FROM ONE TO 9,000 SAMPLES: USING HIGH-DENSITY STREPTAVIDIN-COATED MEMBRANES FOR KINASE DETECTION

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This article describes use of the SAM²® Biotin Capture Membrane for sensitive detection of protein kinases.

Protein kinases modulate the activity of a vast number of proteins, including ion channels, transcription factors, proteases and other kinases, and approximately 2% of the genes in a typical eukaryotic cell are predicted to code for proteins that contain signature motifs of the catalytic domains of protein kinases (1). Protein kinases belong to a family of homologous proteins comprising two major subfamilies: the serine/threonine kinases and the protein tyrosine kinases. Both subfamilies of protein kinases play critical roles in signaling pathways that regulate a variety of cellular functions including cell growth, development, differentiation, membrane transport, and cell death (2,3). Abnormalities in signaling pathways can lead to pathological conditions, including many forms of cancer, making protein kinases important targets for both basic research and drug development.

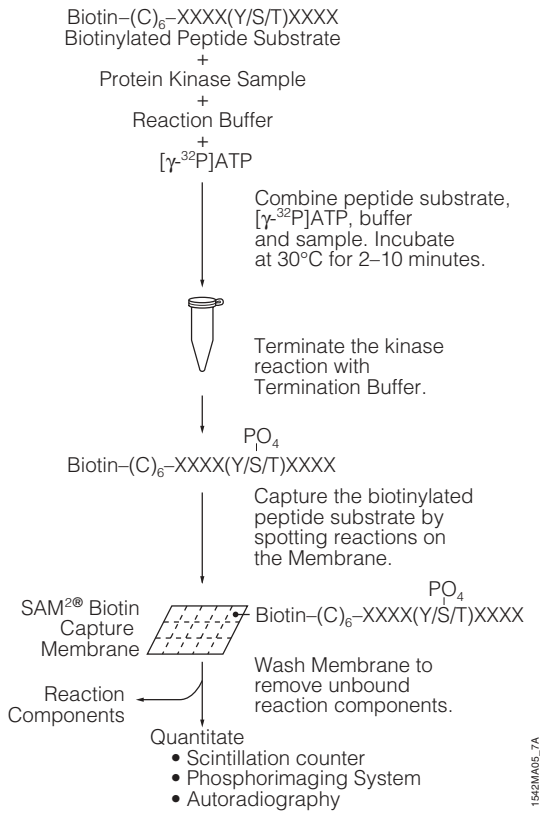

Figure 1. Schematic diagram of the biotin-capture protein kinase assay protocol.

Screening drug candidates for their ability to modulate the activity of a specific protein kinase, or studying protein kinases in general, requires an assay that measures the activity of the kinase of interest. The ideal kinase assay technology would be equally effective with both purified kinases and crude extracts. Additionally, the throughput needs of individual researchers vary greatly, and a technology that allows a researcher to conveniently assay a few samples at a time or thousands in a large screen is desirable. Thus, the ideal assay system is fast and easy to perform, provides a true estimate of enzyme activity under optimal kinetic conditions and is amenable to high-throughput screening.

High-Density Streptavidin-coated Membranes for Measuring Protein Kinase Activity

The most commonly used kinase assay method to quantitate peptide substrate phosphorylation is the P81 phosphocellulose filter assay (4). This method relies on the capture of peptide substrate by phosphocellulose via electrostatic interactions between the positively charged substrate and the negatively charged P81 filter. Although P81 phosphocellulose is widely used, it has several drawbacks including relatively weak binding of labeled substrate, poor signal-to-noise ratios, and nonspecific binding of cellular proteins that are themselves substrates of unrelated kinases (4,5).

Streptavidin-coated membranes used in conjunction with biotinylated protein kinase peptide substrates, such as those used in the SignaTECT® Protein Kinase Assay Systems[a] overcome the drawbacks of P81 phosphocellulose assays. The streptavidin-coated membrane is made using a proprietary process that results in a high density of streptavidin (6). The binding of biotin to streptavidin is rapid and very strong ($K_d = 10^{-15}$M), and the association is unaffected by rigorous washing procedures, denaturing agents, extremes in pH, temperature and salt concentrations. Even crude extracts generate high signal-to-noise ratios, and the high substrate capacity allows for optimum reaction kinetics.

Assays using these membranes allow researchers to measure protein kinase activities using low femtomole levels of purified enzyme or equivalent amounts of crude tissue/cell extracts. As outlined in Figure 1, the assay steps and analysis of results using a biotin-capture system to measure protein kinase activity are straightforward and require only common laboratory

equipment. Following phosphorylation and binding of the biotinylated substrate to the streptavidin-coated membrane, unincorporated [γ-^{32}P]ATP is removed by a simple wash procedure. This procedure also removes nonbiotinylated proteins that have been phosphorylated by other kinases in the sample. The bound labeled substrate is quantitated by scintillation counting, phosphorimaging analysis or using autoradiography in combination with a densitometer.

High-Throughput Applications Using Biotin-Capture Technology

The high-density streptavidin matrix is available in several formats including a perforated sheet, a single sheet (7.6 × 10.9cm), and a 96-well biotin capture plate. To illustrate the performance of the 96-well biotin capture plate in a high-throughput situation, we performed a series of cAMP-dependent protein kinase (PKA) assays (Table 1). We measured PKA activity in the presence of enzyme alone, in the presence of substrate alone and in the presence of both enzyme and substrate (7). The radioactivity measured in the absence of the enzyme or in the absence of the substrate represents <0.02% of input counts. The range of background counts was extremely low, and the assay was time efficient; the full washing procedure required only 5 minutes. The coefficient of variation for enzyme activity did not exceed 8%, indicating highly reproducible results and consistency in the assay performance. When tested with biotin and biotinylated peptides, the binding capacity of the biotin capture plate was linear between 1 and 500pmol/well, and binding was stoichiometric (data not shown, 8). This feature is critical for substrates, such as those for protein tyrosine kinases, that have high K_m values.

Table 1. PKA Activity as Measured Using 96-Well Biotin Capture Plate.

Sample	Substrate	PKA	Substrate + PKA
A	12.0	40.2	26,438.5
B	30.1	36.2	24,518.1
C	24.1	22.1	22,017.8
D	28.1	18.1	23,666.7
E	20.4	10.2	25,403.9
F	22.4	32.6	24,695.9
G	18.3	42.8	24,051.2
H	16.3	40.7	26,886.9
Avg.	21.5	30.4	24,709.9
S.D.	6.02	12.09	1,559.11
%CV	28.06	39.81	6.31

The enzyme reaction was performed with substrate only (Substrate), enzyme only (PKA) or substrate plus enzyme. Reactions were terminated as described (5,10), and sample aliquots (5µl) were added to wells of a 96-well biotin capture plate. The wells were washed using a vacuum manifold (4 washes of 2M NaCl, 6 washes of 2M NaCl/1% H_2PO_4, 4 washes of water). The plates were dried and counted using a MicroBeta® TriLux liquid scintillation counter (EG&G Wallac, Inc.). Letters A–H represent replicate samples placed in random wells to examine well-to-well variations. Results are expressed in counts per minute (cpm). Avg. = average; S.D. = standard deviation; %CV = percent coefficient of variation.

µARCS Technology

Abbott Laboratories (Abbott Park, IL) has expanded the capabilities of the biotin capture membrane technology by developing a high-throughput assay based on gel permeation of arrayed compounds. The microarray compound screening (µARCS) technology allows approximately 9,000 compounds to be screened in a single assay (9). Nanoliter quantities of test compounds are arrayed onto a polystyrene sheet that has the same footprint as a multiwell plate (Figure 2). The compound sheet is dried and then overlaid onto an agarose gel containing the kinase of interest. After allowing the compounds to diffuse into the kinase gel, the polystyrene sheet is removed, and the kinase gel is then layered onto a streptavidin-coated membrane cut to the same dimensions as the gel. The kinase reaction is initiated by overlaying a substrate gel containing the biotinylated peptide substrate and [γ-^{33}P]ATP onto the kinase gel. The peptide substrate and [γ-^{33}P]ATP diffuse through the kinase gel, and the biotinylated peptide is captured on the streptavidin-coated membrane. The membrane is washed to remove nonspecific radioactivity, and signal is detected using a PhosphorImager® (Molecular Dynamics, Sunnyvale, CA, USA). In the absence of inhibitor, the peptide substrate is phosphorylated with [^{33}P], and the output from phosphorimaging analysis appears as a light background (reverse image). Dark spots on a light background suggest the presence of a kinase inhibitor in that region of the assay (Figure 2). This technology allows Abbott Laboratories to screen 50,000–100,000 compounds in a single day. The µARCS technology reduces both reagent consumption and costs compared to standard plate screening and does not suffer from many of the drawbacks common to high-density plate screening, such as complex liquid-handling requirements, evaporation problems, plate-edge effects and extensive automation requirements (9).

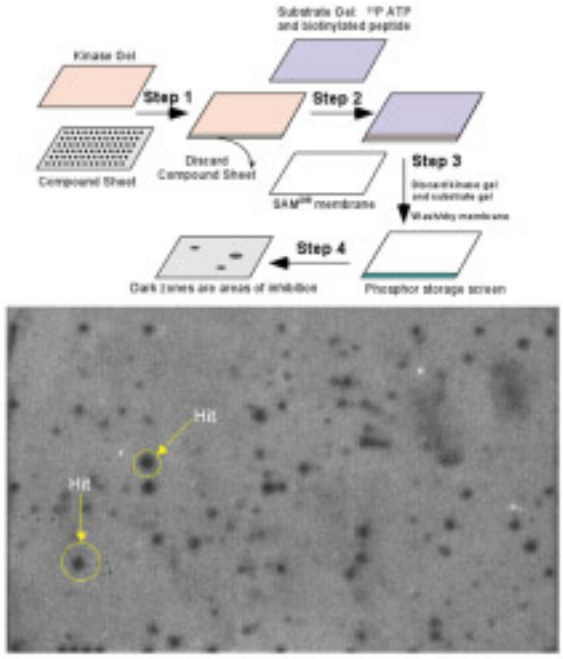

Figure 2. A prototypical protein kinase arrayed compound screen (ARCS).
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Summary

The study of kinases and their role in cellular regulation continues to expand as the human genome is sequenced and new kinases are identified. Reagents and assay systems that allow sensitive, accurate and high-throughput analysis of both purified kinases as well as crude extracts will enhance the characterization of these important cellular components, speed the identification of appropriate therapeutic targets, and drive the development of new and more effective treatments for many diseases. ■

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Protocols

SAM²® Biotin Capture Membrane
Technical Bulletin #TB547
www.promega.com/tbs/tb547/tb547.html

SAM²® Biotin Capture Plate
Technical Bulletin #TB249
www.promega.com/tbs/tb249/tb249.html

Ordering Information

Product	Size	Cat.#
SAM²® Biotin Capture Membrane*[a]	96 samples	V2861
	7.6 × 10.9cm	V7861
	30 × 30cm membrane	Pls. Inq.
SAM²® Biotin Capture Plate*[a]	96-well plate	V7541
	5 × 96-well plates	V7542
SignaTECT® cAMP-Dependent Protein Kinase A (PKA) Assay System[a]	96 reactions	V7480
SignaTECT® Protein Kinase C (PKC) Assay System[a]	96 reactions	V7470
SignaTECT® Protein Tyrosine Kinase (PTK) Assay System[a]	96 reactions	V6480
SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System[a]	96 reactions	V8161
SignaTECT® DNA-Dependent Protein Kinase Assay System[a]	96 reactions	V7870
SignaTECT® cdc2 Protein Kinase Assay System[a]	96 reactions	V6430

*For Laboratory Use.

[a]U.S. Pat. Nos. 6,066,462 and 6,348,310 have been issued to Promega Corporation for quantitation of protein kinase activity. Other patents are pending.