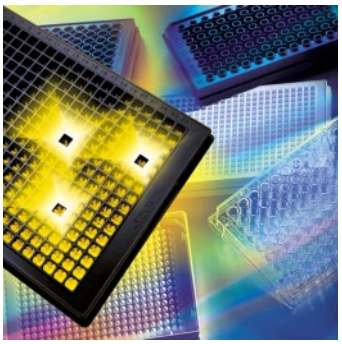
Fluorescence Polarization Kinase Assay Miniaturization in Corning® 96 Well Half-Area and 384 Well Microplates

Application Report

Innovative Techniques in Drug Discovery



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Summary

Numerous homogenous assays have been developed for use in high-throughput screening. While many of these assays address automation, speed, detection and sensitivity issues, cost reduction remains illusive. In an effort to enable rapid, cost effective methods to identify potential therapeutic candidates, assay miniaturization in Corning 96 well, 96 well half-area and 384 well microplates was examined. Fluorescence polarization tyrosine kinase assays were used to assess volume reduction from 100 µL in 96 well microplates, to 50 and 25 µL in 96 well half-area and 384 well microplates. Concentrations of purified human $P60^{c\text{-}src}$ or substrate were varied, and kinase activity was detected at even the lowest concentrations of enzyme or substrate in all formats and volumes. These results suggest that Corning 96 well half-area microplates can be utilized to lower reagent costs while maintaining the common 96 well automation footprint without a significant reduction in detection sensitivity. For high-throughput screening laboratories that have incorporated the 384 well automation footprint, Corning offers an array of microplates in the 384 well format to allow a further reduction in reagent volume while maintaining high detection sensitivity.

Introduction

An elaborate network of tyrosine kinases are required for the regulation of growth, proliferation, and differentiation in eukaryotic cells (1). The number of human genes encoding tyrosine kinases is estimated to be more than 1,000, which could account for as much as 1% of the human genome, and provide an ever increasing number of potential targets for therapeutic intervention (2). Non-receptor (cytosolic) src family protein tyrosine kinases preferentially phosphorylate peptides recognized by their own SH-2 (src homology-2) domains and are key mediators of signal transduction pathways (3). These kinases play an important role in normal cellular physiology, but a growing body of evidence suggests that activated mutants of src, such as v-src, are oncogenic (4). While many tyrosine kinases have been described, src remains a focus of investigation both as a model tyrosine kinase and as an important drug discovery target (5). Hence, a major effort in drug discovery is to ascertain effective tyrosine kinase inhibitors as chemotherapeutic agents (6). Several protein tyrosine kinase activity assays have been used in an effort to rapidly screen libraries of compounds that affect tyrosine kinase activity, including 32PO4-transfer, ELISA (Enzyme-linked immunosorbent assay), and DELFIA® (Dissociation-enhanced lanthanide fluorescence immunoassay) (5). The 32PO4-transfer assays often use radioactive [g-32P] as a PO4 donor and have inherent problems associated with half-life, storage, usage, and disposal of large amounts of radioactive waste. In addition, these assays are not amenable to high-throughput as they include several wash, liquid transfer, and incubation steps, are labor intensive and time consuming. Fluorescence polarization competitive immunoassays have been designed to overcome these problems and can be used to monitor any biochemical reaction that leads to an alteration in the molecular volume of a fluorescently labeled molecule, such as that which occurs through cleavage, binding, or a conformational change (7). In this homogenous fluorescence polarization assay, a substrate molecule phosphorylated by $p60^{c\text{-}src}$ competes with a fluorescent phosphopeptide used as a tracer for immunocomplex formation with anti-phosphotyrosine antibody. Kinase activity will result in the loss of polarized fluorescence as the phosphorylated substrate competitively displaces the fluorescently labeled phosphopeptide from the phosphotyrosine antibody. While use of this competitive immunoassay in high-throughput screening alleviates many of the problems associated with earlier assays, the cost of

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reagents is still considerable. 96 well microplates have remained the standard for high-throughput screening, although robotics for handling the 384 well plate format are becoming increasingly available (8). Assay miniaturization has the potential to deliver many benefits, including more sparing use of precious chemical compounds and biological reagents, reducing the waste stream and increasing the speed and number of compounds screened. These benefits can also lead to an overall cost reduction and decrease in the time for drug discovery. However, assay miniaturization also has drawbacks that include the initial monetary outlay for retooling to accommodate format changes and the potential to miss candidates for therapeutic intervention due to a reduction in sensitivity of the miniaturized assay. Corning has developed the 96 well half-area and 384 well microplates in an attempt to address these concerns.

Materials and Methods

Reactions were modified from the TKX.™ Tyrosine Kinase Exploration Kit (Cat. No. 044-0002, LJL BioSystems) and carried out in Assay Buffer consisting of 50 mM HEPES, pH 7.5 containing 0.1 mM EDTA, and 0.015% IGEPAL CA-630 (Cat. No. I-3021, Sigma®). Reactions included $p60^{c\text{-}src}$ (Cat. No. PKO2, CALBIOCHEM) diluted in Assay Buffer containing 0.1 mg/mL BSA and 0.2% ß-mercaptoethanol to final concentrations of 0.015 to 0.00015 unit/µL, poly-glut/tyr (4:1) substrate (Cat. No. P-0275, Sigma) concentrations from 1.0 µM to 0.001 µM, 1:1000 dilutions of mouse monoclonal anti-phosphotyrosine antibody (Cat. No. P-3300, Sigma) and 1.0 nM concentration of fluorescently-labeled tracer from the TKX Kit mentioned previously. Reactions were started with the addition of $MgCl_2$ and ATP to final concentrations of 3 µM and 20 µM respectively. Reaction mixtures were prepared for all conditions and dispensed in triplicate by manual pipetting appropriate volumes into wells of black opaque Costar® Brand 96 well (Cat. No. 3915), 96 well half-area (Cat. No. 3694) and 384 well (Cat. No. 3710) microplates. Following room temperature incubation for 30 minutes to 3 hours, kinase activity was detected in millipolarization units (mP) using the standard fluorescence polarization protocol in an LJL Biosystems Analyst™ as follows: Digital conversion, attenuator out, units = mP, Plate = Costar 96 well solid, 384 well solid or 96 well half-area (as appropriate), static polarizer (s) = excitation, dynamic polarizer (p) = emission, polarizer settling time = 30 ms, z height = 1.2 mm, integration time: 100 ms, excitation filter 485 nm, emission filter 530 nm.

Results and Discussion

In the past five years, dramatic advances in automation techniques and computer technology have combined with new assay techniques that are both miniaturized and highly sensitive (9). Recently, homogenous fluorescence polarization immunoassays have grown to occupy a significant portion of the high-throughput screening market for determining drug discovery targets (7). Targets, such as non-receptor src family protein tyrosine kinases, have been detected using fluorescence polarization immunoassays and compounds that alter src kinase activity are a major focus of some high-throughput screening laboratories (5). In this homogenous fluorescence polarization immunoassay, $P60^{c\text{-}src}$ phosphorylates a polyglutamate/tyrosine substrate and competitively displaces a fluoresceinated tracer molecule associated with an anti-phosphotyrosine antibody resulting in a loss of fluorescence polarization. From a high-throughput standpoint, this biomolecular assay addresses key features of an automated platform including high speed of reaction, "mix and read" liquid handling and high sensitivity of kinase activity detection. When screening large libraries of compounds, cost of immunoassay reagents can be considerable and restrict both the size and variety of libraries investigated. Whereas assay volumes are commonly 100-200 µL in 96 well microplates, this fluorescence polarization kinase assay was carried out in three different microplate formats, assessing 25 and 50 µL volumes in 96 well half-area and 384 well microplates. These results demonstrate that activity of $p60^{c\text{-}src}$ kinase is detected as a decrease in polarized fluorescence in all formats and volumes at concentrations from 0.015 units/µL to 0.00015 units/µL (Figure 1). Similarly, at a one hundred-fold dilution of substrate, kinase activity was still detectable in all formats and volumes maintaining detection sensitivity of src kinase activity in Corning® 96 well half-area and 384 well microplates following 50% and 75% reductions in reagents, respectively (Figure 2). Even at the lowest substrate concentration, considerable signal is detected when compared to the level of noise for all formats and well volumes (Figure 3).

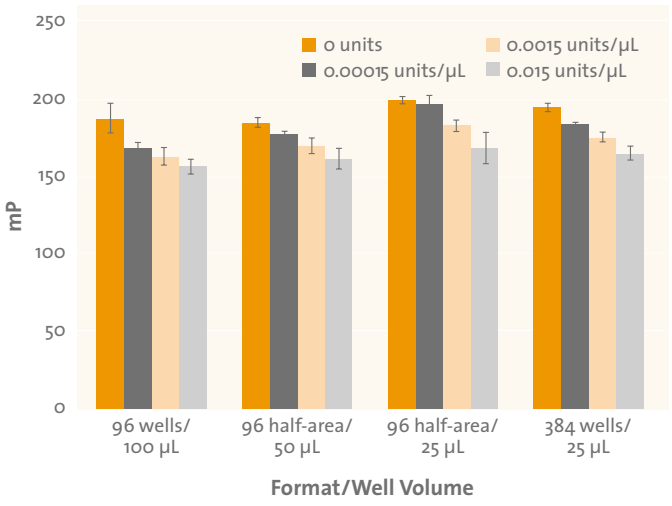

Figure 1. Titration of $p60^{c\text{-}src}$ kinase (no enzyme, 0.00015 units/µL, 0.0015 units/µL, and 0.015 units/µL) were incubated with 1.0 µM substrate for 30 minutes at room temperature in Corning® 96 well, 96 well half-area, and 384 well microplates. As src kinase concentrations increased, a decrease in fluorescence polarization (mP) was detected at all concentrations in 96 well, 96 well half-area and 384 well microplates.

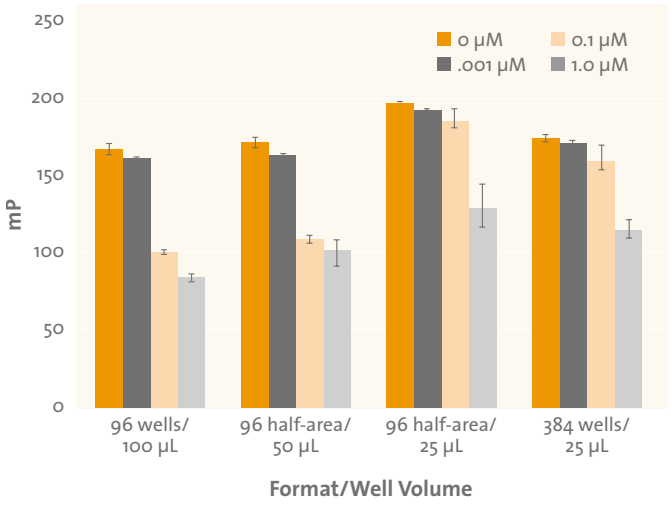

Figure 2. Titration of src kinase poly-glut/tyr substrate (0 µM, .01 µM, 0.1 µM, and 1.0 µM) were incubated with p60$^{c\text{-}src}$ 0.015 units/µL for 3 hours at room temperature in Corning® 96 well, 96 well half-area, and 384 well microplates. As substrate concentrations increased, fluorescence polarization (mP) decreased in all platforms.

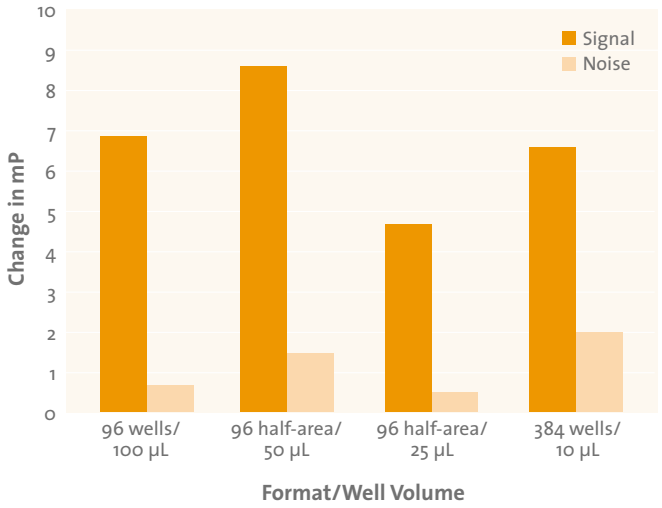

Figure 3. After 3 hours of incubation with 0.015 units/µL of p60$^{c\text{-}src}$, the amount of decrease in polarized fluorescence (signal) is compared to the variation in signal (noise) for replicates at the lowest substrate concentration. Actual ratios of signal/ noise are (from left to right): 9.8, 5.7, 9.4, 3.3.

Conclusions

- Corning® 96 well half-area and 384 well microplates enable assay miniaturization without loss of sensitivity.
- Corning 96 well half-area microplates provide a novel way to conserve chemical compounds and biological reagents by allowing assay miniaturization without costly automation format retooling.
- Corning offers an array of 384 well microplates to augment throughput and facilitate rapid screening of potential therapeutic candidates for laboratories that have incorporated the 384 well automation format.
- Corning 96 well half-area and 384 well microplates help to reduce the waste stream and overall cost of high-throughput screening.

References

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