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Development of an Inhibitor Screening Platform via Mass Spectrometry

RAKESH RATHORE,[1] JAY CORR,[2] GEORGE SCOTT,[2] PAULINE VOLLMERHAUS,[2] and KENNETH D. GREIS[1]

Commonly used methods for isolated enzyme inhibitor screening typically rely on fluorescent or chemiluminescent detection techniques that are often indirect and/or coupled assays. Mass spectrometry (MS) has been widely reported for measuring the conversion of substrates to products for enzyme assays and has more recently been demonstrated as an alternative readout system for inhibitor screening. In this report, a high-throughput mass spectrometry (HTMS) readout platform, based on the direct measurement of substrate conversion to product, is presented. The rapid ionization and desorption features of a new generation matrix-assisted laser desorption ionization-triple quadrupole (MALDI-QqQ) mass spectrometer are shown to improve the speed of analysis to greater than 1 sample per second while maintaining excellent Z′ values. Furthermore, the readout was validated by demonstrating the ability to measure IC_{50} values for several known kinase inhibitors against cyclic AMP–dependent protein kinase (PKA). Finally, when the assay performance was compared with a common ADP-accumulation readout system, this HTMS approach produced better signal-to-background ratios, higher Z′ values, and a reagent cost of about $0.03 per well compared with about $0.60 per well for the fluorescence assay. Collectively, these data demonstrate that a MALDI-QqQ-MS–based readout platform offers significant advantages over the commonly used assays in terms of speed, sensitivity, reproducibility, and reagent cost. (*Journal of Biomolecular Screening* 2008:1007-1013)

Key words: protein kinase A, high-throughput screening, mass spectrometry, matrix-assisted laser desorption ionization (MALDI), label-free screening

INTRODUCTION

HIGH-THROUGHPUT SCREENING (HTS) plays a central role in the drug discovery process starting at the early "hit" discovery stage then continuing through to lead optimization. As such, the screening community has continuously developed new technologies to meet the broadening assay needs and to expedite the work flow of drug discovery. Within the pharmaceutical and biotechnology enzyme screening community, fluorescent-and chemiluminescent-based detection methods continue to be the routine assay platforms of choice for isolated enzyme assays. These assays are desirable because they have been developed to be simple homogeneous assays that often offer universal methods to evaluate a variety of targets using the same reagents. However, 1 of the primary challenges for these approaches continues to be how to maintain the high speed of analysis while minimizing false-positive or false-negative readouts. Inherent to this challenge is the nature of fluorescence and chemiluminescence readout, which can lend itself to false readouts because of the properties of various compound classes that enhance or quench the signals. Thus a comparable approach in speed, robust readout, and cost that further minimizes the potential for false readouts would be a benefit to the field.

One approach that offers promise in this arena is mass spectrometry. A variety of mass spectrometry (MS) techniques have been used to measure enzyme activity, enzyme kinetics, inhibition, and more recently for HTS applications (reviewed in the studies of Greis[1] and De Boer et al.[2]). MS-based assays offer a rapid, sensitive, and direct approach to measure the effects on enzyme activity by simultaneously determining the relative quantity of substrate and product. Unfortunately, the cost and low throughput has typically limited the application of MS to small libraries or to hit follow-up assays.

Previous studies in our laboratory have demonstrated the potential of matrix-assisted laser desorption ionization (MALDI)-MS–based inhibitor screening by using the ratios of substrate and product to investigate the inhibition potency.[3] In addition, others have reported the successful implementation of an electrospray MS-based approach to screen inhibition of enzyme reactions

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based on the same substrate/product ratio principle.[4] Unfortunately, in both of these approaches, the maximum speed of analyses was limited to ~1 sample analyzed every 5 to 10 sec, thus limiting their utility for primary screens from large compound repositories. This report describes the development of a MALDI-triple quadrupole-MS (MALDI-QqQ-MS) approach as a readout method for inhibitor screening. To demonstrate the utility of this method, cyclic AMP–dependent protein kinase (PKA)–catalyzed phosphorylation was evaluated with a series of commercial kinase inhibitors. In addition, this readout is also compared with the PKA assay performance using the commercially available ADP Hunter™ kit from DiscoveRx Corp. (Fremont, CA). Finally, additional advantages of the MALDI-QqQ-MS readout as an alternative tool to current methods of high-throughput inhibitor screening are discussed.

MATERIALS AND METHODS

Materials

The catalytic subunit of cAMP-dependent protein kinase (PKA, V5161) and kemptide (V5601) were purchased from Promega (Madison, WIi). α-Cyano-4-hydroxycinnamic acid (4-HCCA), staurosporine, H-89 dihydrochloride hydrate, and GF109203X were purchased from Sigma-Aldrich (St. Louis, MO). Ammonium monobasic phosphate and formic acid were from EMD Biosciences (Gibbstown, NJ) and J. T. Baker (Phillipsburg, NJ), respectively. Hit Hunter™ ADP Hunter Plus kit was obtained from DiscoveRx Corp.

Liquid handling robotics

All assay reactions were prepared on 384-well plates using a Beckman Coulter Multimek NX (Fullerton, CA) equipped with a 384-well low-volume transfer head. PKA enzyme, compound dilutions, the peptide substrate/ATP, and matrix solution plates were prepared on separate plates using the same workstation.

MS-based PKA assay

PKA control reactions and inhibition assays were prepared on 384-well plates (Corning 3572, Corning, NY) as described previously[3] but with minor modifications. In brief, the reactions were initiated by transferring and mixing 5 μL of the compound dilution series into the assay plate containing 20 μL of enzymes immediately followed by the transfer of 25 μL of the substrate peptide mixture (kemptide and ATP) to the assay plate to start the reaction. The specific assay conditions for PKA enzyme reaction were as follows: 0.2 units per well PKA in 40 mM Tris:HCl, pH 7.5, containing 20 mM $MgCl_2$, 0.1 mg/mL BSA, 5 μM ATP, and 0.5 μM kemptide substrate. After 30 min, the reactions were quenched by removing 5 μL of the reaction mixture into 45 μL of MALDI matrix solution (5 mg/mL 4-HCCA, 5 mM ammonium phosphate monobasic in 50% acetonitrile/0.1% formic acid), then transferring ~1 μL onto the MALDI target plates via the Beckman NX system. The total time from quenching the reaction to spotting the samples onto the MALDI target plate is less than 30 sec.

Fluorescence PKA assay

PKA activity was measured in 384-well plates (Greiner Bio-One 781096, Greiner Bio-One North America Inc., Monore, NC) as ADP accumulation using the ADP Hunter™ Plus Universal Kinase Assay kit from DiscoveRx Corp. by following the vendor's instructions. Reactions were initiated robotically as described for the MS-based assays. The specific reaction conditions were as follows: 15 mM HEPES, pH 7.5, 20 mM NaCl, 1 mM EGTA, 0.02% Tween 20, 10 mM MgC12, 0.1 mg/mL bovine-γ-globulins, 6 μM kemptide substrate, 100 μM ATP, and 0.1 units of PKA enzyme per well. After 30 min, reagents A and B from the kit were added and the fluorescence intensity was measured after 45 min with an Evotec Technologies plate vision detector (λ ex = 530 nm and λ em = 580 nm).

MALDI-TOF and MALDI-QqQ readout

All matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectra were collected on an Applied Biosystems (Foster City, CA) 4800 MALDI-TOF/TOF instrument equipped with a Nd:YAG laser (355 nm, 3 ns pulse width, 200 Hz repetition rate) in positive ion reflector mode. Automated acquisition parameters were adjusted to capture and average only those individual spectra within defined success criteria as described previously.[3]

MALDI-QqQ data were collected on an Applied Biosystems/MDS Analytical Technologies FlashQuant™ MALDI-QqQ system (Concord, ON), which included a diode-pumped solid-state UV laser with an Nd:YLF crystal (349 nm, 5 ns pulse width, 1000 Hz repetition rate) and an API 4000 QTRAP® mass spectrometer system. The 4000 QTRAP® system was operated in selected ion monitoring (SIM) mode with dwell times of 5 ms (four ions monitored) or 10 ms (two ions monitored). Laser raster speed across the MALDI sample plate was typically 4.5 mm/sec or 9.0 mm/sec. Technical details about the FlashQuant™ system are reported elsewhere.[5]

Data analysis

Peak areas for the substrates and products of each enzyme reaction were used to calculate percent product and subsequently percent inhibition as previously reported.[3] IC_{50} values were calculated with 95% confidence interval using the GraphPad Prism 5.0 program, based on a sigmoidal dose-response using a 3-parameter logistic equation and nonlinear regression without fixed parameters. Each experiment was repeated a minimum of 3 times to establish the reproducibility. Data presented in each graph are the mean ± standard deviation of triplicate determinations ($n = 3$ wells per data points). The Z′ values were calculated as previously described.[6]

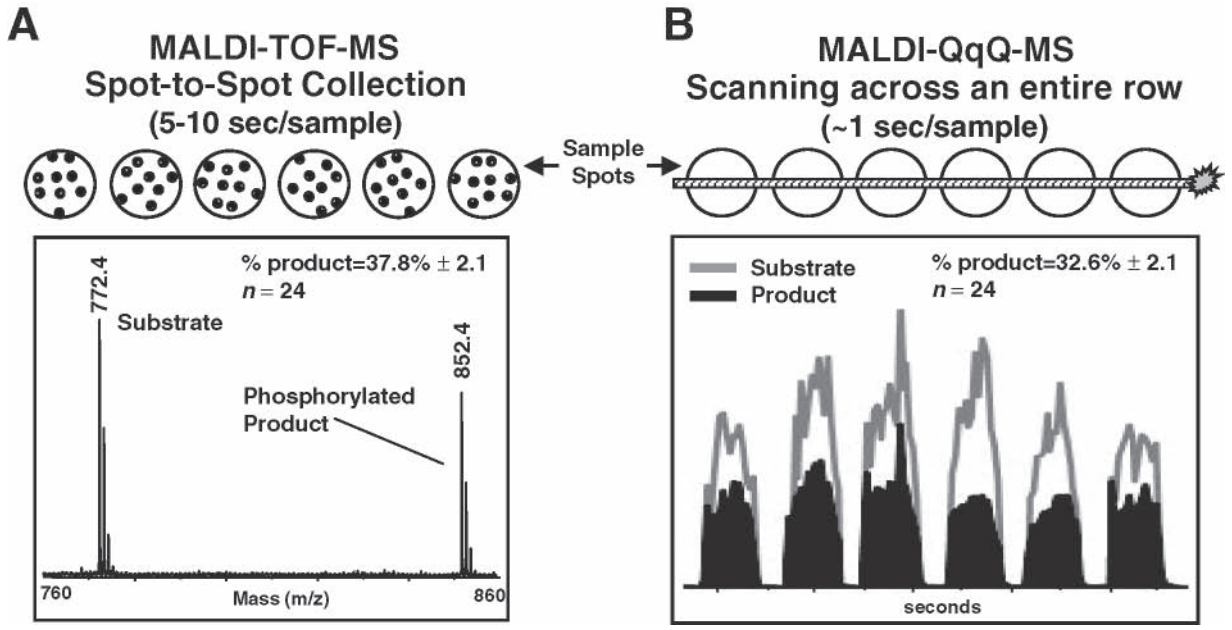

FIG. 1. Comparison of the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) ionization and data output with the matrix-assisted laser desorption ionization-triple quadrupole (MALDI-QqQ) system. (**A**) MALDI-TOF readout of the control cyclic AMP–dependent protein kinase reaction. The data are collected from spot to spot by averaging a few hundred laser shots at multiple positions on each spot (the dots on each sample spot represent the various positions of the laser). The peak area of the substrate and product are then extracted to determine the percent product formed. (**B**) The selected ion readout of the substrate and product masses detected using the FlashQuant™ (MALDI-QqQ) system. The 1-kHz laser allows for near continuous ionization and desorption across an entire row of samples with the data collected on the triple quadrupole to determine the relative quantities of the substrate and product. The line through the spots in the upper portion of panel **B** represents the track of the laser ionization.

RESULTS AND DISCUSSION

The ability to directly quantify the loss of substrate and/or the gain of product during the enzyme reaction is an ideal way to determine enzyme activity and inhibition. The MALDI-QqQ mass spectrometer used in this study provides such a direct readout system with speeds and reproducibility appropriate for HTS.[4] Conceptually, this approach is similar to previous reports on MS-based screening[3,4] in that they all take advantage of the properties of MS to directly quantify the substrate and/or product to reliably measure enzyme activity. The primary advantage being the virtual elimination of false-positive or false-negative readouts, which are prevalent in many coupled and/or chromogenic or fluorogenic readout approaches. For example, a kinase reaction readout using a MALDI-TOF-MS produces a profile in which the ratio of the substrate and product can be used as an accurate measure of the degree of enzyme activity (**Fig. 1A**). When one adds increasing concentrations of kinase inhibitors, the ratio for the percent product formed decreases according to the potency of the inhibitor; thus accurate IC_{50} curves can be generated.[3] Unfortunately, the logistics of data collection on a MALDI-TOF instrument require the averaging of multiple laser shots at various positions on each spot; thus the readout speed is limited to ~5 to 10 sec per sample (**Fig. 1A**). Alternatively, the advantages of combining a new generation MALDI-based ionization system with a triple quadrupole (QqQ) mass spectrometer are illustrated in **Figure 1B**. First, this system takes advantage of a 1-kHz laser to produce a continuous desorption across an entire row of samples at rates that can be set to less than 1 sec per sample. Second, the QqQ readout offers the ability to measure the intensities of multiple ions simultaneously without the need to collect full range spectra, thus improving both the speed of acquisition and the sensitivity of detection. For example, as demonstrated in **Figure 1B**, as the laser scans across each spot on the target plate, the mass spectrometer measures the intensity of the substrate (gray line) and the product (black area) masses across each spot. The area of the substrate and product from each spot are then used to determine the amount of enzyme activity detected—in this case, an average of 32.6 ± 2.1%. The loss of the conversion of substrate to product in the presence of an inhibitor can then be readily detected as the degree of inhibition in either single point assays or as dose-dependent inhibition curves thus fulfilling the critical needs for an HTS assay.[7]

To validate the MALDI-QqQ-MS readout system, a series of reactions using PKA were evaluated. PKA was chosen as the test case because it represents a typical kinase reaction and information from a variety of assay systems is available for this kinase.[8] In addition because protein kinases represent the

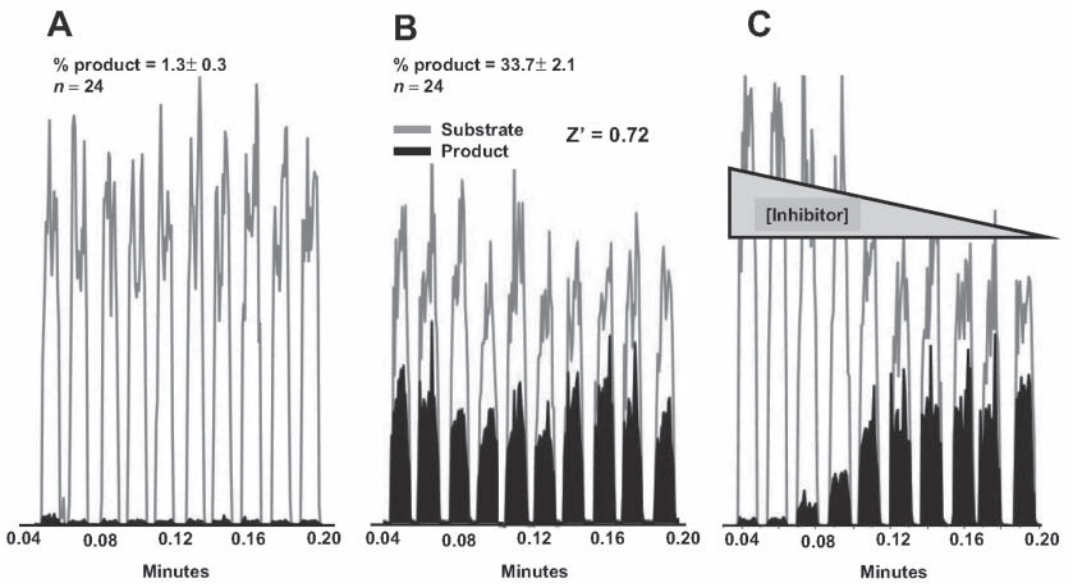

FIG. 2. An example of the raw data readout from a matrix-assisted laser desorption ionization-triple quadrupole (MALDI-QqQ) instrument. Selected ion monitoring from the MALDI-QqQ instrument for substrate and product of cyclic AMP–dependent protein kinase from a single row of a 24 × 16 sample array. Note that only 10 sample positions are shown in the illustration for each figure to improve the clarity of the substrate and product areas detected. (**A**) The background reactions with cofactors and substrate (but no enzyme) demonstrating a minimal background signal. (**B**) The control reaction containing the enzyme and all the necessary components for activity. (**C**) Selected ion monitoring scans from a 10-point inhibitor series with staurosporine starting at 2 μM followed by 5× dilutions.

largest druggable gene family within the human genome[9] and some reports indicate that as much as 24% of all research spending on drug discovery and development is focused on kinase targets,[10] PKA represents an appropriate enzyme in this class for testing a new readout system. Triplicate samples of PKA reactions were prepared—including the appropriate control and background reactions as illustrated in **Figure 2**. For background reactions, the average readout for percent product was 1.3 ± 0.3% (**Fig. 2A**). Upon addition of enzyme and incubation at room temperature for 30 min, the control reaction produced a percent product of 33.7 ± 2.1% (**Fig. 2B**). One advantage of using the ratio of substrate and product to measure enzyme activity is also illustrated here in that the absolute intensity of the signal does change from sample to sample; however, the ratio of the substrate to product is quite constant resulting in excellent Z′ values[5] that average >0.72 across replicates of samples. One concern that may be raised when using the percent product as the measure of enzyme activity relates to the linearity of the response for the substrate and product at variable molar concentrations. Specifically, chemical changes in the molecule when converting a substrate to a product can drastically affect the ionization efficiency and detection of the molecule; thus the concern is that the change in ionization efficiencies will affect the mass spectrometry readout. Evaluation of this effect has been investigated previously and shown to be negligible for phosphorylated peptide products because the response is linear ($r^2 > 0.985$) across the entire range from 0% product to 95% product.[1,3] To date 9 different substrate/product pairs representing kinases, phosphatases, proteases, and a hydroxylase enzyme have been evaluated using MALDI-MS as the readout system and all have shown a linear substrate/product response (unpublished results). Finally, for this readout to be useful for inhibitor screening, dose-dependent inhibition must also produce reliable results. **Figure 2C** shows the direct readout results from a 10-point inhibitor dilution series using staurosporine—a widely used general kinase inhibitor. Note that the data are of sufficient quality for the IC_{50} to be visualized directly within the raw data—in this case at ~32 nM, consistent with previously reported values for PKA inhibition with staurosporine.[3] A more detailed evaluation of additional inhibitors is provided later in this report.

A 2nd advantage of the MALDI-QqQ readout system is the ability to scan an entire 384-well plate very rapidly while maintaining sufficient data quality. From previous reports the fastest rate for electrospray or MALDI-based methods was ~5 to 10 sec per sample.[3,11,12] To test the limit of acquisition speed on the MALDI-QqQ system, an entire 384-well plate of control reactions was scanned at a standard rate of 1.2 sec per sample (<8 min per plate) and then again at the fastest rate of 0.3 sec per sample (<2 min per plate). As is demonstrated in **Figure 3**, even at the highest acquisition rate, the reproducibility of the substrate and product ratios are maintained with a robust Z′ value at 0.65. As described above, even though the absolute intensity of the signals do vary across the plate (specifically at

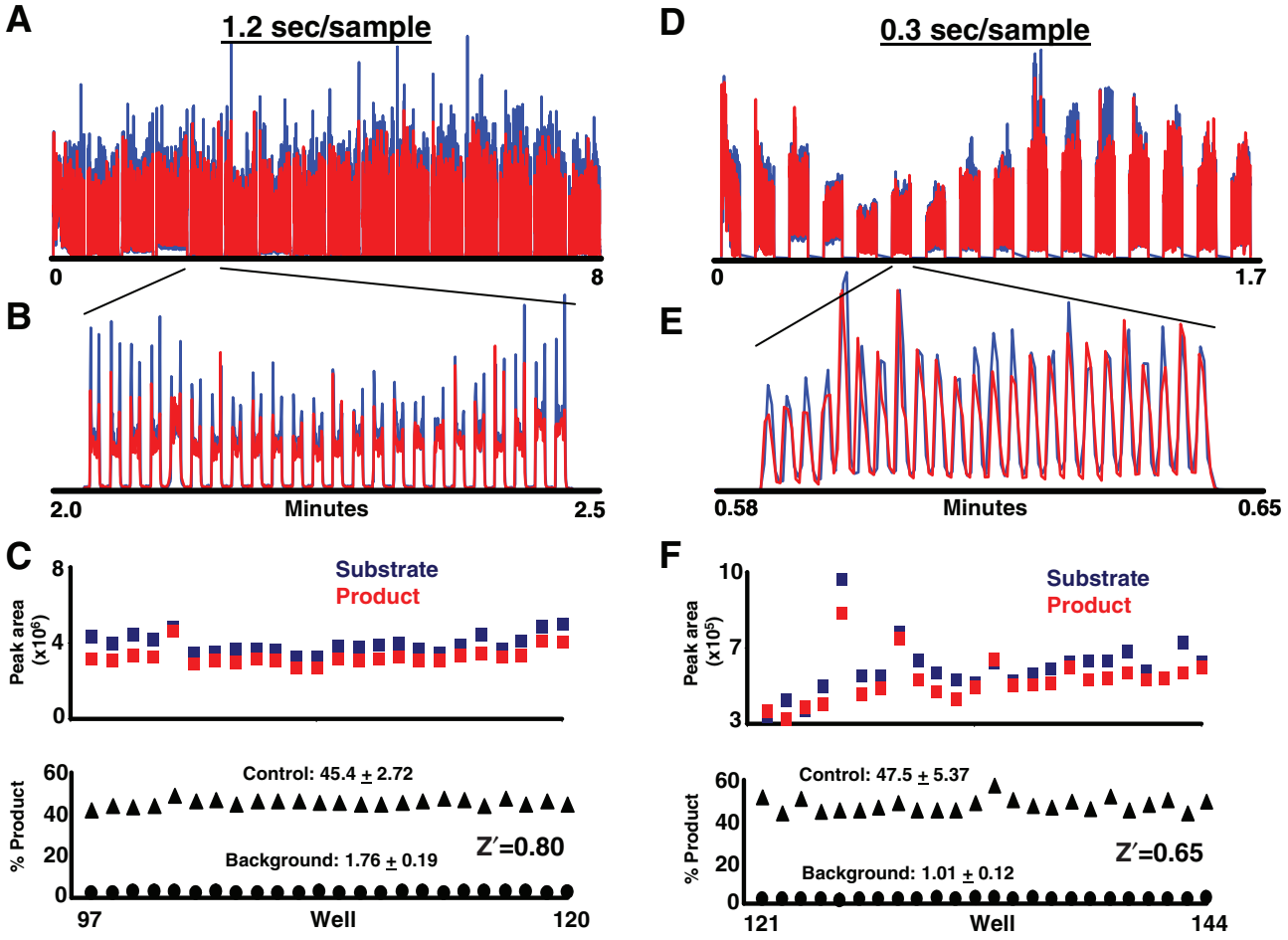

FIG. 3. The effect of acquisition speed on assay robustness. Control cyclic AMP–dependent protein kinase reactions were spotted onto a 384-well plate with the substrate (blue) and product (red) masses evaluated at various data collection speeds in selected ion monitoring mode. (**A**) Scanning across an entire 384-well plate in 8 min. (**B**) The expanded range of a single 24-well row of samples from panel **A** showing the substrate product profiles. (**C**) A plot of the raw substrate and product areas from the indicated row of 24 samples (top) and a plot of the percent product in control reactions (solid triangles) and background reactions (solid circles) to show the normalization of the data when plotted as percent product (bottom). The average and standard deviations of the percent product in control and background reactions are presented in panel **C** and were used to calculate Z′ values. Panels **D** to **F** are the same as **A** to **C**, but with the scan rate ~4 times faster—1.75 min to scan an entire 384-well plate. The background measurement in panels **C** and **F** were collected from a separate plate with no enzyme added to the reaction mixture.

the fastest rate; **Fig. 3D**), the ratio of substrate and product remain constant thus providing a robust and reproducible readout of the enzyme activity (**Fig. 3C** and **F**). At readout speeds of a sample per second or less, the MALDI-QqQ system is clearly on par with other HTS readout systems. The only added time for an MS-based readout compared with other plate readers is the time required to get the sample plate into the mass spectrometer and under vacuum. The current vacuum cycle time for the FlashQuant™ system is ~1.5 min per plate; thus at the normal readout speed of ~1.2 sec per sample the plate-to-plate cycle and readout time is just over 9 min per plate. At the fastest readout speed tested (0.3 sec per sample), the total cycle time for a 348-well plate is just under 3.5 min per plate—a rate that is comparable with those of other HTS readout systems.

As further validation of the MALDI-QqQ readout as a viable alternative to HTS assays, this approach was compared with a popular commercial ADP accumulation assay.[13] One advantage of this fluorescent kinase assay is that it can be used for a variety of kinase reactions inasmuch as the readout is based on the formation of ADP as 1 of the products.[10] However, potential drawbacks include the cost of reagents (~$0.60 per well) and the necessity to use a series of 3 coupled enzyme reactions to go from ADP concentration to fluorescence readout. Thus any compound that might interfere with any of these coupled reactions or the fluorophore could result in false readouts. By comparison, the MALDI-QqQ readout offers the use of label-free substrates (<$0.03 per well) and a direct measure of substrate to product conversion, thus eliminating false readouts due to compound

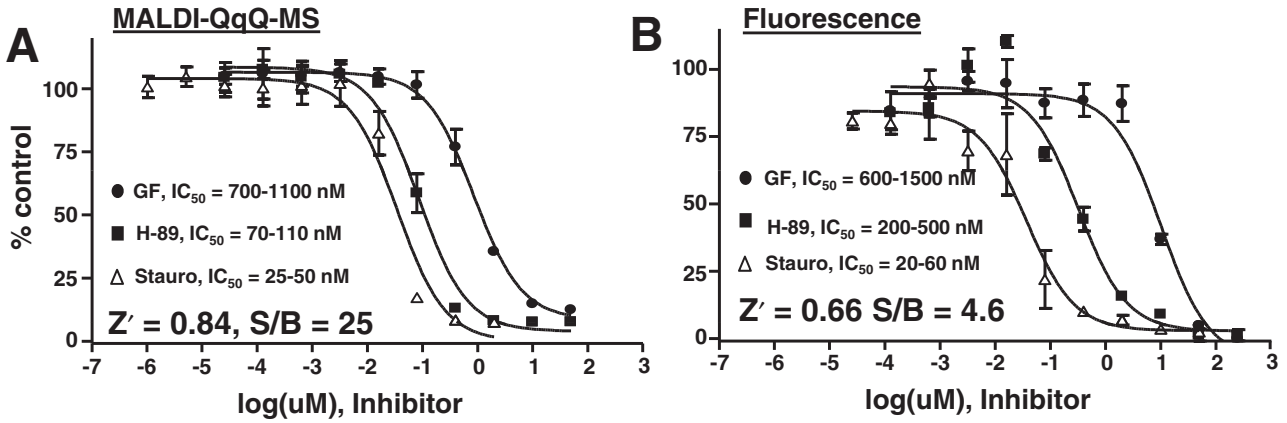

FIG. 4. Matrix-assisted laser desorption ionization-triple quadrupole (MALDI-QqQ) inhibitor readout compared with a fluorescence ADP accumulation assay. The MALDI-QqQ inhibitor readout (**A**) and fluorescence ADP accumulation readout (**B**) for 10-point dose-dependent inhibition assays for cyclic AMP–dependent protein kinase were set up to evaluate the IC_{50} values for a series of commercial kinase inhibitors (GF109203X, H-89 dihydrochloride hydrate, and staurosporine). The inhibitor assays were done in triplicate (mean ± standard deviation and $n = 3$ wells per data points). The Z′ values and the signal-to-background (S/B) ratio are also provided.

quenching or interference. As demonstrated in **Figure 4**, the MS readout compares favorably with the ADP-accumulation assay when 3 different compounds (staurosporine, H-89, and GF109203X) were evaluated as inhibitors of PKA. The IC_{50} curves demonstrated the same rank order of inhibitor potency with overlapping 95% confidence intervals for both staurosporine and GF109293X. Some variation was noted in the IC_{50} values for the H-89 inhibitor between the 2 assays. The origin of this variation is likely related to the different concentrations of ATP used in the 2 assays (5 μM and 100 μM for MS and fluorescence assay, respectively), however, the mass spectrometry readout at 70 to 100 nM is comparable with the reported IC_{50} values for H-89 against isolated PKA between 48 and 135 nM.[14] Overall the MALDI-QqQ method produced a significantly better signal-to-background ratio (25 for MS vs. 5 for fluorescence) and standard deviation, which ultimately produced Z′ values on the order of 0.65 to 0.85 compared with 0.55 to 0.70 for the fluorescence readout. In terms of reagent costs, the MS readout also offers a significant advantage. For example, the reagent cost for a MALDI-QqQ readout is only ~$0.03 per well for the buffer and the unlabeled peptide substrate compared with $0.60 per well for ADP accumulation assay. Although this may not seem all that significant when measuring only a few test validation samples, when scaling up to evaluate a compound repository of 100,000 compounds, the reagent cost difference becomes $3000 for the MS versus $60,000 for the fluorescent readout. At this rate of saving in reagent costs alone, the investment cost for the mass spectrometer ($350,000 to $450,000) could be recovered after only a short time by a productive screening group. Thus with comparable readout speeds, better signal-to-background ratios, a significant reagent cost advantage, and the likelihood of fewer false-positive or false-negative results, the MALDI-QqQ readout represents a major step forward for the HTS readout of isolated enzyme reactions.

Going forward, several other features of this MALDI-QqQ-MS–based screening method may provide even greater potential for HTS. For example, the MS readout enables simultaneous measurement of multiple substrate and product pairs from each reaction well, thus opening the possibility for multiplex enzyme reactions screening. In preliminary studies, we have demonstrated the ability to measure activity and inhibition of a kinase and a protease in the same reaction mixture without interference.[15] Additional evaluation of wider classes of compatible enzyme reactions are needed to determine the scope of this capability, but the ability to multiplex enzyme reactions opens the door to using a single primary screen through a compound repository to get "hits" for multiple therapeutic targets.

In closing, some of the challenges that still need to be addressed for the MALDI-QqQ-MS system include the full scope of enzyme classes that can be measured using this method, the robustness of the instrument, and the integration of the MS system with the liquid handling robotic system for unattended plate loading into the mass spectrometer. First, thus far we have only evaluated enzyme classes that have peptide substrates, including PKA, as representative of kinases. We have begun to evaluate proteases, phosphatases, hydroxylases, and deacetylase enzymes—each of which is readily detected by an MS readout (unpublished data). Additional studies will be directed at other enzyme classes with nonpeptide substrates. Second, because this is the 1st report addressing the HTS potential of this new instrument platform, we have not fully evaluated the long-term robustness of the mass spectrometer in full HTS mode. However, based on sensitivity data collected under continuous operation using a series of 5 diverse compounds on this MALDI-QqQ instrument, a linear loss of sensitivity was recorded down to ~60% of the original activity at the end of 1 week (unpublished data). Inasmuch as the

signal-to-background ratios for these screening assays have been shown to be ~25, this loss of sensitivity could likely be managed down to a specific sensitivity level after which the instrument would need to be cleaned. Noting that continuous collection for 1 week in an HTS mode would be equivalent to about 1000 plates (384K wells), at the standard scan rate or about 2500 plates (960K well), at the fastest rate, we anticipate being able to evaluate a significant number of samples prior to needing to clean the instrument. Clearly, however, significant additional evaluation and validation of how robust this system will be in a full HTS mode still must be investigated. Finally, the system in its current configuration required manual loading of target plates 1 at a time. However, we are continuing to work toward full automation from the enzyme assay through the sample readout. To date, unattended loading of sample plates has been accomplished via a standard robotic arm (unpublished results), but there is still considerably more work to done in the development of a fully automated HTS readout system. Nonetheless, the data presented in this report provide a solid basis for the continued development and application of MS-based technology from HTS.

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