Microarrayed Compound Screening (μARCS) to Identify Activators and Inhibitors of AMP-Activated Protein Kinase

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A novel and innovative high-throughput screening assay was developed to identify both activators and inhibitors of AMP-activated protein kinase (AMPK) using microarrayed compound screening (μARCS) technology. Test compounds were arrayed at a density of 8640 on a polystyrene sheet, and the enzyme and peptide substrate were introduced into the assay by incorporating them into an agarose gel followed by placement of the gels onto the compound sheet. Adenosine triphosphate (ATP) was delivered via a membrane, and the phosphorylated biotinylated substrate was captured onto a streptavidin affinity membrane (SAM™). For detection, the SAM™ was removed, washed, and imaged on a phosphor screen overnight. A library of more than 700,000 compounds was screened using this format to identify novel activators and inhibitors of AMPK. (*Journal of Biomolecular Screening* 2004:112-121)

Key words: microarrayed compound screening (μARCS), high-throughput screening, AMP-activated protein kinase (AMPK), streptavidin affinity membrane (SAM™)

INTRODUCTION

AMP-ACTIVATED PROTEIN KINASE (AMPK), a widely distributed heterotrimeric protein kinase, is considered to be part of a cellular energy-sensing system. It is stimulated by increased levels of 5′-AMP (adenosine monophosphate) and through phosphorylation by an upstream kinase. AMPK substrates include several key enzymes involved in glucose and fatty acid metabolism, including acetyl-CoA carboxylase and 3-hydroxy-3-methylglutaryl-CoA reductase. Net results of increased AMPK activity are inhibition of anabolic adenosine triphosphate (ATP)–consuming pathways (e.g., fatty acid synthesis) and activation of catabolic ATP-generating pathways (e.g., fatty acid oxidation).[1-3] In addition, sustained activation of AMPK in skeletal muscle increases glucose transport and GLUT4 expression,[4] mimicking the effects of exercise. Based on these observations, stimulation of AMPK could be beneficial in type 2 diabetes and obesity and may emerge as a new therapeutic strategy for these diseases.[2,5]

Research on AMPK in cellular and in vivo situations relies heavily on AICAR (5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside),[6] a cell-permeable ZMP (5-aminoimidazole-4-carboxamide-1-β-d-ribofuranotide) precursor. ZMP, an AMP mimic, stimulates AMPK in vivo but has been shown to be nonspecific. Several other enzymes, including fructose-1,6-bisphosphatase and glycogen phosphorylase, which are allosterically regulated by AMP, are also modulated by ZMP;[7,8] therefore, physiological responses observed after AICAR administration are not necessarily due to increased AMPK activity. A specific AMPK activator would be a highly desirable tool for further elucidation of physiological responses caused by increased AMPK activity.

Microarrayed compound screening (μARCS), a well-less, high-density, ultra-high-throughput screening format, has been used at Abbott Laboratories to screen several target classes, including kinases.[9-13] In μARCS, the reagents are incorporated into agarose gels and layered over compounds arrayed on polystyrene sheets,[10,12] mimicking the pipetting steps in a microplate assay. Advantages of μARCS screening compared to traditional plate screening include increased throughput and decreased reagent consumption.[10,12] μARCS is a very flexible format and eliminates the requirement for complex liquid-handling instruments and

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μARCS technology developed at Abbott Laboratories and licensed to Discovery Partners International (San Diego, CA).

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avoids problems associated with evaporation and plate edge effects. In this article, we describe a novel high-throughput μARCS screen specifically designed to discover both inhibitors and activators of AMP kinase.

MATERIALS AND METHODS

Preparation of enzyme

Rat livers (Pel Freez, Rogers, AR) were diced and homogenized in a Waring blender in buffer A (50 mM Tris-HCl [pH 8.0], 250 mM mannitol, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM ethylenediaminetetraacetic [EDTA], 1 mM ethylene glycol-bis-tetraacetic acid (EGTA), 1 mM NaN_3, 1 mM dithiothreitol [DTT]) containing protease inhibitors.[14] The homogenate was centrifuged (Beckman G2-MI) for 15 min at 5500 *g*, and the supernatant was decanted and suspended in 2.5% polyethyleneglycol (PEG, avg. 8000 MW). The crude enzyme preparation was mixed for 30 min, followed by centrifugation at 30,000 *g* for 30 min to clarify the sample. The supernatant from this centrifugation was suspended in 6% PEG and mixed and centrifuged as above, and the pellets were resuspended in buffer B (50 mM Tris-HCl [pH 7.6], 250 mM mannitol, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.02% Brij 35, 1 mM NaN_3, 1 mM DTT) using a glass homogenizer. The enzyme suspension was then applied onto a DEAE-Sepharose fast-flow resin (Sigma, St. Louis, MO) column. This column was washed with buffer B, the protein was eluted in a step gradient of buffer B with 200 mM NaCl, and the eluate was applied to a blue Sepharose resin (Sigma) column. Protein eluted from the Sepharose column using a step gradient of buffer B with 0.8 M NaCl was concentrated, and the buffer was exchanged to buffer B and applied to a Q-Sepharose Hi-Load (Amersham-Pharmacia, Picataway, NJ) column. Fractions were collected using a linear gradient of buffer B to buffer B plus 450 mM NaCl. Active fractions, identified in the 96-well plate assay described below, were pooled and concentrated using a Centricon 10 (Millipore, Bedford, MA), and the concentrate was applied to a Sephacryl-200 column (Amersham-Pharmacia) in buffer C (50 mM Tris-HCl [pH 7.6], 250 mM mannitol, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.02% Brij 35, 1 mM NaN_3, 1 mM DTT). Active material was stored at –80 °C. All steps in the above protocol were carried out at 4 °C, and all chemicals were supplied by Sigma unless otherwise noted.

Preparation of peptide substrate

The peptide substrate used for the AMPK screen, biotin-Aha-HMRSAMSGLHLVKRR (SAMS) (Aha = 6-aminohexanoic acid), was assembled with a 430A automated synthesizer (Applied Biosystems, Foster City, CA) using standard FastMOC™ chemistry on Wang resin preloaded with the first amino acid. Aha was attached as for the proteogenic amino acids, and then biotin was added at the N-terminus using modified FastMOC™ double-coupling cycles to accommodate cartridges containing biotin predissolved in 1:1 DMSO/*N*-methylpyrrolidinone. The peptide was cleaved and deprotected with (trifluoroacetic acid [TFA]/water/thioanisole/phenol/ethanedithiol/triisopropylsilane, 80:5:5:5:2.5:2.5) for 3 h at ambient temperature and purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on an automated HPLC (Gilson, Inc., Middleton, WI) on a 25 × 2.5-mm radial compression Dynamax C18 column (Waters, Inc., Milford, MA) eluted with a linear gradient of 0.1% TFA-water/acetonitrile. The peptide was > 95% pure, as judged by analytical RP-HPLC run on an Agilent 1050 HPLC (Palo Alto, CA) on a YMC AQ-ODS 0.46 × 25-mm column (Waters, Inc.) and identified by matrix-assisted laser-desorption ionization mass spectroscopy (MALDI-MS).

All reagents were used as obtained from the vendor, unless otherwise specified. Peptide synthesis reagents, including diisopropylethylamine (DIEA), N-methylpyrrolidone (NMP), dichloromethane (DCM), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), N-hydroxybenzotriazole (HOBt), and piperidine were obtained from Applied Biosystems (AB, Foster City, CA). In addition, 9-fluorenylmethyloxycarbonyl (Fmoc) amino acid derivatives (Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Val-OH, Fmoc-aminohexanoic acid (Fmoc-Aha-OH)) were obtained from SynPep (Dublin, CA) or ABI. Preloaded Fmoc-Arg(Pbf)-Wang resin was obtained from Novabiochem (San Diego, CA). TFA, thioanisole, phenol, triisopropylsilane (TIS), ethanedithiol (EDT), acetic acid, DMSO, biotin, and methanol were obtained from Sigma-Aldrich Chemical (Milwaukee, WI). HPLC-grade water and acetonitrile were obtained from EM-Science (Gibbstown, NJ). Mass-spectra (MALDI-MS) were recorded on an Applied Biosystems Voyager DE-PRO MS.

96-Well AMPK assay

In a 20-μL reaction volume, 1 μCi ^{33}P-ATP (PerkinElmer, Boston), 200 μM ATP, 20 μM SAMS peptide, AMPK, and AMP or compounds at the indicated concentrations were incubated for 15 min at 30 °C in a buffer containing 40 mM HEPES, 80 mM NaCl, 5 mM $MgCl_2$, 0.8 mM EDTA, 8% glycerol, 0.18% Triton-X-100, and 1 mM DTT.[15] The reaction was stopped by the addition of 10 μL per well of 7.5 M guanidine HCl. The plates were shaken briefly, and 15 μL of reaction mix was transferred to a streptavidin affinity membrane (SAM™) filter plate (Promega, Madison, WI). Then, 100 μL of 2 M NaCl was added per well, and the plates were incubated at room temperature (RT) for 1 to 2 min. Wells were then washed 8 times with 2 M NaCl, 12 times with 2 M NaCl with 1% phosphoric acid, 4 times with water, and 1 time with EtOH before air-drying. Scintillant was added to the plates, and the radioactivity was quantitated in a Topcount (PerkinElmer, Boston). Unless oth-

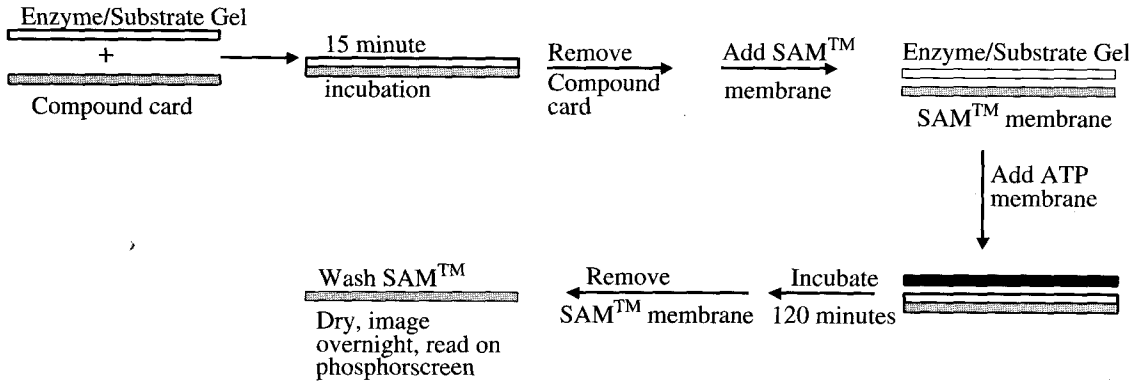


FIG. 1. Schematic representation of the AMP-activated protein kinase (AMPK) assay in microarrayed compound screening (μARCS) detailing the steps in the assay, as described in the Material and Methods section.

erwise specified, chemicals used in the above assay were supplied by Sigma.

Compound preparation

A total of 8640 compounds were arrayed at 200 pmoles of each compound in 40 nL of DMSO on white polystyrene cards using a synQuad 8-tip liquid dispenser.[12,16] The compound cards were dried under vacuum and then stored at –80 °C.

AMP kinase μARCS development

The initial assay development was performed by varying the concentration of 1 reagent at a time in the assay while maintaining the rest of the reagent concentrations constant. Substrates (SAMS, ATP) and enzyme were titered to find the optimal concentrations, which provided high signal-to-background ratios while still allowing the detection of enzyme activation at low AMP concentrations. In addition to varying reagent concentrations, several methods of delivering ^{33}P-labeled ATP using porous membranes were evaluated. These membranes included glass fiber filters (Gelman-Pall Biomedical Products Co., Glen Cove, NY; Millipore Co., Billerica, MA; and Sigma), nylon transfer membranes (Amersham), blotting paper (VWR, West Chester, PA), and common tissue paper (1-ply tissue wrapping paper).

AMP kinase μARCS protocol for high-throughput screening (HTS)

High-gel-strength, low-melt-temperature agarose purchased from BioWhittaker Molecular Applications (Rockland, ME) was prepared as a 1% solution in an assay buffer consisting of 40 mM HEPES, 80 mM NaCl, 8% glycerol, 5 mM $MgCl_2$, and 0.1% Triton-X-100. The agarose suspension, melted by heating in a microwave oven, was cooled to 37 °C in a water bath, and DTT was added to a final concentration of 1 μM. AMP kinase and SAMS were added to the agarose to a final concentration of 7 μg/mL and 40 mM, respectively. The agarose containing the enzyme-substrate mixture was immediately poured into a Protean II gel-casting apparatus (Bio-Rad, Hercules, CA), using spacers yielding a gel thickness of 0.75 mm, and cooled for 15 min to solidify the agarose. After release from the casting apparatus, the gel was placed on Saran Wrap® for 10 min to air-dry to remove excess moisture. The enzyme-substrate gel was then carefully placed over the polystyrene compound card and incubated for 15 min to allow diffusion of compounds into the gel. A SAM™ washed with phosphate-buffered saline (PBS) was air-dried and placed on a transparency. After the compounds diffused into the agarose, the compound card was carefully removed from the gel, and the enzyme-substrate gel was placed on the SAM™ membrane (Fig. 1). Immediately, a membrane (plain tissue paper) soaked in assay buffer containing ^{33}P-ATP was placed over the enzyme-substrate gel to initiate the kinase reaction. Saran Wrap® was placed over the gel-membrane sandwich to prevent the drying of the gel. After a 60- to 180-min incubation at ambient temperature, the reaction was terminated by removing the enzyme-substrate gel and ATP membrane from the SAM™ membrane. The SAM™ membrane was washed once with 2 M NaCl, then with wash solution containing 2 M NaCl and 1% H_3PO_4, and finally with purified water to remove excess salt. The SAM™ membrane was air-dried and exposed overnight on a phosphorscreen (Amersham). Phosphorscreens were read on a Molecular Dynamics 860 Storm unit (Amersham). Increased AMPK activity leads to greater incorporation of ^{33}P into the substrate and produced a darker image. A compound inhibiting AMPK appears as a light-colored circular zone of inhibition surrounded by a darker background, whereas a compound activating AMPK appears as a dark spot surrounded by a lighter background associated with lower enzymatic activity.

Screening data analysis

All images for the AMPK screening assays were obtained from the Molecular Dynamics 860 Storm unit and transferred to a customized database.[17] The database allows the user to pick spots or

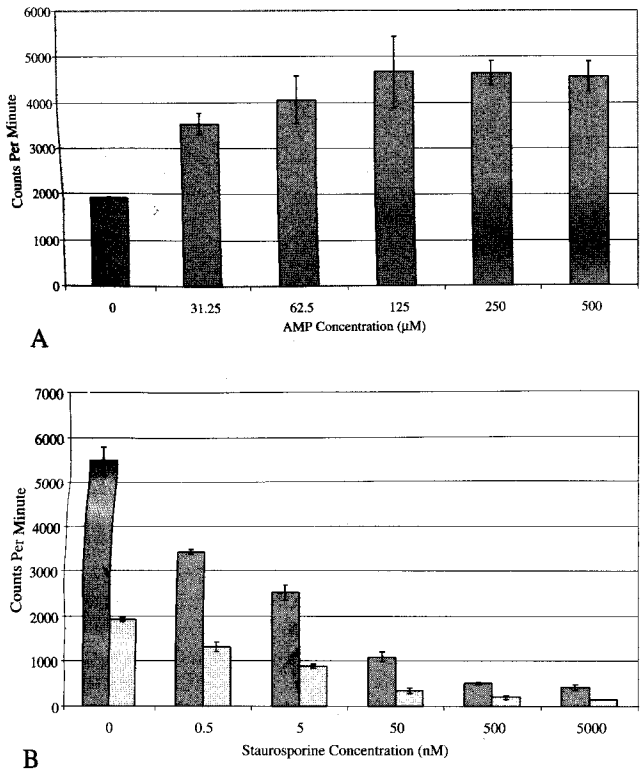


FIG. 2. Measurement, in triplicate ±SD and representative of at least 2 experiments, of AMP-activated protein kinase (AMPK) stimulation and inhibition by known regulators of AMPK activity in a 96-well streptavidin affinity membrane (SAM™) plate assay. (**A**) Activation of AMPK by adenosine monophosphate (AMP). (**B**) Inhibition of AMPK activity by staurosporine in the presence or absence of AMP. With 200 μM AMP (dark-shaded columns); without AMP (light-shaded columns).

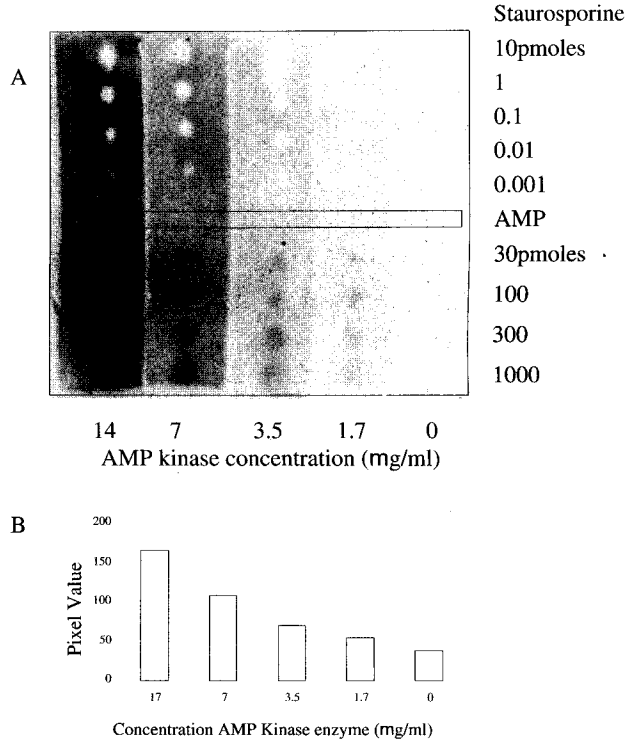


FIG. 3. Titration of AMP-activated protein kinase (AMPK) enzyme concentration in the microarrayed compound screening (μARCS) format. The substrate concentration of SAMS peptide and adenosine triphosphate (ATP) was kept constant at 40 μM and 100 μM, respectively. (**A**) Image showing lowest concentration of adenosine monophosphate (AMP) activating AMPK is 30 pmole, and the lowest concentration of staurosporine inhibiting AMPK is 0.01 pmole. (**B**) Histogram of pixel values, from area within black rectangle in **A**, showing response to AMPK enzyme concentrations.

"zones of activity" from each image and tracks the compounds associated with each spot. To determine the optimal concentration of assay reagents, an enzyme activator (AMP) and an enzyme inhibitor (staurosporine) were used at several concentrations to assess the sensitivity of the assay.

Assay development images were analyzed using Image/J (NIH, Bethesda, MD). Histograms of the pixel values, provided in the figures, demonstrate the concentration-dependent nature of the assay. Graphs generated from the 96-well plate assays were plotted using Excel (Microsoft Co., Redmond, WA). Subsequent IC_{50} analysis was completed using Kaleidograph 3.5 (Synergy Software, Reading, PA).

RESULTS AND DISCUSSION

96-Well plate assay

Both our SAM™ membrane 96-well plate and μARCS assays were developed and validated based on our ability to detect stimulation of AMPK activity by AMP and inhibition by staurosporine using a modification of previously reported assay conditions.[15] Results from 96-well plate assays are shown in Figure 2. Figure 2A demonstrates the dose-dependent activation of AMPK at AMP concentrations ranging from 31.25 to 500 μM, resulting in an EC_{50} of 22.2 ± 6.8 μM. AMPK inhibition, shown in Figure 2B, was observed with staurosporine at the indicated concentrations in the presence or absence of 200 μM AMP. The IC_{50} for staurosporine is 2.1 ± 1.6 nM in the presence of AMP, whereas in the absence of AMP, the observed IC_{50}, 2.9 ± 1.6 nM, is not statistically different.

μARCS enzyme concentration optimization

To evaluate assay conditions for HTS using the μARCS format, we determined the AMPK concentration required for an optimal signal, allowing us to distinguish activation and inhibition from the assay background. Varying concentrations of partially purified rat liver AMPK ranging from 0 to 14 μg/mL were used for the assay

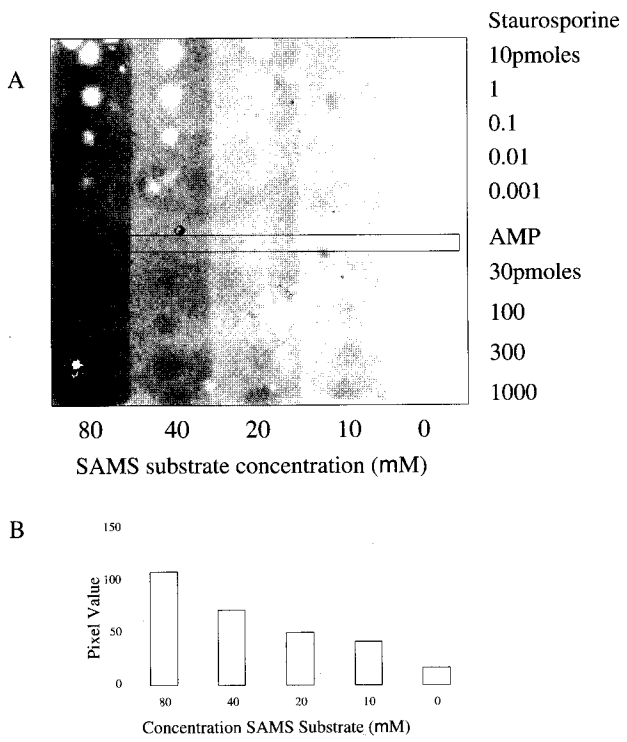


FIG. 4. Titration of SAMS substrate. (**A**) Image demonstrating the lowest concentration of adenosine monophosphate (AMP) activating AMP-activated protein kinase (AMPK) is 30 pmoles, and the lowest concentration of staurosporine inhibiting AMPK is 0.01 pmole. (**B**) Histogram of pixel values, from area within black rectangle in **A**, demonstrating concentration response of assay to SAMS substrate. (AMPK enzyme = 7 μg/mL and adenosine triphosphate [ATP] = 100 μM.)

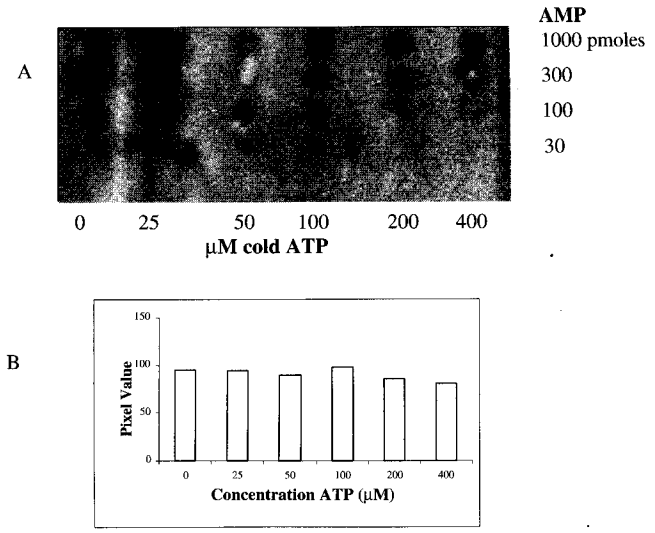


FIG. 5. Titration of cold adenosine triphosphate (ATP) substrate in AMP-activated protein kinase (AMPK) microarrayed compound screening (μARCS format). (**A**) Images illustrate that the lowest concentration of AMP activating AMPK is 30 pmoles at 100-μM ATP concentrations or below. (**B**) Histogram of pixel values from within black rectangle from **A** illustrating lack of dose response of signal to ATP concentrations. (AMPK enzyme = 7 μg/mL, SAMS substrate = 40μM, ^{33}P-ATP = 1.2 μCi/mL.)

while keeping the SAMS substrate constant at 40 μM and ATP at 100 μM. Figure 3A illustrates the image produced using different enzyme concentrations and the stimulatory and inhibitory effects of AMP and staurosporine. Figure 3B shows the dose-dependent response of enzyme concentration verses pixel value. The results shown in Figure 3 demonstrate that a concentration of 7 μg/mL AMPK produced a good signal compared to the enzyme-free controls. At AMP kinase levels of 7 μg/mL, activation can be detected with 30 pmoles of AMP, and inhibition spots with 10 fmoles of staurosporine were visible.

Substrate concentration optimization

We optimized the SAMS peptide concentration for our assays (Fig. 4) while maintaining the enzyme level at 7 μg/mL and ATP at 100 μM and varying substrate concentrations between 0 and 80 μM. Figure 4B illustrates the pixel values for each substrate concentration tested. A peptide concentration of 40 μM produced a sufficient signal and allowed detection of AMPK stimulation at 30 pmoles of AMP.

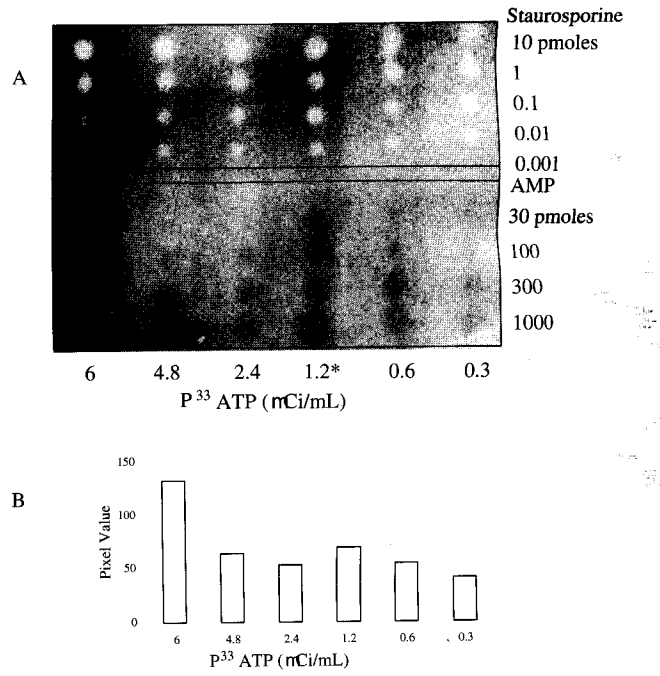


FIG. 6. Titration of ^{33}P-ATP substrate. (**A**) Image demonstrating concentration response to ^{33}P-ATP levels. (**B**) Histogram of pixel values representing assay signal, from within black rectangle, produced by varying levels of ^{33}P-ATP. (AMPK enzyme = 7 μg/mL, SAMS substrate = 40 μM cold ATP = 100μM.) ATP, adenosine triphosphate; AMPK, AMP activated protein kinase.

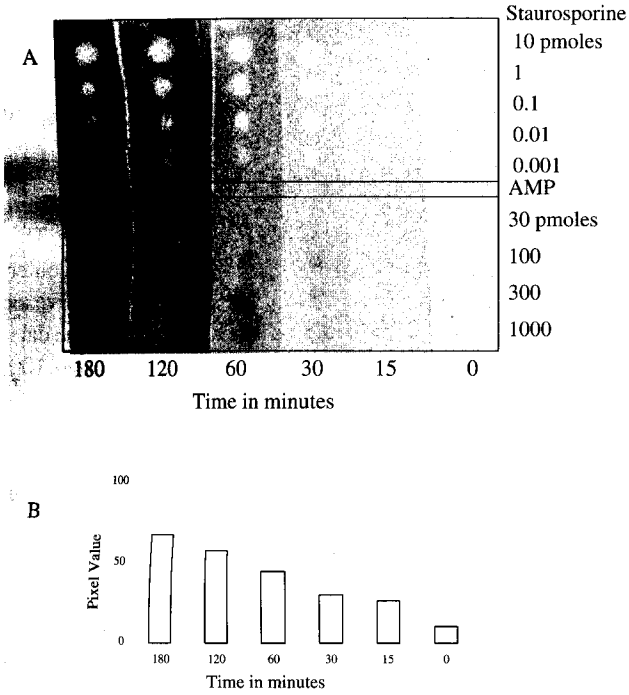


FIG. 7. Time course of AMP-activated protein kinase (AMPK) microarrayed compound screening (μARCS) assay. Times ranged from 0 to 180 min. (**A**) Image showing time course of assay. (**B**) Pixel values, from within black rectangle, of each time point. The concentration of enzyme and substrates was as follows: AMPK enzyme = 7 μg/mL, SAMS substrate = 40 μM, cold adenosine triphosphate (ATP) = 100μM, ^{33}P-ATP = 1.2 μCi/mL.

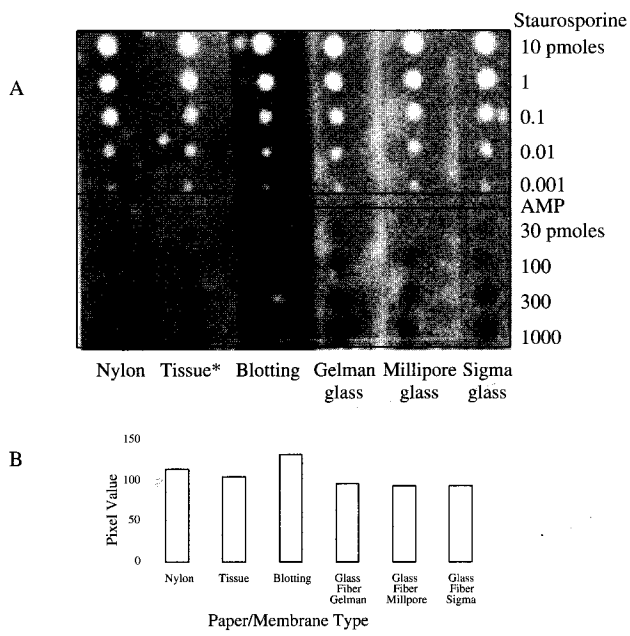


FIG. 8. Tissue paper (TP) addition by various porous matrices. All paper (blotting, nylon, tissue) and nylon and glass fiber membranes were soaked in an identical concentration of adenosine triphosphate (ATP) (cold ATP = 100 μM; ^{33}P-ATP = 1.2 μCi/mL). (**A**) Image of porous matrices. (**B**) Histogram showing pixel values, from within black rectangles, showing similar assay signal from each matrix.

Unlabeled ATP concentrations were titrated from 0 to 400 μM to identify the optimal conditions for detection of AMP-stimulated AMPK activity. Figure 5B shows that the pixel values for each ATP concentration tested remained constant, whereas the stimulatory effect of AMP, demonstrated in Figure 5A, was diminished using high concentrations of ATP in the assay. To screen for novel AMPK activators, we chose to use 100 μM ATP in our assays because at that ATP concentration, AMPK stimulation could be detected down to 30 pmoles of AMP. AMPK inhibition could be detected with 10 fmoles staurosporine at all ATP concentrations tested (data not shown).

Holding unlabeled ATP constant at 100 μM while varying ^{33}P-ATP concentrations from 0.3 to 6 μCi/mL, we identified the optimal ^{33}P-labeled ATP concentration required to obtain good-quality images. Figure 6 shows the response to varying concentrations of ^{33}P-labeled ATP. AMPK activation using as little as 30 pmoles of AMP could clearly be demonstrated with 1.2 μCi/mL of ^{33}P-ATP.

Time course

A time course for the kinase assay was performed by terminating the reaction after incubating the enzyme, substrate, and ATP at ambient temperature from 0 to 180 min. Results shown in Figure 7A demonstrate an increase in signal over 3 h as the images were darker for the longer incubation periods; AMP and staurosporine spots are detected as early as 30 min and become clearer at 180 min. Figure 7B shows the pixel values for each time point demonstrating the assay signal increase over the 3 h. The HTS for AMP kinase activators was conducted using a 2-h incubation.

Method of ATP addition

In the μARCS assay, the addition of a mixture of cold and ^{33}P-labeled ATP initiated the AMP kinase reaction. Several strategies were used to add ATP to the reaction mixture. These strategies included pouring an ATP-agarose gel and laying it over the substrate-enzyme gel, as well as using porous membranes to deliver ATP in an assay buffer. We determined that the membranes (Fig. 8) were much easier to handle than ^{33}P-ATP gels, required less ATP, and produced results equivalent to those obtained using gels. A gel large enough to screen 1 compound card required 10 mL of agarose-ATP, whereas the various membranes absorbed different volumes of buffer-ATP ranging from 7.5 mL for blotting paper to only 1 mL for tissue paper. Because tissue paper produced results (Fig. 8B) similar to those observed for gels or blotting paper

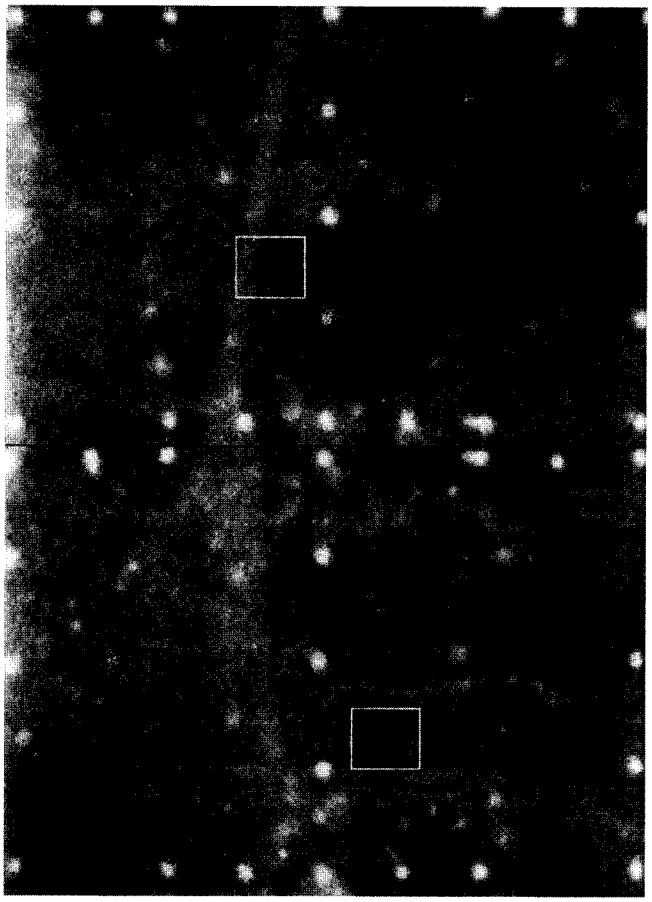


FIG. 9. Primary screening set example illustrating potential AMP-activated protein kinase (AMPK) activator. Each card contains the identical 8640 compounds arrayed in a normal and deconvoluted pattern. The concentration of enzyme and substrates was as follows: AMPK enzyme = 7 μg/mL, SAMS substrate = 40μM, adenosine triphosphate (ATP) = 100μM. Twenty-one white spots on the periphery and through the center of cards are constellation points used to orientate the image in the database. Adenosine monophosphate (AMP) controls (dark spots) are seen on the bottom of each card. A dark spot surrounded by a rectangle indicates a screening hit.

while requiring 10-fold less ATP than agarose, we chose to use tissue paper to deliver ATP and initiate the AMP kinase reaction.

Summary of AMPK screening conditions

From the experiments used to optimize μARCS screening conditions, 7 μg/mL AMPK enzyme and 40 μM SAMS substrate were used in all primary and retest screening assays. Then, 1% agarose containing enzyme and substrate was used to absorb compounds and layered over the SAM™ membrane, and tissue paper containing 100 μM ATP and 1.2 μCi/mL ^{33}P-labeled ATP was used to initiate the enzyme reaction. Assays were allowed to incubate 2 h before the SAM™ membranes were washed, terminating the reaction.

Primary screen for AMPK

A total of 717,120 compounds in duplicate on 166 compound cards (normal and deconvoluted) were screened for their ability to either activate or inhibit AMP kinase activity. Normal and deconvoluted sheets contain the same compounds but were spotted in differently arrayed patterns.[16] Figure 9 shows gel images from both a normal and a deconvoluted sheet from 1 screening set. The white spots in the center of the compound card and on the outer edges were produced by 0.5 pmoles of staurosporine and serve as constellation points for the orientation of the gel image in the data analysis software. Control spots of AMP at 1000, 300, and 100 pmoles were also applied to the bottom of each compound card to determine the sensitivity and robustness of the assay run. The same images were loaded under 2 different assay codes to identify both activators and inhibitors of AMPK, and the spots were picked by clicking in the center of the spots. Initially, the most prominent spots were picked and assigned a designation of quality 1 (Q1), followed by the quality 2 (Q2) and 3 (Q3) spots. After the picks for normal and deconvoluted sets were made and saved, the database analyzed the selected spots and assigned matched status to spots that had compounds in common in normal and deconvoluted sheets. Any spots with no matching compounds on the other sheet were considered unmatched spots, and the screener tested up to 25 compounds to confirm the activity on retest sheets.[17] All matched and unmatched compounds were requested for retesting. During retesting, potential activators were kept separate from compounds selected as AMP kinase inhibitors.

Matched and unmatched compounds selected as described above were retested for confirmation of AMPK activation or inhibition at a density of 384 compounds per sheet. Figure 10 shows a retest sheet, completed in duplicate, of activating compounds identified in the primary screen. One compound that originated as a matched pick (Fig. 9) that was reconfirmed on both retest sheets indicated the reproducibility of the assay. Figure 11 illustrates an example of primary screening sheets, normal and deconvoluted, showing 1 compound with apparent inhibitory activity that reconfirms on both sheets as well as on the retest sheet. After reconfirming the activity in retesting, the compounds were ordered, and their dose-dependent activities in both the μARCS and the plate format were determined.

Screening of more than 700,000 compounds to identify activators of AMPK resulted in the identification of 3 novel compounds. As shown in Figure 12, these 3 compounds activate the kinase in a dose-dependent manner in the μARCS format. Compound 1 appears to be as potent or more potent than AMP in activating AMPK, whereas compounds 2 and 3 seemed less potent. At high concentrations, compound 3 demonstrated some inhibitory activity.

The screen identified 208 compounds with inhibitory activity. We determined dose-dependent inhibition, in the μARCS format, over a 4-log range of compound levels starting at 1000 pmoles (Fig. 13). Nine compounds demonstrated activity at 1 pmole, the

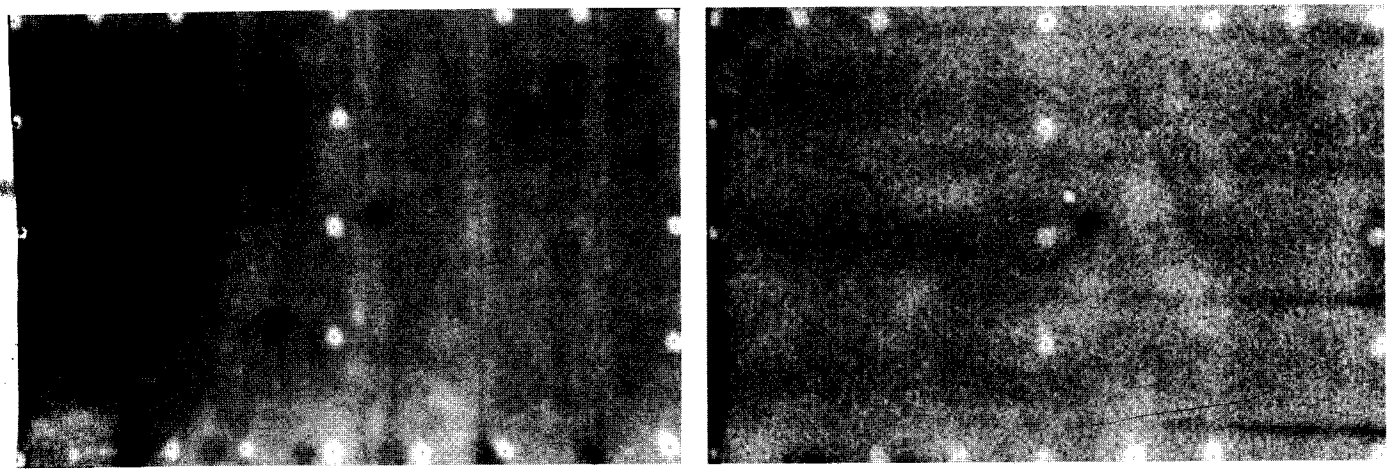
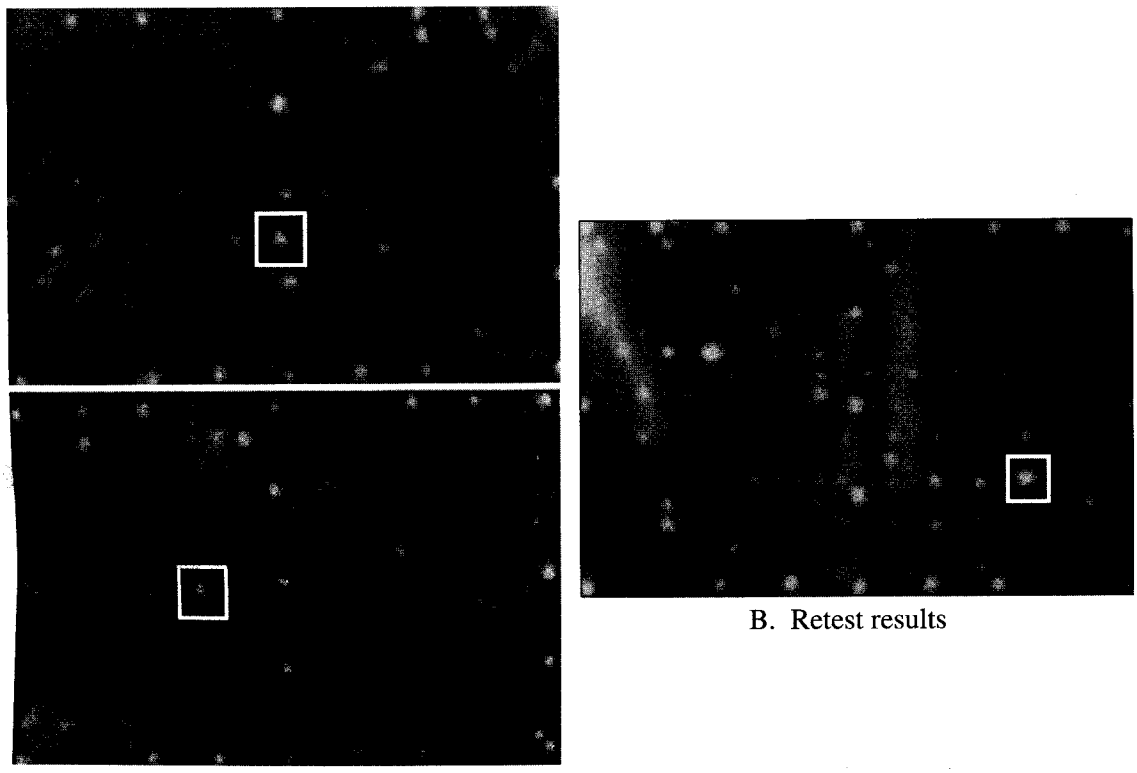


FIG. 10. Retest sheets completed in duplicate for AMP-activated protein kinase (AMPK) activators. Retest sheets are arrayed at a density of 384 compounds per compound card and completed in duplicate. The spot on each image is the compound picked from the primary sheet shown in Figure 9.



A. Primary screening results

B. Retest results

FIG. 11. Primary (**A**) and retest (**B**) sheet example for AMP-activated protein kinase (AMPK) inhibitors. The picks in the primary screen in the rectangular boxes illustrate a matched pick—active in both normal and deconvoluted sheets. The single box shown in the retest sheet demonstrates the activity of the same compound marked by the rectangles in the primary screen, demonstrating confirmation activity.

lowest quantity tested; 22 compounds showed activity at 10 pmoles; and 177 compounds showed inhibitory activity only at 100 pmoles or greater.

We evaluated the potencies of activators and inhibitors identified in the μARCS screen in the 96-well SAM™ plate assay (Fig. 14). Of the 3 AMPK-stimulating compounds identified in the

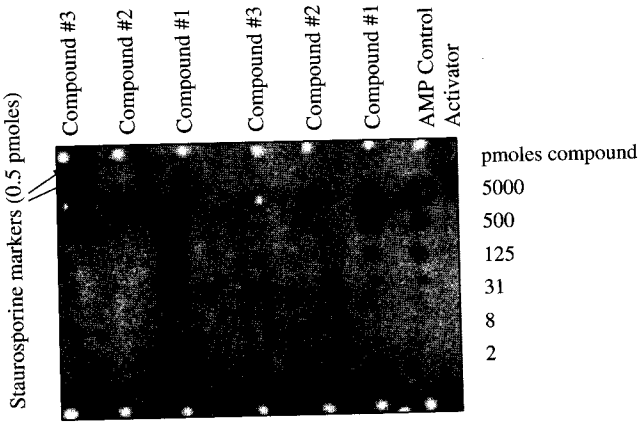


FIG. 12. Dose response of adenosine monophosphate (AMP) kinase activators in the microarrayed compound screening (μARCS) format. Compounds were hand-spotted in duplicate at amounts between 5000 and 2 pmoles. Staurosporine spotted on the top and bottom of the compound card was used to assist in locating the test compounds.

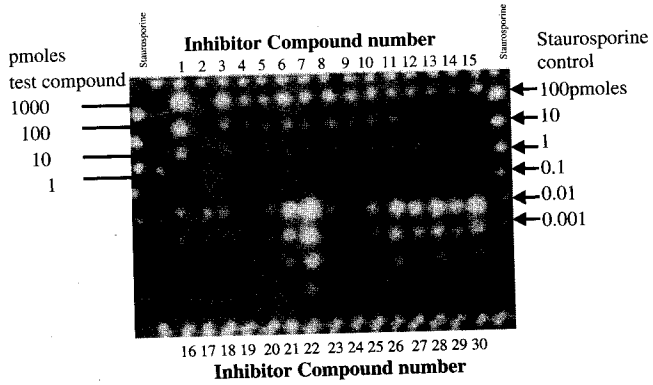


FIG. 13. Dose response of adenosine monophosphate (AMP) inhibitors in the microarrayed compound screening (μARCS) format. Compounds were hand-spotted starting at 1000 pmoles at log dilutions. Staurosporine spotted on either side of the compound card served as an activity reference. Thirty test compounds from the screen were spotted per card.

high-throughput assay, 2 produced dose-dependent AMPK activation in the 96-well assay. Figure 14A shows that activator 1 (compound 1) at 10 μM increased the activity of AMPK to an extent equivalent to that achieved by 200 μM AMP. Eight of the most potent AMPK inhibitors identified from the μARCS format were tested in the plate assay, and 4 displayed inhibitory activity in a dose-dependent manner. In Figure 14B, we demonstrate concentration-dependent AMPK inhibition using one of these inhibitors. With these data, we confirmed that both AMPK activators and inhibitors were identified using a μARCS screening format and show that the activity of these compounds were confirmed in the standard 96-well SAM™ membrane assay.

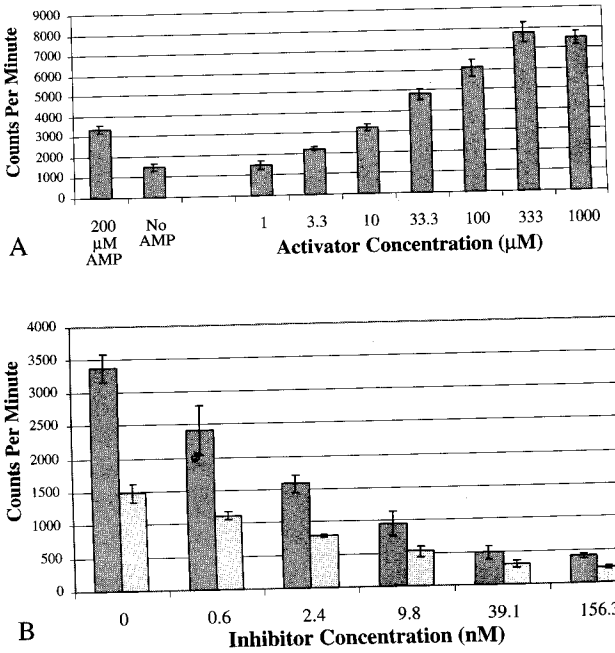


FIG. 14. Measurement of AMP-activated protein kinase (AMPK) stimulation and inhibition ±SD by compounds detected in microarrayed compound screening (μARCS) using the 96-well streptavidin affinity membrane (SAM™) membrane assay. These assays, performed in triplicate, are representative of at least 2 similar experiments. (**A**) Stimulation of AMPK by novel activator—compound 1 from Figure 12. (**B**) Inhibition of AMPK activity in the presence or absence of adenosine monophosphate (AMP) by inhibitor detected in the screen. With 200 μM AMP (dark-shaded columns); without AMP (light-shaded columns).

CONCLUSION

The μARCS AMPK assay can be used to screen large numbers of compounds in a simple, robust fashion, making it possible to rapidly evaluate a library of 700,000 low–molecular weight compounds. The assay demonstrated a good signal-to-noise ratio as well as good reproducibility. Both activators and inhibitors of AMPK were discovered in this screening campaign. The μARCS format provided a necessary substitute for a plate-based AMPK assay because a plate format would have been prohibitively expensive due to the high cost of SAM™ filter plates, ^{33}P-ATP, and excess enzyme required for a 96-well format. Disregarding the cost of enzyme and substrate, the cost of reagents (ATP, streptavidin membranes) used for the AMPK μARCS assay was under $15,000, compared to the cost of screening plates using our standard 10-compound mixture format, which was estimated to be more than $250,000.

In this article, we also report on the first use of a porous membrane rather than another agarose gel to introduce reagents. By

eliminating gel casting (the rate-limiting step in μARCS assays), the throughput of the assay was significantly improved. Because the membrane introduced ^{33}P-ATP into the assay and eliminated the need to pour radioactive gels and the associated cleanup, the throughput of the kinase assay has at least doubled. It was actually possible to screen more than 200,000 compounds (100,000 in duplicate) in an 8-h day.

Two observations made during the screen will need to be addressed in the future. Using the AMPK μARCS assay, we were able to discover novel activators and inhibitors that did not confirm in the plate format. It appears that the μARCS format may be able to identify low-potency compounds that would not be detected using the plate format. For example, compound 1 in our plate format had an EC_{50} of 25.6 ± 6.4 μM. If a plate screen had been attempted at 10 μM, the screen would most likely have missed this compound as a possible activator of AMPK. Likewise, many of the less intense spots (picks) selected from the primary screen for AMPK inhibitors were inactive in the plate format but reproduced very well in the dose-dependent μARCS format. We are currently conducting a study to investigate the comparison of dose-dependent activities in the plate format versus the μARCS format in several μARCS assays. In addition, it was surprising that we obtained similar results using a porous membrane versus agarose even though 10-fold less ATP was contained in the membrane. This calls into question the quantity of ATP we are delivering into the assay using the μARCS format. These phenomena will require further investigation. Most important, the AMPK assay presented here demonstrates the utility of μARCS radiometric kinase assays, showing the ability to identify novel activators and inhibitors.

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