Automation of Full-Kinome Profiling Using a Continuous Kinase Activity Platform

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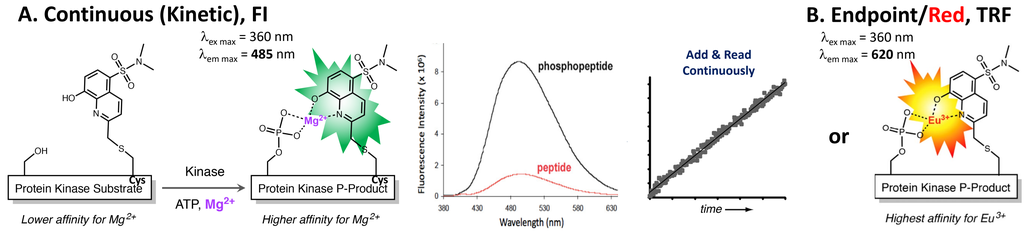
ABSTRACT

Automated high throughput assessment of the kinase activity of lead compounds for optimization and selectivity assessment is essential for developing successful drug candidates. The typical high-throughput screen involves a primary assay of a huge number of compounds against a single target to determine lead hits that are then characterized further in selectivity and potency assays to validate and prioritize these hits as part of a drug discovery cascade. The primary assay and even the follow-up assays are typically run as an end point format to reduce the complexity. Determining the full-kinome selectivity profile for kinase inhibitors is a common need, but this can be very challenging since this requires the screening of lead compounds against hundreds of kinases run at different concentrations, with varying stability and that require different substrates or additives, as part of specific assay conditions including measuring inhibition at the ATP Km concentration for each kinase in the panel.

We will present the development of a semi-automated assay and then to fully automated assay workflow to address the complexity encountered with selectivity profiling. Using the PhosphoSens® continuous assay format for profiling provides a more robust and informative readout of the target biology (detection of any enzyme lag or instability) and inhibitor function (determination of initial and final reaction rates provides a more accurate assessment of inhibition and allows detection of time-dependent inhibition). This involves the automation of three types of samples: enzymes, substrates, compounds, and their individual properties and processing needs. It also involves new process development and the automation of unique liquid handling. New techniques for stability and solubility of the samples and reagents were paramount to achieve consistent and highly reproducible results. We will present data on full-kinase profiles of known specific and non-specific inhibitors validating the continuous assay format as well as the fully automated process.

THE PHOSPHOSENS® ASSAY – CONTINUOUS & ENDPOINT/RED FORMATS

A. Continuous (Kinetic), FI



B. Endpoint/Red, TRF

Peptide sequences are synthesized using solid-phase methods with the Sox fluorophore coupled through the sulfhydryl group of a cysteine residue proximal to a protein kinase phosphorylation site, such as a tyrosine, serine or threonine. Upon addition of a kinase, the peptide is phosphorylated. In the presence of magnesium ion, a chelation complex is formed with the phosphate group, resulting in fluorescence enhancement of the Sox fluorophore that can be **monitored continuously** as fluorescence intensity **(A)**. Kinase inhibitors prevent phosphorylation and thus fluorescence. At any point, Europium ion can be added, to displace the magnesium ion, resulting in a long wavelength, time-resolved fluorescence (TRF) **endpoint/Red format (B)** that is useful for high-throughput or structure activity relationship (SAR) applications.

THE ASSAY

MANUAL

Reaction Conditions:
54 mM HEPES, pH 7.5
1 mM ATP
1 mM AMP [4]
1 µM t-RNA [6]
1X DNA-PK Activator [7]
1.2 mM DTT [9]
0.012% Brij-35
0.5% glycerol
0.2 mg/ml BSA
0.52 mM EGTA [2]
10 mM $MgCl_2$ or 20 $MgCl_2$ [5]
10 mM KCl [1] or 50 mM KCl [8]
140 µM Phosphatidylserine [3]
3.8 µM Diacylglycerol [3]
0.3 mM $CaCl_2$ [3]
10 or 15 µM Sox-based substrates
1% DMSO
N µM compound, or 1% DMSO
Kinase as indicated

Reaction Set Up:
2 µL 10X Sox-based substrate
2 µL DMSO (10%) or Compounds (C µM in 10% DMSO)
12 µL Reaction Mix with ATP & DTT [9]

30-minute pre-equilibrate at RT
4 µL 5X Enzyme dilution buffer or 5X Kinase in EDB
OR if needed depending on kinase
- **2 µL** 10X PS/DAG/Ca [3]
- **2 µL** 10X AMP [4]
- **2 µL** 10X $MgCl_2$ [5]
- **2 µL** 10X t-RNA [6]
- **2 µL** 10X DNA [7]

And
2 µL 5X Enzyme dilution buffer (EDB) added to all odd numbered rows and 10X Kinase in EDB added to all even numbered rows

20 µL Final reaction volume run at 30˚C for 120 minutes.

Notes:
- Enzyme Dilution Buffer (EDB): 20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 1 mM DTT [9] 1 mg/ml Bovine Serum Albumin is added to all odd numbered rows.
- Reactions were run in PerkinElmer, ProxiPlate-384 Plus, white shallow well microplates (Cat. #6008280) after sealing using optically-clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185]) in a Biotek Synergy Neo 2 microplate reader with excitation (360 nm) and emission (485 nm) wavelengths.
- **Special Conditions:** [1] Only for PDHK1-4 , [2] No EGTA for CaMK Family or PKC Family, [3] Only for PKC Family, [4] Only for AMPK Family , [5] Only for RIPK1,2,3, & S , [6] for GCN2, [7] for DNA-PK , [8] for BCKDK , [9] no DTT for MAP3K6 (ASK2)

SEMI-AUTOMATED

Reaction Conditions:
54 mM HEPES, pH 7.5
1 mM ATP
1.2 mM DTT [9]
0.012% Brij-35
0.5% glycerol
0.2 mg/ml BSA
0.5 mM EGTA [2]
10 mM $MgCl_2$ or 20 $MgCl_2$ [5]
10 or 15 µM Sox-based substrates
1% DMSO
N µM compound, or 1% DMSO
Kinase as indicated

Reaction Set Up:
2 µL 10X Sox-based substrate
2 µL DMSO (10%) or Compounds (C µM in 10% DMSO)
2 µL Water Or 10X Additive (Plate based and kinase dependent):
- 10 mM KCl [1]
- 5 ng/uL of Calmodulin [2]
- 0.4 mM CaCl2 [2]
- 140 µM Phosphatidylserine [3]
- 3.8 µM Diacylglycerol [3]
- 0.3 mM $CaCl_2$ [3]
- 1 mM AMP [4]
- 20 mM $MgCl_2$ [5]
- 1 µM t-RNA [6]
- 1X Cytiva DNA (Calf Thymus), activated (Cat#:/Lot#: 27-4575-01/01194329)
- 50 mM KCl [8]

10 µL Reaction Mix with ATP & DTT [9] or if needed:
- Without EGTA [2]
- Or Without DTT [9]

5-minute pre-equilibrate at 30˚C
4 µL 5X Enzyme dilution buffer (EDB) added to all odd numbered rows and 10X Kinase in EDB added to all even numbered rows

20 µL Final reaction volume run at 30˚C for 120 minutes.

Notes:
- Enzyme Dilution Buffer (EDB): 20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 1 mM DTT [9] 1 mg/ml Bovine Serum Albumin is added to all odd numbered rows.
- Reactions were run in Corning, low volume 384-well, white flat round bottom polystyrene NBS microplates (Cat. #3824) after sealing using optically-clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185]) in a Biotek Synergy Neo 2 microplate reader with excitation (360 nm) and emission (485 nm) wavelengths.
- **Special Conditions:** [1] Only for PDHK1-4 , [2] No EGTA for CaMK Family or PKC Family, [3] Only for PKC Family, [4] Only for AMPK Family , [5] Only for RIPK1,2,3, & S , [6] for GCN2, [7] for DNA-PK , [8] for BCKDK , [9] no DTT for MAP3K6 (ASK2)

Fig-1

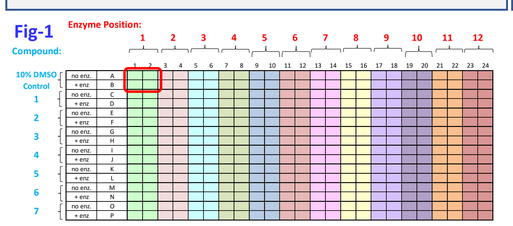


Fig-2

The red squares above highlight 4 wells with 1 compound tested in two conditions in duplicate.

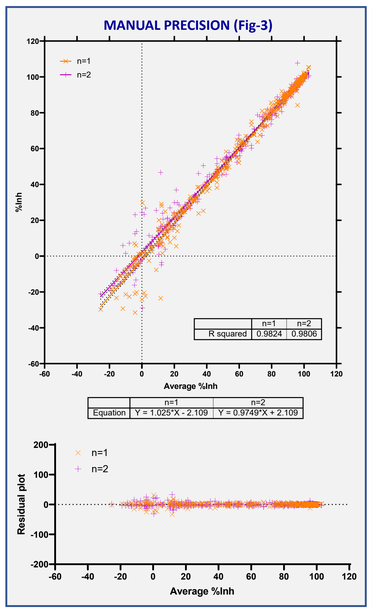
The differences between manual and semi-automated:
- Manual assay was run in 384-w plates from strips of micro-centrifuge vials. **Fig-1** shows the plate layout.
- Semi-automated assay took advantage of identical conditions of the quadrants of the 384-w plate except for the final addition of kinase and dilution buffer. An intermediate 96-w plate was used to combine the components of the assay including the compounds and then compressed into the 384-w assay plate. The final addition is then done from 96-w kinase and buffer plates into the assay plate to start the reaction. **Fig-2** shows a progress curve in every well. Odd rows are blanks with only EDB added and even rows are reactions with enzyme.
- The pre-equilibrate step was reduced from 30-min to 5-min and temperature increase from RT to 30°C. This helped reduce the noise at the start of the reaction.
- The ProxiPlate Plus (10 - 20 µL working volume) was replaced with Corning (5 - 40 µL working volume) improving the accuracy of the assay (final volume 20 µL) by reducing liquid overflow and sealing issues.

VALIDATION

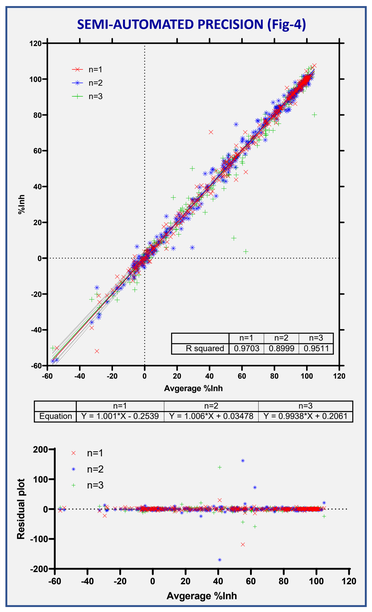
The validation method involved using a full Kinome Profile with ~ 380 Wild-Type Kinases in 32 x 384-w assay plates. Staurosporine was used as a control at 1 µM final concentration. The manual assay was run directly into PE 384-w assay plates and the semi-automated assay was run using Biomek i7 Hybrid.

The method was run in a 96-w intermediate plate up to the Kinase addition step. Then the plate was compressed into a Corning 384-w assay plate and kinase was added to start the reaction. Each 4 wells (for example A1,A2,B1,B2) have the same compound. See **Fig-1** and **Fig-2**. The manual assay was run N=2 and the semi-automated was run N=3. Both assays were read for 120 minutes in a kinetic continuous assay to monitor enzyme activity. The reading was done in Synergy Neo2 reader by BioTek Agilent Technologies

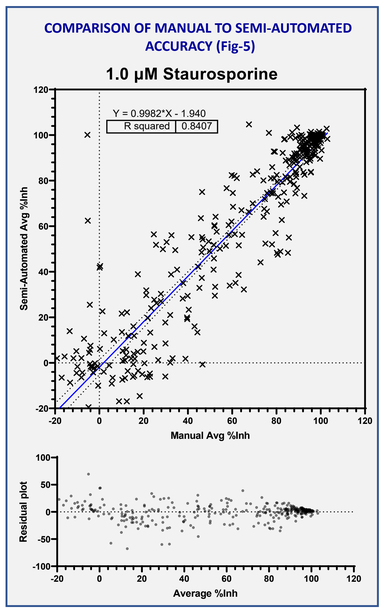
MANUAL PRECISION (Fig-3)



SEMI-AUTOMATED PRECISION (Fig-4)



COMPARISON OF MANUAL TO SEMI-AUTOMATED ACCURACY (Fig-5)



Staurosporine will inhibit more than 200 of the ~380 tested wild-type kinases. % Inhibition was calculated and plotted in **Fig-3** with average % Inh of N1 and N2 in the X-axis and both N1 & N2 in the Y-axis. The residual plot of both % Inh is below that. The data clearly show that manually, the kinome profiling assay was precise. The same was done to the % Inh data generated by the semi-automated system. **Fig-4** showing average % Inh of N1, N2, & N3 and all replicates in the Y-axis. The residual plot of all % Inh replicates is below that. The semi-automated system is as precise as the manual run. Finally, the average % Inh of the manual run and the % Inh of the semi-automated run in **Fig-5** show a very good correlation between the two systems, demonstrating the accuracy of the semi-automated system. All replicates were run on separate days.

IMPROVED CURRENT THROUGHPUT AND FUTURE THROUGHPUT

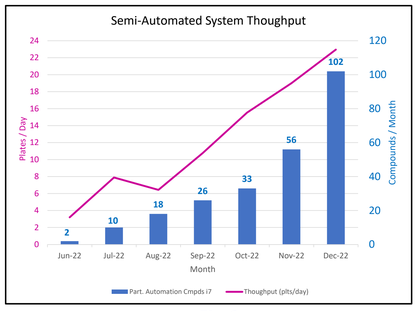


Fig-6

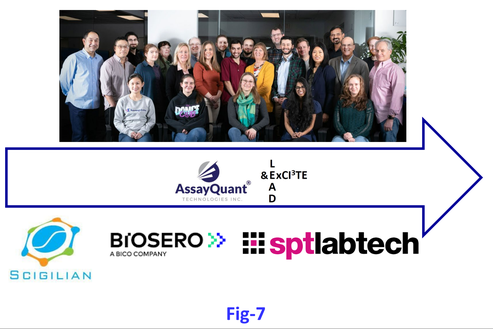


Fig-7

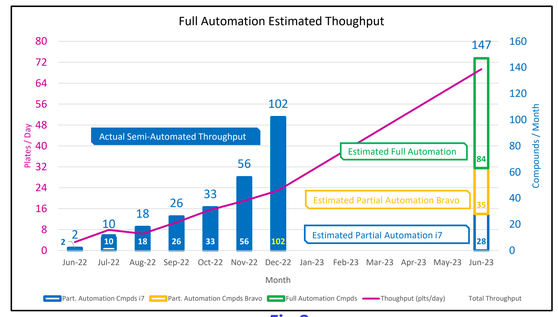
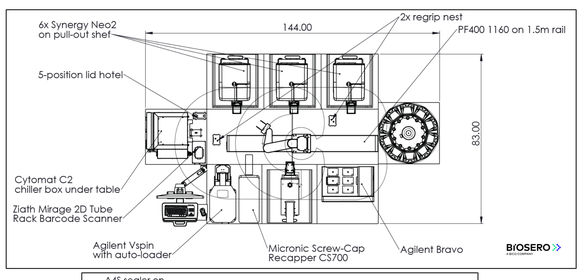
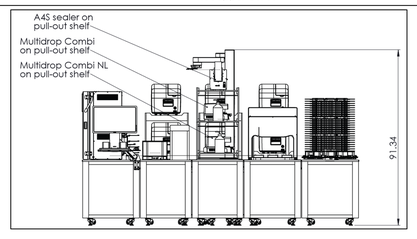


Fig-8

FULL AUTOMATION





Our current semi-automated capacity of 84 compounds/month (or an average of 20 plates/day) has been established by the automation team. While **Fig-6** shows that we have completed a record 102 compounds in December 2022, that is only sustainable for the short term. The goal is also to provide full kinome results in 3 days which will require a multifaceted approach.

Fig-7 Shows three improvements to enable full automation:
1. Sample management and data analysis software provided by Scigilian's Store and Analyze.
2. Automated storage and retrieval managed by SPT Labtech's arktic® -80˚C freezer.
3. Automated profiling run on Biosero's fully automated system with GBG dynamic scheduler.

➢ But most important of all are the people at AssayQuant Technologies managing the automation.

The theoretical throughput of 1 fully automated system assuming 3 assay plates/hour (due to liquid handling limitation) is 260 compounds/month (60 assay plates/day). In order to achieve the estimated throughput of ~150 compounds/month in June 2023 as shown in **Fig-8**, we need to be able to run ~35 assay plates/day. To sustain this throughput, we need to run under 75% capacity and invest in redundancy and backup processes. The system also need to be scalable.

Investing in multiple fully automated systems, additional automated storage and retrieval capacity, more efficient processes that adhere with Lean Six Sigma principles, and highly skilled people will help the automation team meet AssayQuant Technologies projected growth. Additionally, investigating in new technologies like acoustic dispensing, 1536-w plate formats, assay miniaturization, and faster fluorescence readers, will help meet future needs in Kinase Profiling.

SUMMARY

- ✓ The information rich Full-Kinome Profiling assay was successfully converted to a semi-automated system including improved sample management and assay analysis. The semi-automated system and new assay was validated by using a promiscuous standard, Staurosporine, and comparing multiple manual runs to multiple semi-automated runs.
- ✓ The manual assay was modified to allow for better and more efficient processing and automation.
- ✓ The manual throughout was increased from 14 to 84 compounds/month by using semi-automation and will be increased further by using full automation.
- ✓ Redundancy in automation, efficiency in processing, and adapting new technologies will improve the chances of meeting projected AssayQuant Technologies growth.
- ✓ Most importantly, having the team's dedication and support at all levels helped achieve our automation goals.

Full Kinome Profile of Staurosporine at 1 µM final concentration.