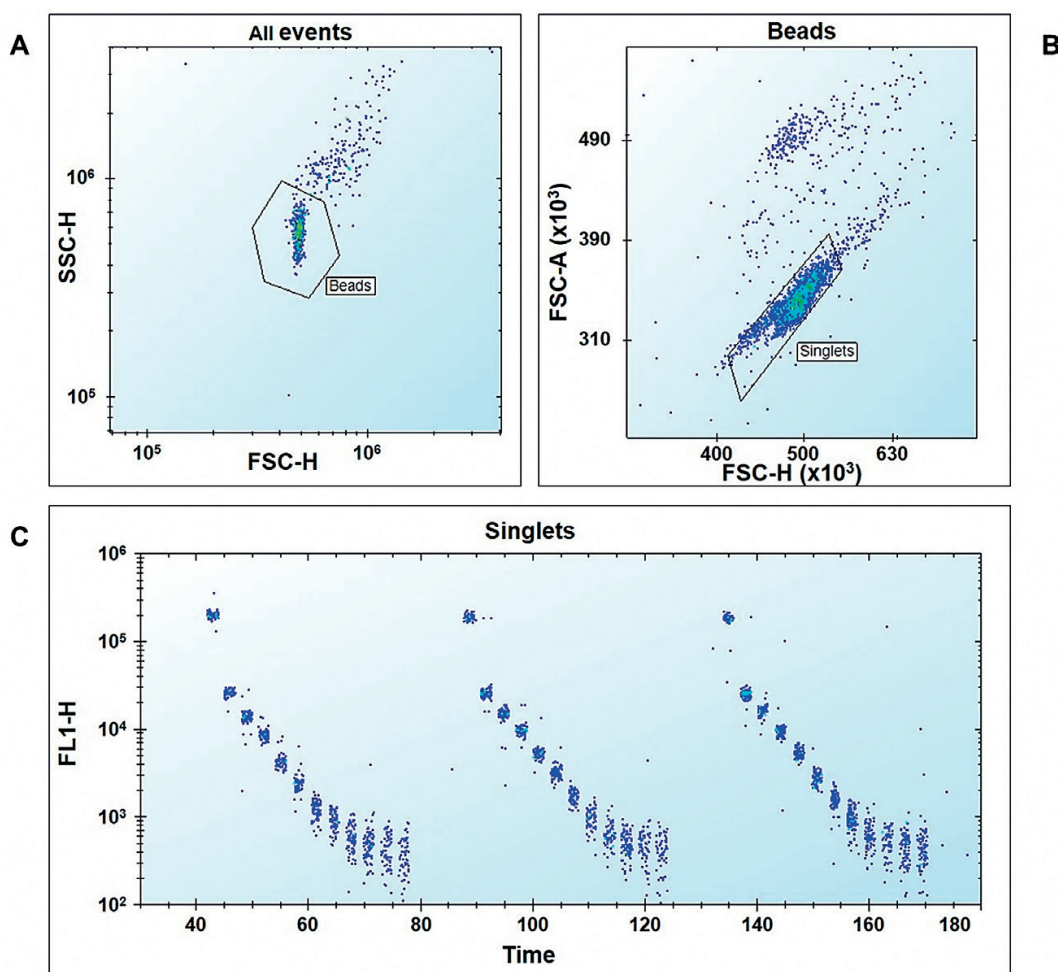


Figure 4. Gating scheme for Rac1 assay. (A) Beads gated on FSC-H versus SSC-H, (B) then on FSC-H versus FSC-A for singlet beads, (C) time-resolved serial dilution curves (time versus log(Median FL1-H fluorescence)) of singlet beads. Wells treated with 0–333.3 μ M unlabeled GTP show decreasing FL-GTP bound to beads (data in triplicate).

Addition of unlabeled GTP was able to compete with FL-GTP under all assay conditions in a concentration-dependent manner (**Figure 5**). Samples incubated at 4°C and read immediately afterward showed the highest binding of FL-GTP as well as the greatest decrease in fluorescence in the presence of unlabeled GTP. All other conditions showed a slight increase in FL-GTP intensity, but very similar competition curves. Assay Z' was greater than 0.75 for all conditions. These data in a final volume of 15 μ L demonstrated that the assay was adequate to move forward with miniaturization, so the assay was tested again in small volumes using 2 μ L of GST-Rac1 coated beads, 2 μ L of buffer containing unlabeled GTP in serial dilutions, and 2 μ L of FL-GTP for a total assay volume of 6 μ L in low volume 384 well plates. The miniaturized assay was comparable to the preliminary tests (data not shown) so we moved forward with miniaturization to 1536 well plates.

The challenge for us was to perform the assay in a 1536-well format in order to minimize both cost and timelines of the screen.

Miniaturization to 1536 well format was first performed by using blank GSH beads to test dispensing, inter-well shake speeds, and sip times. Runs were repeated under various conditions

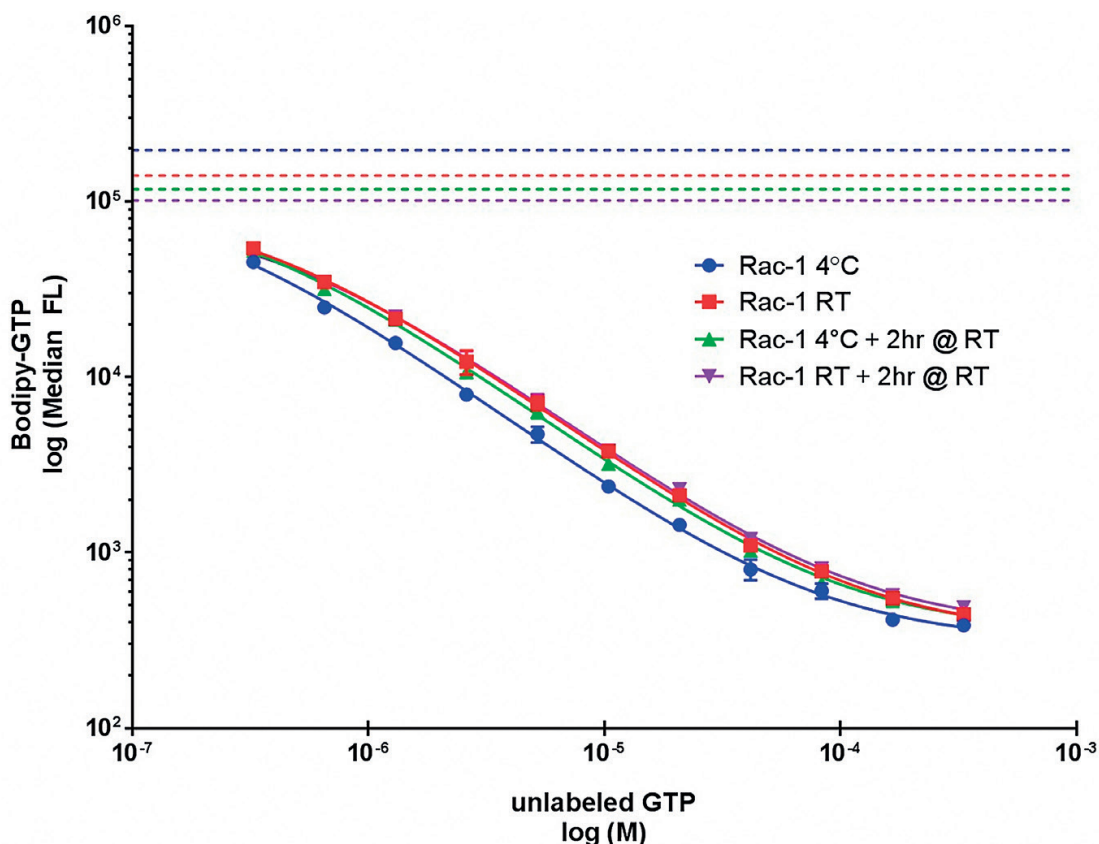


Figure 5. Comparison of incubation on competition of FL-GTP. Bead fluorescence measured by competition of FL-GTP by unlabeled GTP. GST-Rac1 coated beads with FL-GTP were incubated in the presence of varying amounts of unlabeled GTP in triplicate. Dotted lines indicate bead fluorescence in the presence of no unlabeled GTP. ●: incubated at 4°C for 30 min, ■: incubated at RT for 30 min, ▲: incubated at 4°C for 30 min then stored for 2 h at RT and ▼: incubated at RT for 30 min then stored for 2 h at RT.

to determine the optimum protocol. During these runs it was determined that it was best to use the bead sample buffer 0.1% (w/v) BSA in NP-HPS (30 mM HEPES pH 7.2, 100 mM KCl, 20 mM NaCl, 0.01% NP-40) supplemented with 1 mM DTT to prime the system before acquisition and during inter-well shaking. We found that we could decrease the sip time during which the sample probe aspirates from the well to 0.5 s per well, resulting in the acquisition of an entire 1536 well plate in under 50 min. Once the iQue® Screener HD protocol was optimized with blank GSH beads, we ran 3 separate plates with ½ of the plate with negative control (sample buffer) and ½ of the plate with positive control (unlabeled GTP). Briefly, the assay was performed by dispensing 2 µL of GST-Rac1 coated beads to each well of a 1536 well plate, followed by either 2 µL of sample buffer (negative control) or 2 µL of 500 µM unlabeled GTP (positive control; final concentration 166.7 µM). Finally, 2 µL FL-GTP was added to each well, then the plate was heat sealed with a pierceable foil seal and incubated at RT for 30 min. Each plate's Z' factor was calculated as greater than 0.5 with an average of 0.68 across the three plates (**Figure 6**).

We optimized the coating of Rac1 on the beads by testing the signal strength and assay window achieved over a 15 fold range of initial protein concentration from saturation down to zero (**Table 1**).

The aim was to identify the optimum concentration to use while maintaining best signal to background ratio. Following optimization i.e. check that the assay was suitable for running

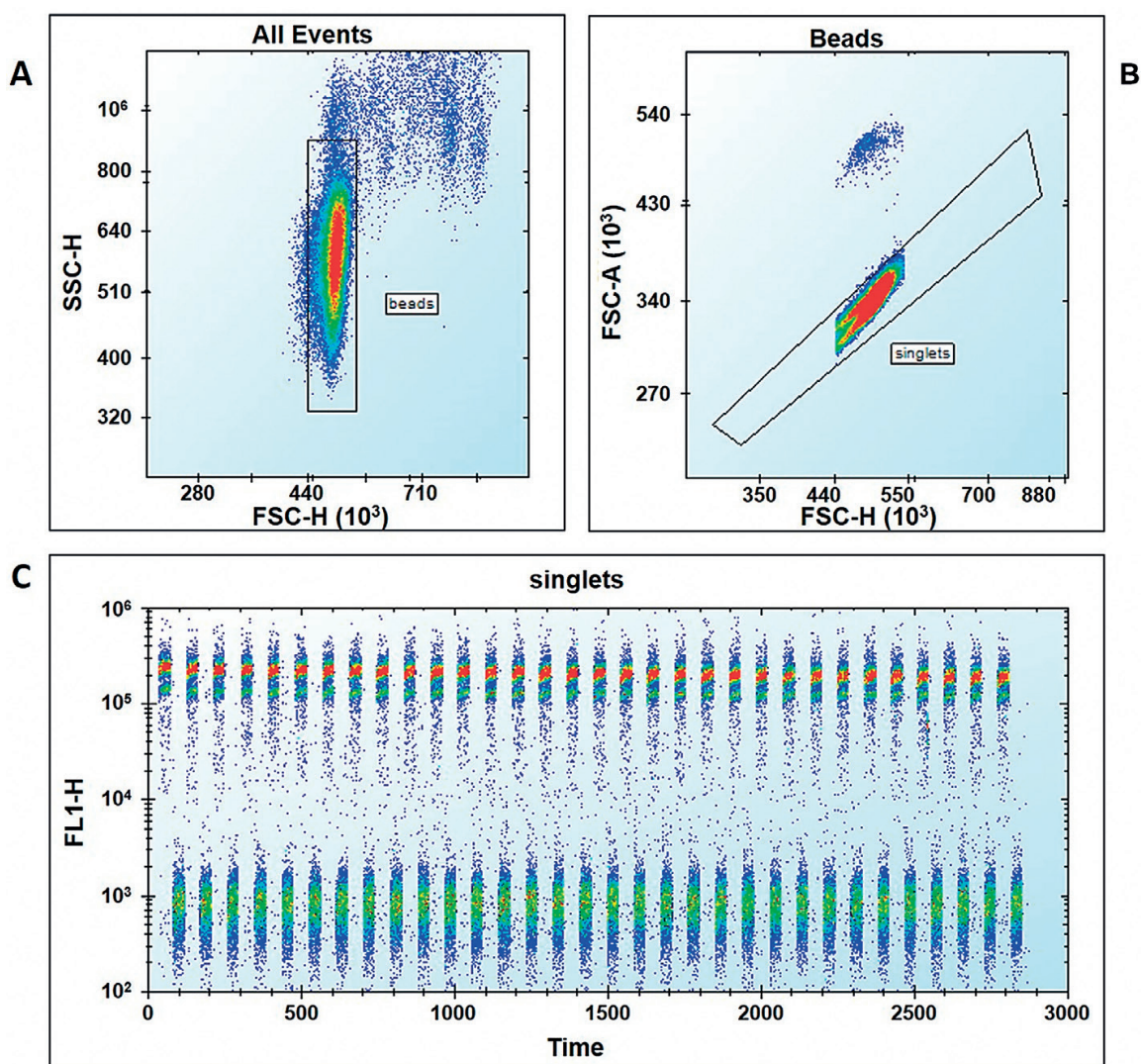


Figure 6. Representative data from 1536 well Rac1 assay. (A) Beads gated on FSC-H versus SSC-H, (B) then on FSC-H versus FSC-A for singlet beads, (C) time-resolved data (time versus log(Median FL1-H fluorescence)) of singlet beads. Wells treated with 0 or 166.7 μM unlabeled GTP show decreasing FL-GTP bound to beads.

Rac1 concentration (μM)	Max signal	Min signal	Z'	S/B
5	107,304	15,623	0.57	6.87
2.5	106,718	14,885	0.82	7.17
1.25	66,630	8559	0.79	7.79
0.625	39,836	5630	0.60	7.08
0.3175	21,087	3518	0.24	5.99

Table 1. Optimization of bead coating. Beads were coated with various concentrations of Rac1, incubated with FL-GTP and the bead fluorescence measured on the iQue[®]. Max refers to signal in the absence of 0.5 mM unlabeled GTP, min refers to signal in the presence of 0.5 mM unlabeled GTP.

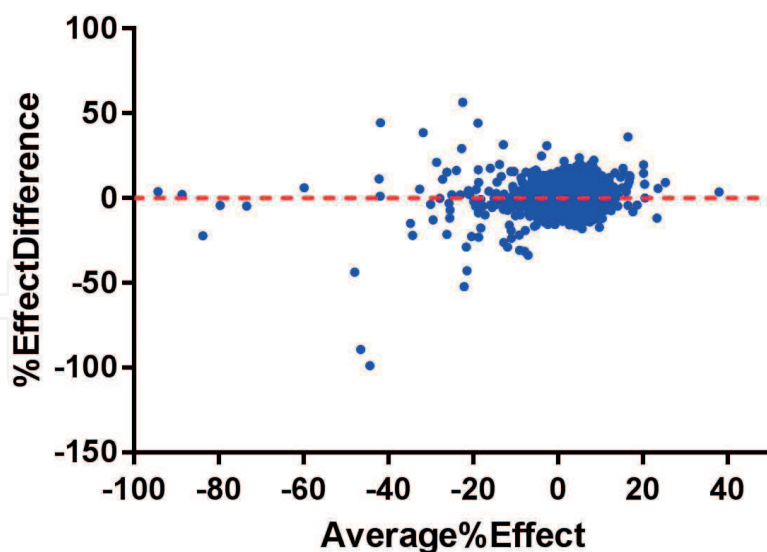


Figure 7. Bland-Altman plot of validation data.

at large scale, the assay was validated by running a set of 7000 core compounds and a set of 3000 low molecular weight compounds (LMW) (mol.wt. below 325) (see Section 3) on 2 separate occasions to assess variability. These compounds encompass the diversity of the collections that will be screened. Running an HTS campaign is a costly and fairly demanding process. For this reason we need to ensure an assay is robust (Z' factor greater than 0.5, signal to background greater than 3, low coefficient of variation across whole plate) and reproducible i.e. active compounds will be found repeatedly if the assay was run multiple times. We therefore defined a set of criteria we need to satisfy prior to embarking on a screen [14]. Core compounds were run at 12.5 μM and LMW at 100 μM . We use an in-house designed Tibco Spotfire[®] tool to analyze the data and visualize the repeatability of the assay. Validation showed the assay was performing well resulting in good agreement between the repeat runs as depicted in Figure 7 which shows a Bland-Altman plot comparing data from the 2 runs; the average difference is 0.3 and the standard deviation of the difference around 7%. We were confident that this assay was now amenable to full scale screening.

3. Screening

The iQue[®] Screener HD increases the capabilities of flow cytometry with rapid sampling and assay miniaturization with a final assay volume of 6 μL per well, using as little as 1 μL per sample with no dead volume. The iQue[®] Screener HD has an autosampler that delivers samples in an air gap delimited stream to the cytometer engine. The plate loading area is accessible to robotic integration and an easy to use interface allows integration of laboratory automation. The entire plate of data is processed at one time. ForeCyt[®] software that controls the iQue[®] Screener HD allows plate level annotation, analysis, and visualization of the acquired data, enabling rapid, high content, multiplexed analysis of cells and beads in suspension. To enable the throughput required, we have linked two iQue[®] Screener HD to an

ACell benchtop automation system (HighRes Biosolutions), as shown in **Figure 8**, comprising a Nanoserve plate carousel and an ambient nest integrated by the Cellario[®] scheduling software. This simple small footprint automation system allows unattended operation as well as automatically clean the cytometers at the end of each run and has a capacity of up to 28 plates per working day. We also used a third iQue[®] Screener HD as a standalone instrument for backup and additional throughput.

Prior to first use, a daily clean was run on the iQue[®] consisting of 5 min in decontamination solution (Intellicyt), a mildly basic solution, followed by 5 min in cleaning solution (Intellicyt), a mildly acidic and surfactant solution that neutralizes the decontamination solution and a final 10 min in water. This ensures cleaning of the entire fluidics path from probe to detector. Following this procedure a QC test was run to check the performance of the iQue instruments consisting of a 2 min water test to check cleanliness of the system followed by an 8 peak (Intellicyt) and 6 peak (Intellicyt) beads test to check laser alignment and performance of the 4 fluorescence channels. The 8 peak product contains a mixture of several similar size particles with 8 different fluorescence intensities. Every particle contains a mixture of fluorophores that enables their excitation with the blue laser (488 nm) to validate the FL1, FL2 and FL3 channels of the iQue. Similarly, the 6 peak product contains a mixture of particles with 6 different fluorescence intensities excitable by the red laser to validate the FL4 channel.

All compounds for HTS in AstraZeneca are delivered in what are known as assay ready plates. Assay ready plates have the correct volume of compound in them such that on addition of



Figure 8. Automation platform used with our iQue[®] Screener HD. (A) and (B) iQue[®] Screener HD, (C) ACell robotic arm, (D) NanoServe[™] stacker carousel.

the assay reagents the correct final compound concentration is achieved. Compound volumes are in the nL range and are added by a Labcyte Echo[®] acoustic dispenser. Using assay ready plates removes the need for intermediate dilution steps to get compounds and vehicle to the correct concentration making plate processing much simpler and faster in HTS screens.

Within the AstraZeneca HTS Centre we have 2 main collections of compounds that we screen. We have what we call the core collection that is screened at a single final concentration of 10 μM or a concentration close to this balancing solubility of the compounds with detecting weaker activity. We also have a low molecular weight collection [15] where we have separated out compounds with molecular weights below 325 Da that pass solubility filters. These compounds are screened at higher concentrations (typically 100 μM) to ensure we detect the activity of these compounds that potentially have better ligand efficiencies than hits that may be found in the core collection.

Rac1-coated beads were prepared by vigorously vortexing the glutathione beads, then transferring 7 mL into a 50 mL centrifuge tube. The beads were centrifuged ($1000 \times g$, 5 min) and the supernatant removed, taking care not to disturb the pellet. 7 mL of wash buffer (0.1% w/v BSA in NP-HPS) were added and vortexed vigorously to wash the beads. Beads were then incubated at room temperature for 30 min. Lyophilized Rac1 protein was reconstituted to 0.25 mg/mL final concentration ($\sim 5 \mu\text{M}$) by adding 25 μL of wash buffer and mixing gently until the protein is completely dissolved. After the 30 min incubation of the beads they were pelleted by centrifugation ($1000 \times g$, 5 min) and the supernatant discarded, taking care not to disturb the pellet. Beads were resuspended in 700 μL of wash buffer +175 μL of Rac1 protein to a final concentration of 1 μM . The sample was vortex mixed gently and incubated overnight at 4°C. After incubation at 4°C, beads were washed twice with 14 mL sample buffer (wash buffer +1 mM DTT) pelleted by centrifugation ($1000 \times g$, 5 mins) between washes and finally resuspended in 14 mL wash buffer. Labeled beads were stored at 4°C. An accurate bead count was made using the iQue[®] Screener HD with beads diluted to a density of 1.33×10^6 beads/mL prior to running the assay. The method described here should provide enough beads for 16 plates a day for 6 days.

The primary screen was performed at compound concentration of 12.5 μM (100 μM for LMW) against a diversity collection of 500,000 compounds. All assay ready plates of compounds were prepared by our compound management team. The plate layout included on-board controls in the four central columns (64 wells of either 12.5 μM unlabeled GTP or 1% (v/v) DMSO as inhibitor and neutral controls, respectively). Each remaining well contained a single compound from the library, resulting in single shot screening of 1408 compounds per plate.

To assemble the assay, the Rac1-coated beads were vigorously vortexed to mix and dispensed 3 μL /well to each well of the 1536 well assay ready plate (polypropylene deep well V-bottom microplates, Greiner Bio-One) using a Multidrop[™] dispenser and a small tube cassette. Assay ready plates for this assay had 7.5 nL (60 nL for low molecular weight compounds) of a 10 mM stock of compound dispensed to each well. 3 μL /well of 200 nM Bodipy-FL GTP in sample buffer was added and plates were centrifuged ($250\text{--}500 \times g$, 10 s) to ensure that samples are at the bottom of the plate and not adhered to the walls of the wells. Plates were sealed using a pierceable heat seal (recommend Thermo Cat No AB-1720 or equivalent) and incubated for 120 min

at room temperature shielded from light. Samples were acquired on the iQue® HD Screener using the “Rac1 template” acquisition and analysis template. Rac1 Sample Buffer (0.1% BSA in NP-HPS supplemented with 1 mM DTT) in the iQue HD Buffer station gave the best sampling results. The cytometer protocol was set up with 0.6 s sip time, 0 s up time, standard pump speed, and medium detector speed as can be seen in **Figure 9**. Reading time per plate is 45 min.

In the Intellicyt ForeCyt® software, we acquired flow cytometric light scatter as well as fluorescence emission in the FL1 channel (Ex 488 nm, Em 530 ± 30 nm). We gated on forward scatter and side scatter to identify the bead populations followed by forward scatter area vs. height to identify the singlet bead subpopulation. The median fluorescence of the singlet bead population was then extracted. Data files were exported from the ForeCyt® software as multiple plates in 1536 grid format as a comma-delimited text file. The files were manually imported into Genedata Screener® for analysis.

Data were normalized on a plate by plate basis using the on-board controls to calculate a % effect value and by robust Z Score. Robust Z Score is a derivation of the original Z Score calculation [16] substituting median for average and robust standard deviation for standard deviation and is used routinely for normalization and hit calling in the AstraZeneca HTS Centre alongside the more common 2-point normalizations using positive (inhibitor) and negative (neutral) control wells. By using robust statistical measures the calculation is resistant to outliers which are, of course, the hits in an HTS which can be missed if using the

Prepare

- Enable Automatic Prime
- Duration (s): 30
- Enable Pre-Plate Shake
- Duration (s): 30
- RPM: 5000
- Synchronize Shake Speeds

Sample

Sample Order:

- Sip Time (s): 0.6
- Additional Up Time (s): 0.0
- Pump Speed: Standard (15 RPM)
- Plate Model: Greiner 782261 1536 PP V bottom dec

Clean

- Enable Mid-Plate Clean
- After every 384 wells: 384

Station	Time (s)
Decon	3.0
Clean	3.0
Water	3.0
Buffer	3.0
Buffer	3.0

Shake

- No Shake
- Inter-well Shake
- Continuous Shake
- RPM: 5000
- Probe station: Buffer
- After every 24 well(s): 24
- Duration (s): 3

Flush and Clean

- Flush Duration (s): 60
- Enable Post-Plate Clean

Station	Time (s)
Decon	60
Clean	60
Water	120

Detector

- Speed: Medium
- Enable Clog Detection
- Thresholds:
 - FSC-H < 80000
 - None < 80000

Figure 9. ForeCyt® protocol on iQue® Screener HD.

non-robust version of the equation. Robust Z Score is calculated according to the equation below (Eq. (1)) and normalizes individual wells by calculating the number of robust standard deviations the well value is away for the median of the plate central reference. In most HTS screens this central reference is the median of all the compound wells on a plate as >99% of the wells will be inactive and will define the central reference.

$$N(x) = \frac{x - \langle CR \rangle}{\langle\langle CR \rangle\rangle} \quad (1)$$

x is the measured raw signal value of a well; $\langle CR \rangle$ is the median of the measured signal values for the central reference values on a plate; $\langle\langle CR \rangle\rangle$ is the robust standard deviation of the measured signal values for the central reference values on a plate.

Hits were identified using a cut-off of -10 in robust Z score; this corresponded to an approximate change of fluorescence greater than 30% (50% for LMW) from controls.

The primary screen of 500,000 compounds took 4 weeks to complete and some summaries of the data are presented in **Figure 10**.

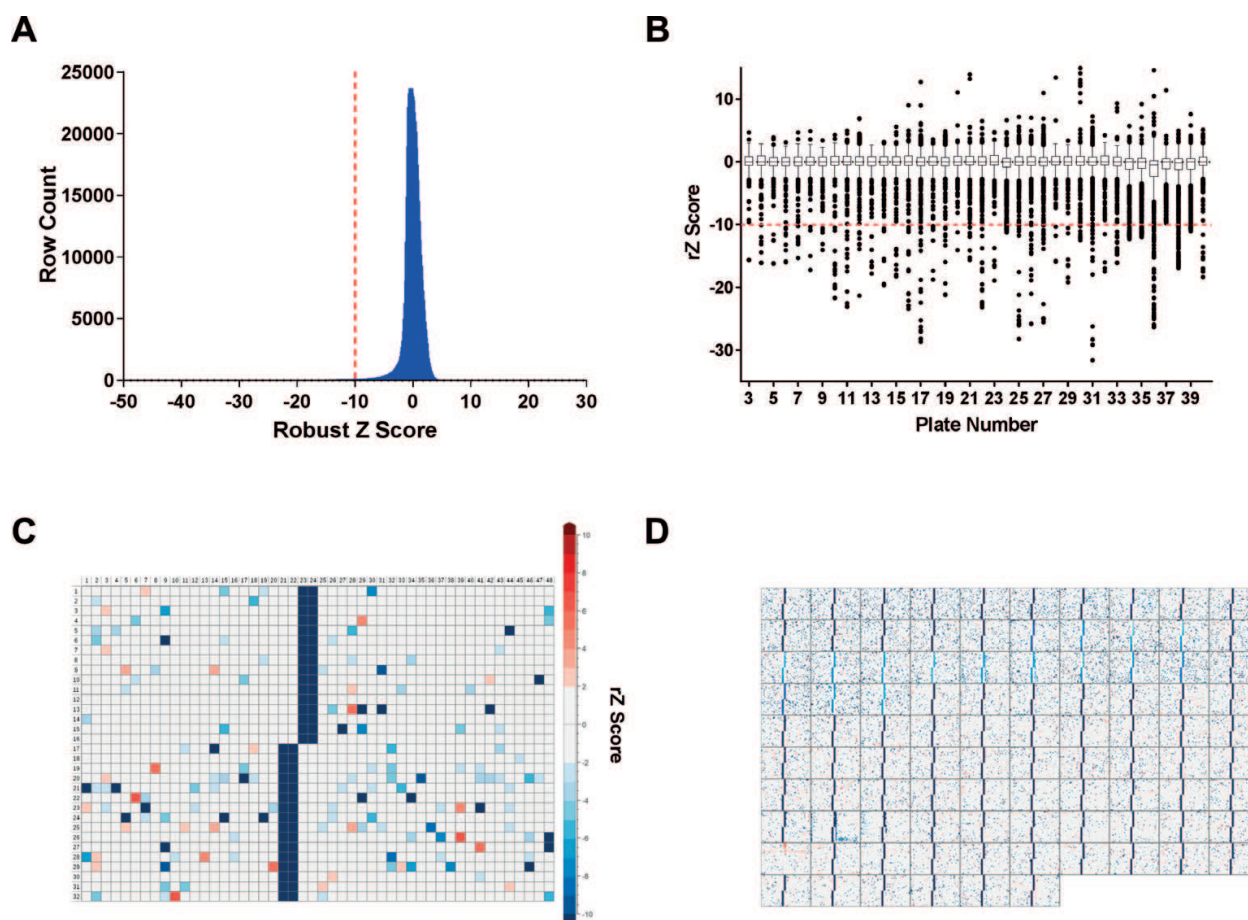


Figure 10. Primary screen data. (A) Distribution plot, (B) box plot, (C) plate view in Genedata Screener® and (D) assay overview in Genedata Screener®.

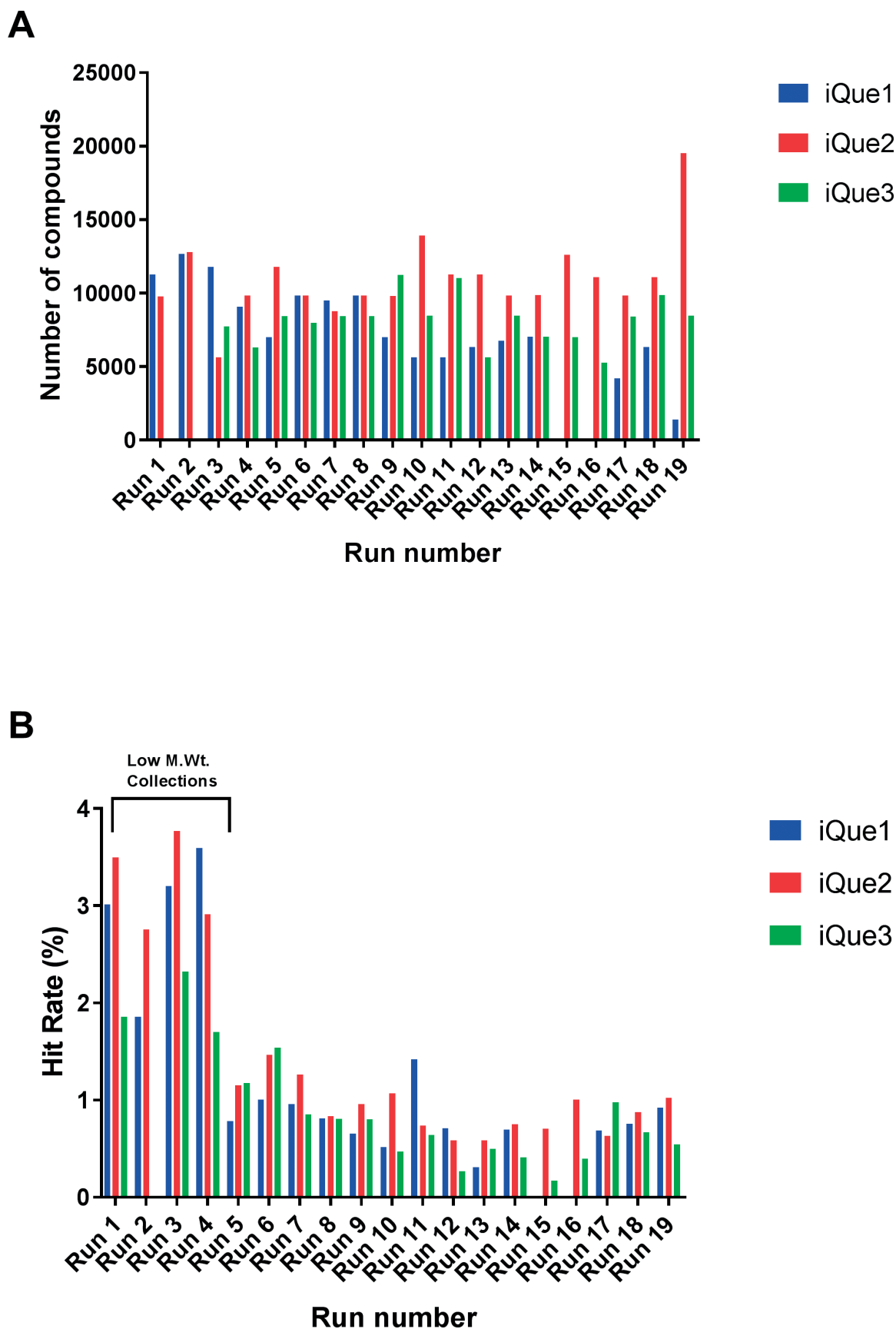


Figure 11. Primary screen data analysis plots. (A) Number of compounds analyzed per run and (B) hit rate per run.

The screen ran at a throughput of 20 plates per day equivalent to 31,000 compounds per day. The overall distribution in **Figure 10** shows a narrow central peak centered on zero with the left tail containing potential hits. This is a typical distribution shape seen in HTS screens and clearly shows that the assay was capable of identifying hits. The box plots shows the stability and consistency of the assay and tightness of the data across multiple plates and also allows visualization of the hits represented as the outliers on the plot. A typical plate analysis is shown depicting the controls in the central columns and a few hits scattered across the plate as dark blue wells with the histogram showing the heat map scale of the robust Z score. The assay overview (**Figure 11**) shows the whole set of plates (with the low molecular weight collection highlighted) run on one iQue® Screener HD highlighting the hit rate variation with the collection subset. It can be clearly seen that the hit rate in the low molecular weight set, found in the first 32 plates, is elevated above that of the core collections. This can occur due to the higher screening concentration leading to more non-specific inhibition due to common contaminants such as metals which are then ruled out by further downstream assays and SAR analysis. Although not shown, the assay signal to background as well as Z' factor [17] are monitored throughout the screen as markers of data quality and again indicated that this assay was of high quality.

5870 hits were identified after the primary screen representing a 1.2% overall hit rate. All hits were then tested as 10-point concentration responses (100–0.003 μM) against Rac1 as well as RhoA and CDC42 so we could assess selectivity of the compounds for Rac1. The assay is performed as described for the primary screen. All hits identified in the primary

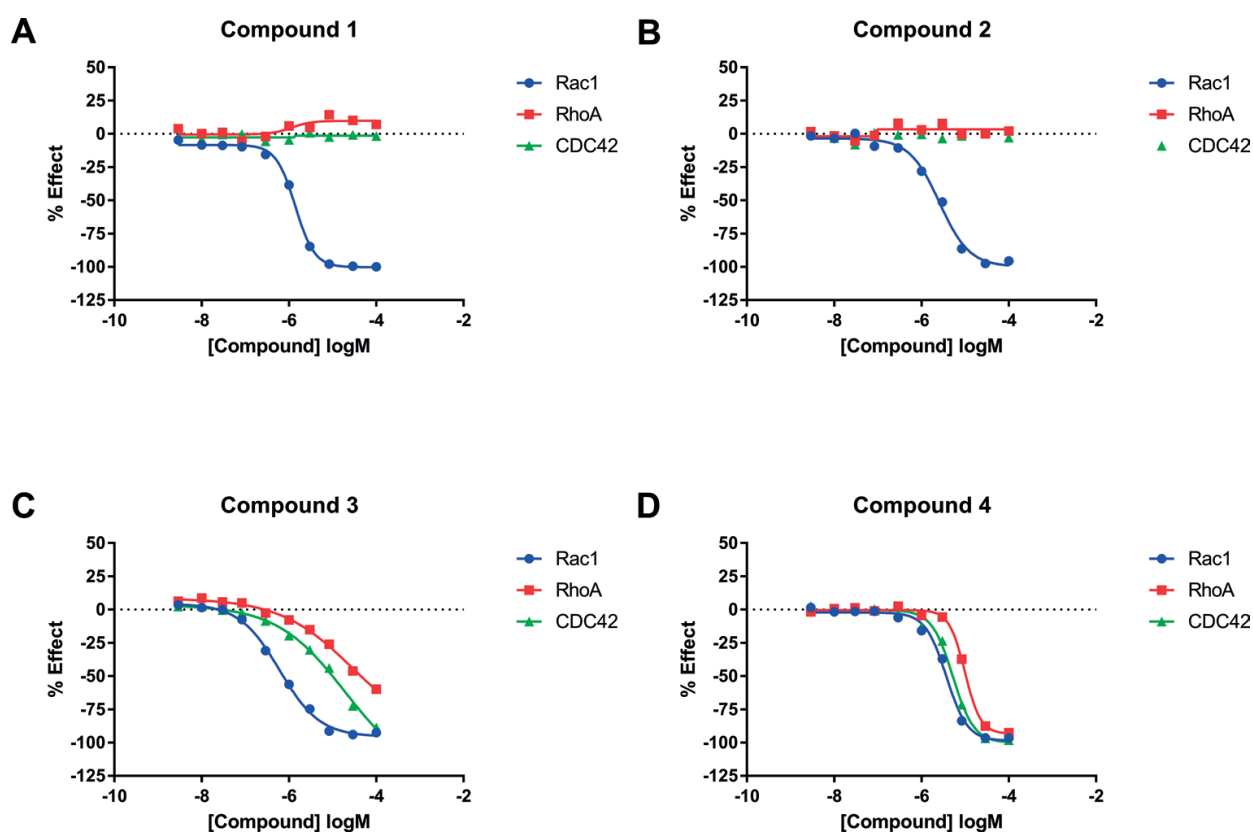


Figure 12. Representative curves of selective and non-selective compounds.

screen were serially diluted 1:3 to construct a 10-point concentration response curve. Compound concentration curves are dispensed using acoustic dispensing into assay ready plates at 150 compound curves per plate. Wells are backfilled with DMSO to keep the DMSO concentration constant in all wells. The plates were sealed and stored in the controlled atmosphere and temperature working plate store before use as for primary screen plates. 1 mM MgCl₂ was added to the buffer for the RhoA assay. 82% of the tested compounds were confirmed as active with 44 identified as Rac1 selective using a ≥ 10 fold ratio of Rac1 IC₅₀ vs. RhoA and Cdc42 as a cutoff. These compounds encompass a variety of chemical structures. **Figure 12** shows representative curves of selective and non-selective compounds. Selected hits are being further characterized through a panel of cellular assays by the team at INSERM.

4. Conclusions

Until recently flow cytometry was considered a powerful technology dedicated to bespoke assays where just a few samples were to be measured. No medium throughput let alone high throughput screen could be run on this technology due to lack of plate handling features, time needed per sample and therefore cellular imaging was the preferred method for any high content screen. Increases of interest in phenotypic screening combined with a desire to access relevant cell models, often suspension cells, and development of plate-based sampling flow cytometers have led to the opportunity to use flow cytometry as a drug screening technology platform. The ability to perform multiplexing where different cells or particles labeled with distinct fluorophores are analyzed in parallel and multiple endpoints are measured leads to in-depth analysis of subpopulations within a sample. Such an approach results in increased productivity by decreasing timelines and cell requirements, two critical parameters for high throughput screening.

We chose to run a screen for Rac1 inhibitors on our iQue® Screener HD as it enabled us to miniaturize and run a simple and robust assay on one of our automation platforms.

We have run a successful 500,000 compound screen using the iQue® HD screener as summarized in **Figure 13**. By optimizing the protocol, we were able to retain high-quality data while decreasing read time per 1536 well plate to under 50 min. This allowed us to run the primary screen within 4 weeks. Although time is not the most critical criteria we consider it remains an important factor as we need to maintain the overall flow of projects through our portfolio and therefore we must decide on a suitable time frame for completion of each screen.

High throughput screening is a key method for the identification of hit and lead compounds and remains at the start of most of our drug discovery programs. As we pursue a wide range of targets we also use a wide range of assays and technologies. We have invested into several iQue® Screener systems as we updated and replaced previous detection systems and to fill a capability gap for screening suspension cells or primary cells in small sample volumes. We have successfully used the iQue® Screener systems in a variety of applications from phenotypic screening to more complex multiplex profiling. The technology has enabled several projects due to the ability to reduce cell requirement, cost and timelines.

Screen Summary

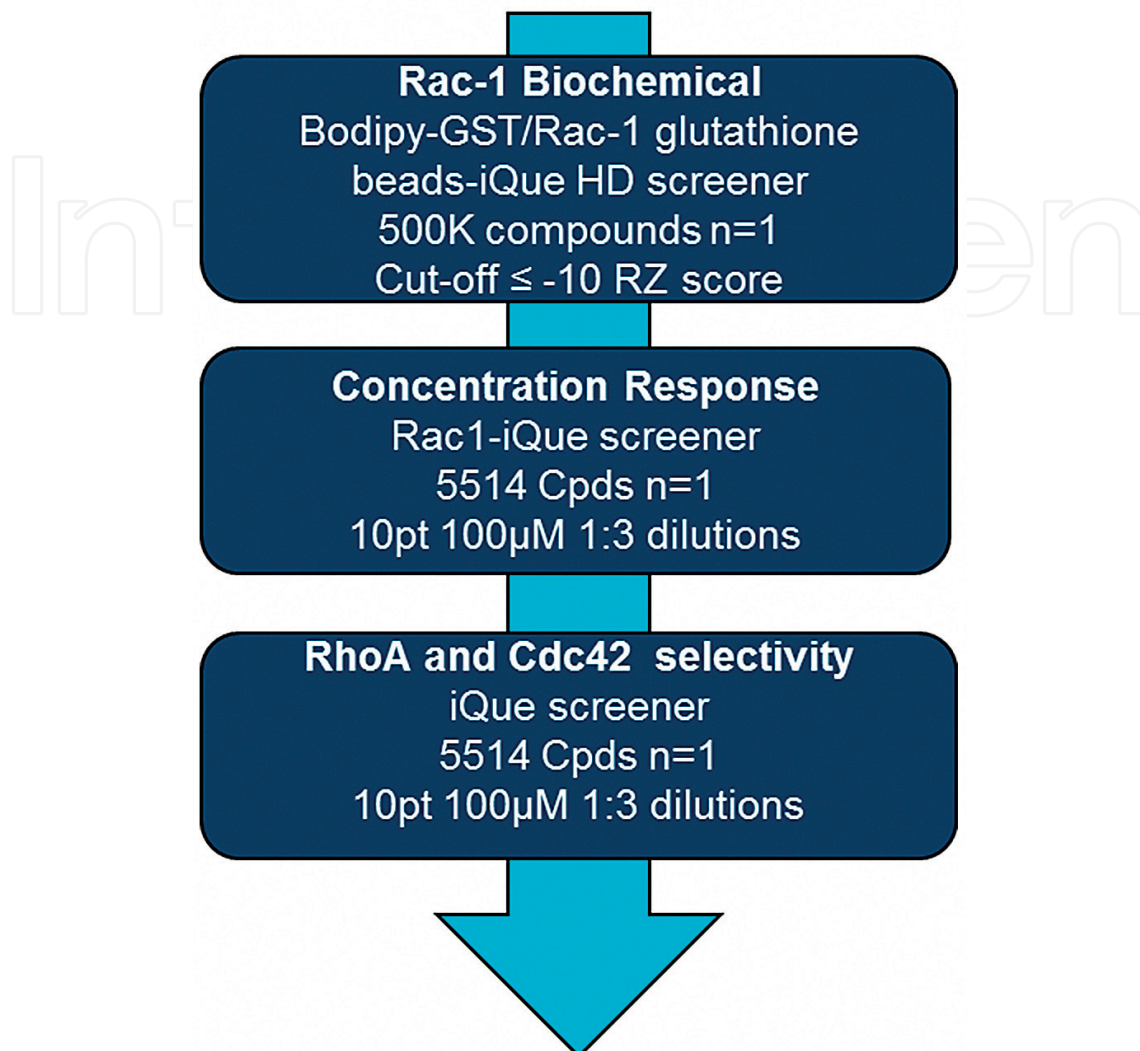


Figure 13. Screen summary.

The screen we have described here would have not been possible a few years ago but, thanks to the development of high throughput capable instruments, flow cytometry has found its place within the drug discovery process and high throughput screening in AstraZeneca. Although we decided not to multiplex our concentration response assays, it should be noted that the use of flow cytometry technology does allow for such multiplexing and indeed has been described in the literature [12].

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