Miniaturization of Protein Kinase Assays in the MultiScreen®HTS 384-Well P81 Phosphocellulose Filter Plate

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Abstract

The protein kinase superfamily contains a significant number of potential targets for pharmaceutical discovery. High throughput screening using filter binding has successfully identified a number of kinase inhibitors. A new 384-well P81 phosphocellulose (PH) filter plate allows for quantitative protein kinase assays in a high throughput format.

In this study, using Protein Kinase A (PKA) as a representative assay system, peptide phosphorylation reactions carried out in 384 PH filter plates showed dependence on enzyme and substrate concentrations, and on time. Kinase inhibition studies demonstrated IC50 and Z' values equivalent to those obtained in 96-well plates. The higher throughput 384 well format offers a miniaturized version of the 96 well assay with equivalent sensitivity and reproducibility. In addition to providing for substantial reagent cost savings, kinase screening assays can be performed directly in the plate with equivalent performance to assays carried out in a separate reaction plate, thereby reducing radioactive waste and assay time.

Introduction

The protein kinase super-family represents a vast opportunity for drug discovery. Protein kinases play a fundamental role in cellular signal transduction and aberrant kinase activity has been observed in many diseases. In recent years, kinase inhibition has become a major area for therapeutic intervention and high throughput screening has successfully identified a number of kinase inhibitors.

High throughput screening by means of filter binding is an important method to identify kinase inhibitors. Phosphocellulose filter plates are used to perform such protein kinase assays. The ability of PH paper to retain phosphorylated peptides containing basic amino acid residues is a central element of filter binding assays for kinase activity. PH filter binding is a proven and reliable methodology, directly measures phosphorylation and can be quickly automated.

The MultiScreen$_{HTS}$ 384-well phosphocellulose filter plate is used to perform both quantitative and screening kinase assays. The higher throughput 384-well format offers a miniaturized version of the 96-well assay with equivalent sensitivity and reproducibility. In addition to providing for substantial reagent cost savings, kinase screening assays can be performed directly in the plate with performance equivalent to assays carried out in a separate reaction plate, thereby reducing radioactive waste and assay time.

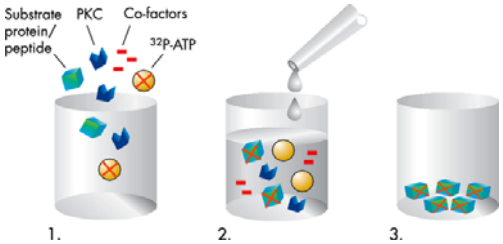
Methods

Protein Kinase A Phosphorylation Reactions
Unless otherwise indicated, phosphorylation assays were run directly in the phosphocellulose (PH) filter plate. For in-plate reactions, the wells were pre-treated with 100µL 1M Tris-HCl pH 7.4. This step is not required for out-of-plate reactions, which only require that the filter plate be pre-wet with 1X reaction buffer. Two reaction mixtures were assembled in 96-well V-bottom plates: buffer A contained cAMP (80nM), Kemptide (Sigma-Aldrich cat. # K1127 - conc. as indicated) and PKC/CaMK inhibitory cocktail (Upstate Inc. cat. #20-119) in 1X ADB Buffer (4mM MOPS, pH 7.2, 5mM β-glycerol phosphate, 1mM EGTA, 0.2mM sodium orthovanadate, 0.2mM DTT); buffer B contained Mg/ATP (6mM/40µM) with ^{33}P-γATP (0.1uCi/reaction), inhibiting peptide or drug (conc. as indicated) or buffer and Protein Kinase A (Upstate Inc. cat.# 14-114) (amount as indicated) in 1X ADB buffer. The pre-wet filter plates were vacuum evacuated at 8" Hg without being allowed to dry. 15µL of reaction buffer A was added to each test well. To start the reaction, 15µL of reaction buffer B was added to each test well in a timed fashion. Unless otherwise indicated, reactions were incubated for 15 minutes. The reactions were stopped by the addition of 100ul 100mM Phosphoric acid. The plates were washed 5 times under vacuum at vacuum levels form 4-8" Hg with 100 µL phosphoric acid.

Liquid Scintillation Counting and analysis of Reaction Product
10ul (384 well plate) or 25 to 50ul (96 well) Packard Opitphase™ Supermix was added to each test well and allowed to incubate for at least 2 hours before counting. Counting was performed in a Wallac MicroBeta® Trilux in coincidence counting mode. In order to prevent ^{33}P crosstalk, a crosstalk correction and normalization protocol was performed as recommended by the manufacturer (PerkinElmer lit. #14501016)

MultiScreen$_{HTS}$ 384 well Phosphocellulose Filter Plate

Overview of In-plate Kinase Phosphorylation Assays

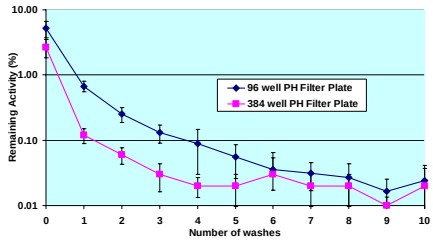


❶ Pre-wet the PH plate with 1M Tris-HCl (7.4 pH; 100 µL/well). Vacuum evacuate filter plate without drying. Mix cofactors and reaction buffer. Add this mixture to the plate. Mix enzymes and unknowns, add to plate. Incubate for 10 to 30 minutes at room temperature.

❷ Add phosphoric acid (100 mM) to stop the reaction. (Incubate 5 minutes.) Wash plates, under low vacuum, with phosphoric acid. (use 4-5 x 100 µL washes; The vacuum must be turned off between each wash step.)

❸ Count reacted components in a microplate scintillation counter

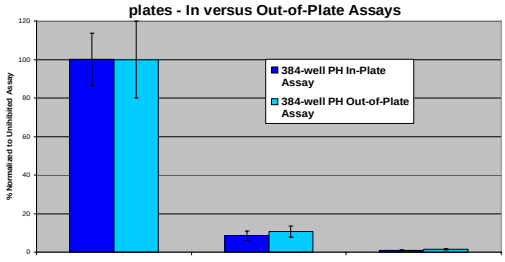
Reduced surface area of 384-well PH Filter Plate reduces non-specific binding and number of required wash steps



Non-Specific Binding of ^{33}P-γ-ATP was determined by adding approximately 100,000 DPM of labeled ATP to each test well (n=8 for each point). Wells were washed with 100µL 100mM phosphoric acid for indicated number of washes (0 washes indicates that these test wells were filtered by vacuum then counted without being washed). In order to determine % remaining, test wells were compared to wells which were not evacuated or washed

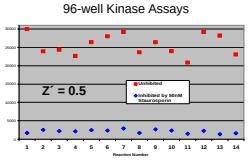
In-Plate and Out-of-Plate Kinase Phosphorylation

Protein Kinase A Assays on Phosphocellulose (PH) filter plates - In versus Out-of-Plate Assays

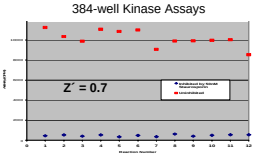


Screening Capability

96-well Kinase Assays



384-well Kinase Assays



Phosphorylation reactions of Kemptide by Protein Kinase A were run in adjacent columns of the filter plate in the presence or absence of the kinase inhibitor, Staurosporin (50nM). Each reaction was washed 5 times with 100mM phosphoric acid after assay termination. Z' values were determined by the formula $Z' = 1-((3\sigma_u+3\sigma_i)/(\mu_u-\mu_i))^2$ where σ = standard deviation, µ = mean, u and i = uninhibited and inhibited, respectively.

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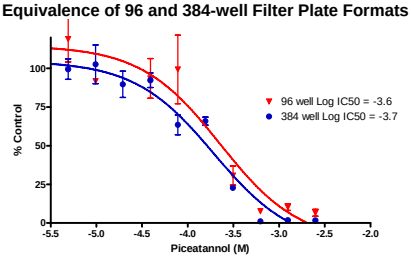
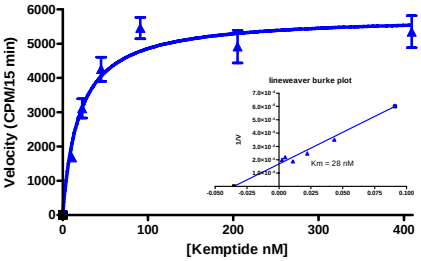
Kinase Inhibition Studies (IC50 Determination): Equivalence of 96 and 384-well Filter Plate Formats



Inhibition of Protein Kinase A phosphorylation of Kemptide assays were used to determine the IC50 inhibition value of the drug Piceatannol under. Assays were initiated then terminated at 15 minutes in synchrony. IC50 values were determined by plotting of the velocity data was fit using Graphpad Prism Software.

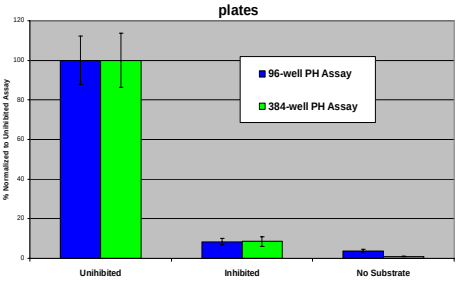
Determination Of Kinase Assay Kinetic Parameter 384-well Filter Plate Format



Protein Kinase A phosphorylation of Kemptide assays were used to determine kinetic parameters of the reaction. Enzyme–limited reactions were initiated then terminated at 15 minutes in synchrony. CPM/assay time was used to determine reaction velocity. Km was determined by Lineweaver-Burke plotting of the velocity data. Curve was fit using Graphpad Prism Software.

Kinase Assays Miniaturized to 384-well format: Comparison of MultiScreen$_{HTS}$ 96 and MultiScreen$_{HTS}$ 384

Protein Kinase A Assays on Phosphocellulose (PH) filter plates



Summary

- Kinase phosphorylation assays can be performed in a 384-well filter plate format with the same robustness and reproducibility as the 96 well filter plate format.
- Using Protein Kinase A as a model system, IC50 and Km values of were measured. Values performed on 96-well and 384-well PH plates were in agreement.
- Due to decreased surface area, the 384-well format exhibits lower non-specific binding background and requires fewer washes than the 96-well format.
- Calculated Z' values demonstrate that higher screening densities can be achieved in the 384-well format compared to the 96 well format without sacrificing sensitivity or reproducibility.
- Kinase assays can be performed directly in the filter plate decreasing the number of manipulations, and reducing radioactive solid waste by eliminating the need for a separate incubation plate.

[1] Zhang, J., Chung, T., and Oldenburg, K., J. Biomolecular Screening 4:67-73, 1999