

Advances in the SAM²™ Membrane Technology: High Throughput Biotin Capture Systems for Use in Rapid Screening



By Said A. Goueli*, Kevin Hsiao* and Claire Ruzicka*
Promega Corporation

*Corresponding authors: e-mail to sgoueli@promega.com, khsiao@promega.com or cruzicka@promega.com

The ability of Promega's SAM²™ Membrane to efficiently capture a variety of biotinylated molecules from solution makes it useful for a number of applications. This article describes the development and use of the SAM²™ Membrane and new products and applications of this technology, particularly for high throughput systems. A plate-to-plate comparison demonstrates the strengths of the SAM²™ Membrane in the SAM²™ 96 Biotin Capture Plate, as compared to other commercially available biotin capture products.

INTRODUCTION

Promega has developed a unique streptavidin membrane for capturing biotinylated molecules. The proprietary process by which the SAM²™ Membrane** is prepared results in a high density of streptavidin linked to the membrane surface, and thus a high binding capacity for biotin. The binding of biotinylated molecules to the SAM²™ Membrane is rapid and strong; it is unaffected by extremes in pH (2.0-10.0), temperature, organic solvents, ionic and nonionic detergents (SDS, CHAPS, Triton[®] X-100, Tween[®] 20 or Tween[®] 80) and other denaturing agents (5M guanidine hydrochloride and 2M urea) (1). The tenacious nature of the bound biotinylated molecules greatly minimizes their removal from the membrane during the washing procedure, allowing accurate assessment of the total amount of biotinylated molecules present. This high affinity, highly selective binding also results in a very low degree of nonspecific binding, and thus low background counts, when using the SAM²™ Membrane technology. A remarkable feature of this affinity-based binding is that binding is independent of the primary amino acid sequence of the peptide or the nucleotide sequence of the oligonucleotide.

** Patent Pending.

The SAM²™ Membrane has recently been introduced in two new formats for use in drug discovery and other high throughput screening applications: a single SAM²™ Membrane measuring 7.6 x 10.9cm and a white, 96 well plate containing the SAM²™ Membrane. Both formats demonstrate linear binding of biotinylated molecules to the SAM²™ Membrane over a wide range of biotin concentrations, with a maximum binding capacity of 2.5nmol/cm² for the membrane and 500pmol/well for the SAM²™ 96 Biotin Capture Plate.

Because of its ability to efficiently capture a variety of biotinylated molecules from solution, the applications for the SAM²™ Membrane are numerous. This article describes the use of the SAM²™ Membrane technology in high throughput kinase assays, provides comparisons against other available methods and suggests other potential applications for the SAM²™ Biotin Capture Membrane.

SIGNIFICANCE OF PROTEIN KINASES

Protein kinases and phosphatases play an important role in a variety of cellular functions such as cell growth, development, differentiation and cell death (2), and are popular targets in the search for novel therapeutic agents (3). It is estimated that one-third of the proteins in a typical mammalian cell are phosphorylated and about 200 protein kinases and 100 protein phosphatases have been identified. Approximately 2-3% of the genes in a typical eukaryotic cell are predicted to code for protein kinases and as many as 5% of human genes may encode protein kinases and phosphatases (4). Not only are there hundreds of protein kinases and phosphatases, but these enzymes have multiple substrates *in vivo*, which may explain their diverse physiological functions. Thus, there is a strong interest in developing an assay system that can detect the activity of individual protein kinases in crude cellular or tissue extracts. The ideal assay should be fast, easy to perform, give a true estimate of enzyme activity under optimal kinetic conditions and provide the sensitivity required for detection of low amounts of enzyme activity in samples. These features are of critical importance to scientists screening for kinase inhibitors or activators in a large volume of samples and have been achieved with the SAM²™ Membrane and SAM²™ 96 Biotin Capture System.

EVOLUTION OF THE SAM²™ MEMBRANE

Promega first developed the SAM²™ Membrane for use in the SignaTECT™ Protein Kinase Assay Systems (5,6). These assays utilize kinase-specific biotinylated peptide substrates that can be phosphorylated using [γ -³²P]ATP and the cognate protein kinase (either in pure form or in crude cellular or tissue extracts). The phosphorylated, biotinylated peptide is captured on the SAM²™ Membrane. Upon washing of the membrane, unincorporated radioactivity and other endogenously phosphorylated proteins in the extract are removed. This method is a significant improvement over the traditional ion exchange-based methods such as phosphocellulose (P81) (7) because it offers high sensitivity and selectivity for accurate quantification of kinases in crude cell extracts.

The SAM²™ Biotin Capture Membrane has now been scaled up for use in high throughput formats. The newly resized SAM²™ Membrane and the SAM²™ 96 Biotin Capture Plate, which contains the SAM²™ Membrane, are identical in composition to the original SAM²™ Membrane and are used in the same manner as the original membrane. After the kinase assay is performed and terminated, a multichannel pipettor or robotic liquid delivery system is used to transfer 1-25 μ l of sample to the solid membrane or plates containing the membrane. The membrane and the SAM²™ 96 Biotin Capture Plates are washed with consecutive washing solutions (5,6); a vacuum manifold is used to remove the wash solutions and radioactive waste from the plate. The plates and membrane are then dried. Radioactivity retained on the membrane is quantified by PhosphorImager™ (Molecular Dynamics) analysis or conventional autoradiography. When using the SAM²™ 96 Biotin Capture Plates, radioactivity can be quantified by using a MicroBeta® TriLux liquid scintillation counter (EG&G Wallac, Inc.) or the TopCount™ microplate scintillation counter (Packard Instrument Company).

To illustrate the utility and superior performance of the SAM²™ 96 Biotin Capture Plate as compared to other commercially available systems for high throughput screening, we have performed kinase assays using several protein kinases. Here we demonstrate the features of the system using the enzyme cAMP-Dependent Protein Kinase (PKA) and assaying for its activity in the absence of substrate (enzyme control), in the presence of substrate alone (biotinylated Kemptide control) and in the presence of the complete system for assay of PKA.

PERFORMANCE OF THE SAM²™ 96 BIOTIN CAPTURE PLATE

We previously demonstrated, using SAM²™ Membranes supplied in the Promega SignaTECT™ PKA Assay System, that biotinylated Kemptide not only serves as an optimal substrate for purified PKA, but also gives an accurate assessment of PKA activity in the extracts tested due to the specific binding of the phosphorylated biotinylated peptide to the membrane (5,6,8). In addition, using the SAM²™ Membrane, there was low to nonexistent binding of endogenously phosphorylated proteins present in the extract, and low nonspecific binding of [γ -³²P]ATP. Here we demonstrate that enzyme activity of PKA can be determined with the 96 well plate format in a manner identical to that determined with the SignaTECT™ PKA Assay System using the SAM²™ Membrane. Furthermore, the radioactivity determined in the absence of the enzyme (substrate control; "Sub") or in the absence of the substrate (enzyme control; "PKA") represents less than 0.02% of input counts of 400,000cpm (Table 1). Counts varied from 12 to 36.7cpm for the substrate control and between 10 and 42.8cpm for the enzyme control; PKA activity for the complete system ("PKA + Sub") was 22,017.8 to 28,139.2cpm. The assay was carried out with maximal time efficiency, where the complete washing procedure took only 5 minutes. Percent coefficient of variation ("% CV") for the enzyme activity ("PKA + Sub") did not exceed 8%, indicating highly reproducible results. Similar percent coefficient of variation values were obtained when assays were carried out at various times and on different plates (data not shown).

Table 1. PKA Activity as Measured Using Promega's SAM²™ 96 Biotin Capture Plate.

	Sub	PKA	Sub	PKA + Sub	PKA + Sub	PKA + Sub
A	12.0	40.2	30.1	26,438.5	28,139.2	27,414.2
B	30.1	36.2	22.1	24,518.1	22,141.8	24,507.8
C	24.1	22.1	26.1	22,017.8	24,339.8	28,132.8
D	28.1	18.1	18.1	23,666.6	22,707.2	22,633.5
E	20.4	10.2	14.3	25,403.9	24,356.9	23,825.4
F	22.4	32.6	30.5	24,695.9	23,028.3	25,019.1
G	18.3	42.8	22.4	24,051.2	24,315.3	23,263.9
H	16.3	40.7	36.7	26,886.9	25,741.5	24,070.7
AVE	21.5	30.4	25.0	24,709.9	24,346.3	24,858.4
STD	6.02	12.09	7.29	1,559.11	1,913.01	1,948.73
% CV	28.06	39.81	29.10	6.31	7.86	7.84

The enzyme reaction was performed with substrate only (Sub), enzyme only (PKA) or substrate plus enzyme (PKA + Sub) present. Reactions were terminated as described (7,8) and sample aliquots of 5 μ l were added to the wells of a SAM²™ 96 Biotin Capture

Plate. The wells were washed using a vacuum manifold (4 washes of 2M NaCl, 6 washes of 2M NaCl/1% H₃PO₄, 4 washes of water). The plates were dried and counted using a MicroBeta[®] TriLux liquid scintillation counter (EG&G Wallac, Inc.). Letters A-H are replicate samples placed in various wells to examine well-to-well variations in results for the SAM²™ 96 Biotin Capture Plate. Results are expressed in counts per minute (cpm). Average (AVE), standard deviation (STD) and percent coefficient of variation (% CV) were calculated for each column.

As with the SAM²™ Membrane, the binding capacity of SAM²™ 96 Biotin Capture Plate was linear between 1 and 500pmol/well, and for each molecule of biotinylated peptide added to a well one molecule was bound. This correlation between added and bound molecules was consistent from 1-500pmol of biotinylated peptide (Figure 1).

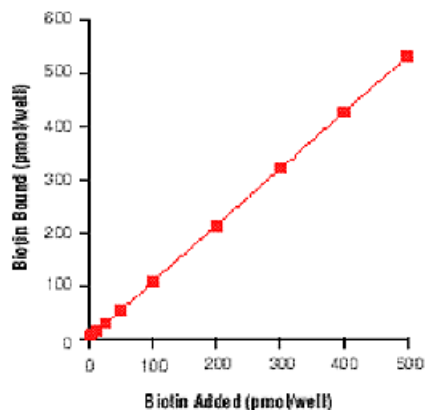


Figure 1. Linearity of binding of [¹⁴C]biotin to the SAM²™ 96 Biotin Capture Plate. Samples (5μl) of various concentrations of [¹⁴C]biotin were added to individual wells of a SAM²™ 96 Biotin Capture Plate. The plate was washed, dried and counted as described for Table 1.

PKA BINDING TO SAM²™ 96 BIOTIN CAPTURE PLATES VERSUS OTHER AVIDIN-COATED 96 WELL PLATES

The SAM²™ 96 Biotin Capture Plates were compared to 96 well biotin capture plates from two major suppliers of streptavidin and neutravidin plates, for their ability to bind biotinylated, phosphorylated peptide (Figure 2). The first plate type, L, (from supplier A), is a streptavidin-coated plate that captures biotinylated peptides or proteins. The second set of plates, M, N, O and P (supplier B), are either streptavidin- or neutravidin-coated plates (Table 2). Both suppliers' plates were compared to SAM²™ 96 Biotin Capture Plates for their ability to capture biotinylated peptides. According to the manufacturers, the maximum binding capacity of plate L is 1pmol/well, while the other plates, M, N, O and P, bind up to 25pmol/well. In comparison, the SAM²™ 96 Biotin Capture Plate has binding capacity of up to 500pmol/well (Table 2).

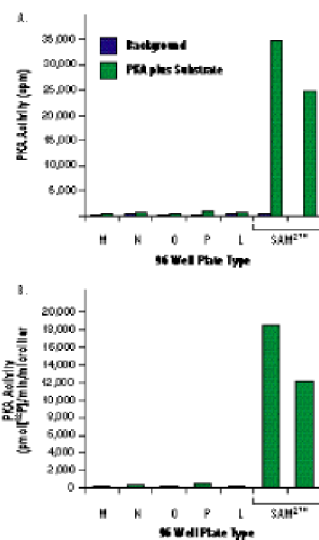


Figure 2. PKA activity assayed with the SAM²™ 96 Biotin Capture Plate and several other commercially available streptavidin and neutravidin plates. Incubation of 5μl samples in each of the plate types (L, M, N, O and P) was carried out for 120 minutes, as recommended by the manufacturers; the plates were washed according to the manufacturers'

instructions. Samples were incubated for 5 minutes (similar to the SAM²™ Plate protocol; 11), 30, 60 and 120 minutes. Plates were washed with a vacuum manifold and dried. Radioactivity was determined using a MicroBeta[®] TriLux liquid scintillation counter (EG&G Wallac, Inc.). PKA activity was measured in counts per minute (cpm). No differences were observed for bound biotinylated peptide at the different time points, so only the results for the 120 minute time point are shown. **Panel A:** Background counts per minute and PKA activity for each type of plate tested. **Panel B:** PKA activity minus background counts for each plate type tested. The SAM²™ 96 Biotin Capture Plate results are shown on the far right of Panels A and B. SAM²™ Plates were counted using two different scintillation counting techniques. The left-hand bar of the pair, in both panels, was data collected using a scintillation counter that measures using the upper photomultiplier tube. The right-hand bar was activity determined using coincidence counting, in which both upper and lower photomultiplier tubes are used. Coincidence counting mode prevents counts due to extraneous light sources, resulting in lower, yet truer results.

Table 2. Specifications for Promega's SAM²™ and Other Commercially Available Biotin Capture Plates.

Supplier	Plate	Avidin Type	Plate Color	Binding
A	L	streptavidin	white	1pmol/well
B	M	streptavidin	black	25pmol/well
	N	streptavidin	white	25pmol/well
	O	neutravidin	black	25pmol/well
	P	neutravidin	white	25pmol/well
Promega	SAM ² ™ 96	streptavidin	white	500pmol/well

Plates manufactured by suppliers A, B and Promega are designated as biotin binding plates. Plates are listed as binding either streptavidin or neutravidin binding, as described by the manufacturer. The amount of biotin reportedly bound by each plate type is listed. Plate colors are either white or black, as shown; black plates are often used in bioluminescence determinations, while white or opaque plates are often used in radioactivity assays. In these studies, black and white plates performed similarly.

We confirmed the binding capacity of plate L to be approximately 1pmol/well, as reported by the manufacturer, but the other plates (types M, N, O and P) were found to have a maximum binding capacity of only 10pmol/well (not the 25pmol/well reported by the manufacturer). The binding of plates M, N, O and P was not linear in this range (1-10pmol), making it difficult to assess the overall binding capacity of the plate. Our examination found that the binding capacity of the SAM²™ Membrane was 50-fold higher than plate types M, N, O and P and 500-fold higher than plate L (data not shown).

The background radioactivity counts were also compared for plate types L-P and the SAM²™ 96 Biotin Capture Plates (Figure 2). Background counts obtained with the SAM²™ 96 Biotin Capture Plates were very low, and in combination with the remarkably efficient capture of biotinylated peptide, resulted in a very favorable signal-to-noise ratio. The sensitivity of the SAM²™ plates is thus high (Figure 2A). On the other hand, the background counts observed with streptavidin or neutravidin plates (types L-P) were high in relation to the signal obtained with the complete system (Figure 2A). When the background counts were subtracted, the remaining signal, attributed to the enzyme, was significantly lower than that obtained with the SAM²™ Plates (Figure 2B). This resulted in a signal-to-noise ratio for plates L-P that was so low that the detection of enzyme activity was limited to only 5-10pmol of bound peptide. Consequently, the sensitivity of the assay using plate types L-P did not allow for estimation of optimal enzyme activity when used with substrates that have high K_m values. For instance, the K_m value for most of the substrates used to assay enzymatic activities of protein tyrosine kinases are in the high micromolar to low millimolar range. This requires the use of substrate concentrations in the millimolar range, a level of sensitivity that would not be attainable with the competitors' avidin-coated plates.

COMPARISON WITH PHOSPHOCELLULOSE (P81) 96 WELL PLATES

Another competitor plate type, Q, is an ion exchange based system that makes use of phosphocellulose filter disks (P81) (Table 3; reference 7). The P81 disks are seated on the top of another supporting membrane in the wells of the 96 well plate. Since capture of peptides to P81 is based on electrostatic interaction, the peptides must have an overall positive charge in order to efficiently bind to the membrane. Protein kinase assays were performed (5,6) and aliquots of the terminated reactions were applied to the plate wells. Incubation and washing protocols were carried out as suggested by the manufacturers.

When PKA reactions were applied to the phosphocellulose membrane in the type Q 96 well plates, we experienced difficulties in achieving consistent drainage of individual plate wells during the washing procedure; some wells took up to 2 hours to drain. This interfered with the assay of multiple samples and multiple plates; lack of consistent drainage time caused problems in synchronizing the wash protocols. In addition, because the peptide substrate is bound to disks that are loosely set onto a supporting membrane, excessive clogging of membranes and trapping of [γ -³²P]ATP occurred, resulting in high background counts and inconsistent results. Endogenously phosphorylated proteins also bound to the membrane, because binding was based on ionic interaction. The results obtained with type Q plates showed high background counts that were variable amongst the control wells. For example, the background

varied from 892.1 to 2,931.8cpm (substrate control) and from 626.5 to 2,831.8cpm (enzyme control) and the PKA activity for the complete enzyme system varied from 9,604.0 to 11,723.7cpm (Table 3).

Table 3. PKA Activity as Measured Using a Phosphocellulose (P81) Plate.						
	Sub	PKA	Sub	PKA	PKA +Sub	PKA + Sub
A	2,016.4	2,062.9	1,849.1	1,512.5	10,138.1	10,872.9
B	1,803.2	1,695.0	2,007.2	1,168.3	10,040.6	11,046.7
C	2,115.5	2,831.8	2,931.8	2,487.3	11,202.6	10,462.0
D	1,314.0	2,363.8	1,745.7	1,143.5	10,949.7	10,873.8
E	2,146.0	1,088.7	1,000.8	1,342.8	11,723.7	10,476.0
F	1,057.3	2,756.1	2,207.9	2,014.6	10,594.8	10,746.4
G	892.1	2,465.8	2,598.3	2,120.7	10,531.1	11,095.0
H	1,622.9	1,477.5	2,282.7	626.5	9,604.0	9,669.2
AVE	1,620.9	2,092.7	2,077.9	1,552.0	10,598.1	10,655.3
STD	485.95	626.17	583.91	612.74	683.60	461.65
% CV	29.98	29.92	28.10	39.48	6.45	4.33

The PKA assay was performed with substrate only (Sub), enzyme only (PKA) or substrate and enzyme (PKA + Sub) present. Samples of 5µl of the terminated PKA kinase reactions were applied to individual wells of the phosphocellulose plate. The plate was washed according to the protocol used for the SAM²™ 96 Biotin Capture Plates (Figure 1; 11). Radioactivity was quantified as described for Figure 1. PKA activity is expressed in counts per minute (cpm). Letters A-H are replicate samples. Average (AVE), standard deviation (STD) and percent coefficient of variation (% CV) were calculated for each column.

ADDITIONAL APPLICATIONS OF THE SAM²™ BIOTIN CAPTURE MEMBRANE

Due to the high biotin binding capacity and the low nonspecific binding of the SAM²™ Membrane, we envision a variety of applications for the membrane and the SAM²™ 96 Biotin Capture Plates. A paramount feature of the membrane is that the binding of the biotinylated peptide substrate to the SAM²™ Membrane is independent of the amino acid sequence of the substrate. Therefore, several peptides and protein substrates can be assayed simultaneously and valid comparison of their kinetic properties for the enzyme can be made with confidence. Due to the sequence independent binding there is no requirement for alteration of the consensus sequence of the peptide substrate for it to bind to the membrane, as is required when using phosphocellulose. Alterations to the peptide sequence can result in changes in the specificity of the substrate for the enzyme, making it a preferred substrate for other enzymes. For example, the addition of arginine residues to the peptide substrate for the CDT kinase makes it a good substrate for PKA as well (10).

We have examined the use of the SAM²™ Membrane and SAM²™ 96 Biotin Capture Plates for binding of biotinylated nucleic acids (data not shown) and we believe that both SAM²™ Membrane products will be useful for other applications, such as binding of biotinylated polysaccharides and lipids. The remarkable binding capacity of the membrane allows for options such as use with a high density of samples (i.e., in a pin array). Customized sizes of membrane are available to accommodate hundreds to thousands of samples. This is a valuable feature for scientists who are searching for new drugs, as thousands of compounds can be screened for their inhibitory or stimulatory effect on an enzyme or for the ability to interrupt protein-protein interactions.

SUMMARY

The SAM²™ Membrane and SAM²™ 96 Biotin Capture Plate developed by Promega offer the novel feature of high density, membrane-linked streptavidin that makes them superior to other assay systems, including other commercially available avidin-coated 96 well plates. Due to the specificity of the interaction between the phosphorylated biotinylated peptide substrate and the SAM²™ Membrane, the amount of bound radioactivity represents a true estimate of the amount of enzyme activity present. Endogenously phosphorylated, nonbiotinylated proteins do not bind to the SAM²™ Membrane, allowing quantification of only the specific kinase activity. Because of the low nonspecific binding of interfering compounds such as [γ -³²P]ATP and endogenous substrates, the background counts are extremely low, making it possible to assay for kinase activity present at very low concentrations in a sample. This highly sensitive assay enables one to examine the kinase activity of nanogram amounts of enzymes such as PKA, Protein Kinase C, cdc2 Protein Kinase, Protein Tyrosine Kinase and DNA-Dependent Protein Kinase.

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11. SAM²™ 96 Biotin Capture Plate Technical Bulletin #TB249, Promega Corporation.

Ordering Information

Product	Size	Cat.#
SAM ² ™ 96 Biotin Capture Plate	96 samples	V7541
	5 x 96 samples	V7542
SAM ² ™ 96 Biotin Capture Membrane	96 samples	V2861
	7.6 x 10.9cm	V7861

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