Luminescent Assay Optimization, Kinase Profiling, and uHTS Application

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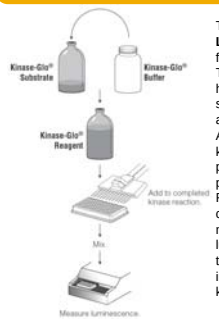
1. Abstract

Kinases play a crucial role in regulating complex cellular processes. Their involvement in a number of diseases makes them a target class of interest for the pharmaceutical and biotechnology industries. For this reason, kinases are the most screened target class to date.

Successful kinase screening campaigns require precise liquid handling, robust assays, reliable plates, and sensitive detection methods. With the increasing number of options available to the screener, it is becoming more of a challenge to choose which are best suited for a particular application. By partnering together, we demonstrate the complementarity of diverse technologies for a 1536-well kinase assay application.

Using a luminescent kinase technology platform from Promega, we demonstrate the optimization of kinase assays with the Deerac Fluidics™ Equator™ dispenser, using assay plates from Corning® and with luminescent detection by BMG LABTECH's PHERAstar. Following assay optimization, the Labcyte® Echo® 555 liquid handler is used for ultra low-volume compound dispensing for screening applications. Data show that miniaturization does not compromise quality when lower volume assays are required.

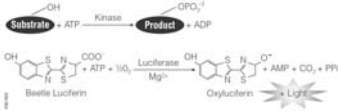
2. Luminescent Assay



The **Promega Kinase-Glo® Luminescent Kinase Assay** was used for all experiments shown in this poster. The Kinase-Glo platform provides a homogeneous, high-throughput screening method for measuring kinase activity by quantitating the amount of ATP remaining in solution following a kinase reaction. Kinase-Glo Assays are performed in a single well of a multiwell plate by adding a volume of Kinase-Glo Reagent equal to the volume of a completed kinase reaction and measuring luminescence. The luminescent signal is correlated with the amount of ATP present and is inversely correlated with the amount of kinase activity.

Advantages to using the Kinase-Glo Assay in this study include:

- Use higher ATP concentrations: Linear response up to 500 µM ATP
- Use any kinase and kinase-substrate combination, including peptide, protein, lipid and sugar substrates
- No substrate modifications required
- Batch plate-processing capable: Highly stable luminescent signal, with over 50% signal remaining at 5 hours
- Distinguish between ATP competitive and noncompetitive kinase inhibitors
- Quickly screen large numbers of library compounds
- Homogeneous, non-radioactive reagents
- Reliable, reproducible data: Z´-factor values routinely >0.7



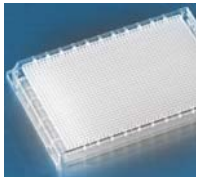
The kinase reaction is conducted under the appropriate conditions. ATP remaining at the time that the reagent is added is used as a substrate by the Ultra-Glo™ Luciferase to catalyze the mono-oxygenation of luciferin. The luciferase reaction produces one photon of light per turnover. Luminescence is inversely related to kinase activity.

3. Instrumentation

Corning 1536-well Microplates were used in the experiments described in this poster. Two specific plate types utilized were polystyrene white assay plates and cyclic olefin copolymer Echo-qualified clear source plates.

Advantages to using Corning microplates for this study include:

- Superior flatness to minimize dispensing variability
- Suitability for use with automated dispensers
- Ideal for homogeneous assays
- Working well volumes up to 10 µL



The **Deerac Fluidics Equator** reagent dispenser was used to dispense Kinase-Glo reagents in the experiments described in this poster. The Equator uses an 8-channel pipetting system to dispense liquids ranging in volumes from 50 nL to 50 µL. Reagents are washed thoroughly from the system between dispensing steps via the Active Wash Station.

Advantages to using the Equator for this study include:

- Speed and accuracy
- Low dead volumes
- Ease of use
- Independent channel control for dispensing regions and gradients

The **BMG LABTECH PHERAstar** was used to capture all luminescence (RLU) data in this poster. The PHERAstar is a multi-detection HTS microplate reader for non-isotopic detection technologies. This standard reader has five photomultiplier tubes optimized for specific reading modes and is capable of simultaneous dual emission in all modes.

Advantages to using the PHERAstar for this study include:

- Fast reading speed
- Auto Z-height focal adjustment
- Low background signal
- Dynamic range

The **Labcyte Echo 555** acoustic liquid handler was used to dispense library compounds and prepare 16-point IC_{50} curves. The Echo 555 uses acoustic energy to transfer droplets of compounds in 2.5 nL increments directly from source plates into 1536-well assay plates. Direct transfer creates concentration curves with high accuracy and zero contamination.

Advantages to using the Echo 555 for this study include:

- High precision and accuracy (< 8% CV and <10% error)
- Minimal compound consumption (2.5 - 50 nL per assay well)
- DMSO backfill option for IC_{50} curves

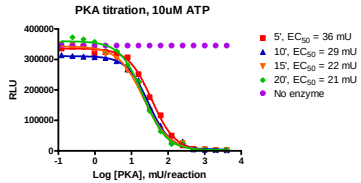
4. Kinase Selection & Assay Optimization

Kinase	Substrate
PKA	Kemptide
Src	Peptide 2
MAPKAP K2	MAPKAP
PI3K	L-A-phosphatidylinositol ammonium

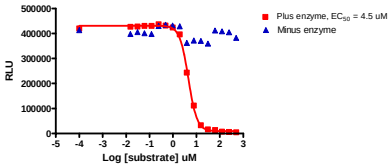
Compound	Target Kinase
Staurosporine	Universal kinase inhibitor
H 89	PKA
PKI	PKA
Rottlerin	MAPKAP K2
Wortmannin	PI3K
LY 294002	PI3K
Quercetin	PI3K
PP2	Src
SU 6656	Src
Src inhibitor #1	Src

Kinases used in this profiling study (shown above) were chosen for the roles they each play in a variety of disease states. PKA was selected as a control and focus of this poster for its ease-of-use in automation and characterization in previous automation experiments. Known kinase inhibitor compounds (shown above right) were also used in this profiling application.

Reaction Time & Kinase Concentration optimization experiments were performed. Kinase concentration was varied in a 1:2 titration as well as reaction stop time via the addition of the Kinase-Glo Plus reagent at 5, 10, 15, and 20 minutes. Optimal reaction time was selected to be the time point in which EC_{50} no longer changed. Optimal kinase concentration was determined to be the smallest amount of enzyme needed to give the largest dynamic range with the no enzyme control. For PKA, optimal reaction time was 15 minutes and optimal kinase concentration was 130mU per reaction.



Substrate Concentration optimization experiments were also performed. Substrate concentration was varied in a 1:2 titration. Optimal substrate concentration was determined to be the smallest amount of substrate needed to give the largest dynamic range with the no enzyme control. Optimal kinase amount and reaction time from the previous experiment were used in determining optimal substrate concentration. For PKA, optimal substrate concentration was 30µM kemptide.



Z'-Factor Analysis was performed using the Kinase-Glo Plus reagent. Z'-factor values were determined for each kinase assay at optimal reaction conditions. Z'-factor of 0.5 or greater is considered an excellent assay. All assays were above 0.5.

Enzyme	Z'-factor
PKA	0.84
MAPKAP K2	0.85
PI3K	0.84
Src	0.78

10. Kinase Profiling – Selectivity and Potency

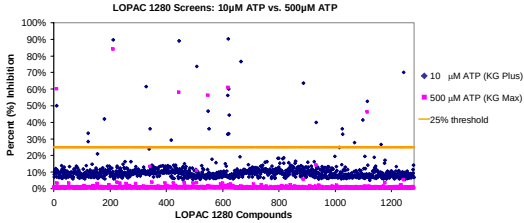
Selectivity and Potency Profiles were assessed by performing 16-pt dose response curves. Compound concentration was varied in a 1:2 titration (100 µM to 0.01 nM). Results indicate the potency and selectivity of each compound against the kinase panel. Light blue combinations represent inhibition (IC_{50}) <1µM, while yellow combinations represent inhibition >1 µM, no effect, or IC_{50} >100 µM (some inhibition noted at higher doses).

Compound	PKA	Src	MAPKAP K2	PI3K
Staurosporine	23 nM	22 nM	12 µM	> 100 µM
PKI	27 nM	> 100 µM	No effect	No effect
H 89	186 nM	510 nM	> 100 µM	No effect
Rottlerin	1 µM	3 µM	14 µM	> 100 µM
Wortmannin	37 µM	> 100 µM	No effect	14 nM
LY 294002	19 µM	No effect	No effect	9 µM
Quercetin	> 100 µM	13 µM	No effect	4 µM
PP2	> 100 µM	331 nM	No effect	> 100 µM
SU 6656	> 100 µM	179 nM	No effect	No effect
Src inhibitor #1	> 100 µM	196 nM	No effect	No effect

Compounds inhibited their target kinase with the expected potency. Some inhibitors were selective for not only their target kinase. PKI, Wortmannin, PP2, SU 6656, and Src inhibitor #1 showed the greatest selectivity for their target kinases.

11. Kinase Screening – LOPAC[1280] Compound Library

LOPAC[1280] Compound Library Screenings were performed against PKA using optimal conditions from previous experiments. Kinase-Glo Plus and Kinase-Glo Max assays at two ATP concentrations (10µM ATP and 500µM ATP, respectively) were used with 10µM LOPAC[1280] compound per well. Blue and pink data points above the yellow line represent 27 hit compounds with kinase inhibition greater than 25%. Six of the 27 hits appear as ATP competitive kinase inhibitors, including H 89. The remaining 22 hits appear as ATP non-competitive and universal kinase inhibitors.



12. Conclusions

- Together the Promega Kinase-Glo Assay platform, Deerac Fluidics Equator, Corning 1536-well Microplates, BMG LABTECH PHERAstar, and Labcyte Echo 555 liquid handler provide an unique integrated solution for luminescent assay optimization, kinase profiling, and screening in uHTS formats.
- Profiling with enzyme inhibition and compound screening at different ATP concentrations using the Kinase-Glo Assay platform can help determine selectivity and potency, as well as help to distinguish between ATP competitive and noncompetitive kinase inhibitors.

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