

Protein Kinases as Drug Targets in High-Throughput Systems

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There are an estimated 2,000 kinases and 500 phosphatases in the human genome, and almost one-third of all proteins in a cell can be phosphorylated. The completion of sequencing of the human genome and other genomes may lead to the discovery of additional kinases and phosphatases, many of which could be implicated in the regulation of cell growth. With these discoveries comes the need for novel reagents and specific assays to identify potential gene products and to characterize these enzymes and their functions in the cell.

INTRODUCTION

Protein phosphorylation is implicated in cellular processes such as proliferation, differentiation, secretion and apoptosis. Protein kinases and phosphatases play key roles in regulating these processes and thus affecting the cellular response to a variety of hormones, cytokines, and neurotransmitters (1). Changes in the level, subcellular location and activity of kinases and phosphatases have consequences on normal cell function and maintenance of cellular homeostasis (1,2). For example, the inactivation of both copies of nonreceptor protein tyrosine kinase (PTK) ZAP70 causes severe combined immunodeficiency. In addition, a mutation in the X-linked BTK gene results in agammaglobulinemia. Other examples include: i) inactivation of the X-linked Rsk2 protein serine kinase gene, suspected to be involved in the development of Coffin-Lowry syndrome; ii) overexpression of the aurora2 protein serine kinase, implicated in colon carcinomas; and iii) activation of MAP kinases, thought to play a role in the etiology of breast and colon cancer. It has also been shown that insulin-independent diabetes and peripheral neuropathies are caused by insufficient receptor PTK signaling, further evidence that the balance between protein phosphorylation and dephosphorylation is critical in maintaining normal cell growth and function.

PROTEIN KINASES AND KINASE ASSAYS

Protein kinases have become major targets for the development of novel, designer drugs, thus it is imperative to develop enzyme assays that are specific for individual kinases (3). It is essential to have an enzyme assay that is specific for the targeted kinase in order to screen a chemical library of inhibitors and be able to identify an inhibitor that meets the requirement of a therapeutic drug. The assay should not only be specific but also scalable to a high-throughput level in order to utilize it in screening for thousands to millions of compounds. In addition, for screening an expression library in search of a protein kinase target, the assay has to be highly sensitive, as the expression level of the target enzyme may be very low and thus difficult to detect.

Promega first developed the SAM²® Biotin Capture Membrane (Cat.# V2861, V7861) for use in the SignaTECT® Protein Kinase Assay Systems^(4,5). The membrane is prepared by a proprietary process resulting in a high density of streptavidin, which provides rapid, quantitative binding of biotinylated substrates. The SignaTECT® assays utilize

kinase-specific biotinylated peptide substrates, phosphorylated with [γ -³²P]ATP and the cognate protein kinase (either in pure form or in crude cellular or tissue extract). The phosphorylated biotinylated peptide is captured on the SAM²® Membrane, and unincorporated radioactivity and other phosphorylated proteins in the extract are removed by a simple washing procedure. This method is a significant improvement over traditional ion exchange-based methods, such as phosphocellulose (P81) detection (6) because the SAM²® Membrane provides high sensitivity and selectivity for accurate quantification of kinases in crude cell extracts.

The prototype of the SignaTECT® Kinase Assay Systems (7) has been scaled-up for use in high-throughput formats with the development of a larger membrane format (7.6 × 10.9 cm; Cat.# V7861) and the SAM²®96 Biotin Capture Plate (96 wells; Cat.# V7541, V7542) (8). After the kinase assays are performed, samples (1–25 μ l) are transferred to the solid membrane or to plates using a multipipettor or robotic liquid delivery system. Radioactivity incorporated on the membrane is quantitated by Phosphor-Imager® analysis or conventional autoradiography. For the SAM²® 96 Biotin Capture Plates (Cat.# V7541, V7542), radioactivity is quantified using a MicroBeta® TriLux liquid scintillation and luminescence system (EG&G Wallac, Inc.) or the TopCount® microplate scintillation and luminescence counter (Packard).

Table 1. PKA Activity as Measured Using Promega's SAM²® 96 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 (6,7) and sample aliquots (5 μ l) were added to 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% HPO₄, 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.

To illustrate the performance of the SAM²® 96 Biotin Capture Plate we performed a protein kinase A assay (Table 1), measuring PKA activity in the presence of enzyme only, in the presence of substrate only and in the presence of both. The radioactivity determined in the absence of the enzyme (“Substrate”) or in the absence of the substrate (“PKA”) represents <0.02% of input counts. The range of background counts was extremely low and the assay was carried out with maximum efficiency of time; the full washing procedure took only 5 minutes. It is also noteworthy that 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 SAM²® Plates was linear between 1 and 500pmol/well and binding was stoichiometric, as demonstrated in Figure 1 (8). This feature is critical for enzymes such as PTK whose substrates have high K_m values.

The SAM²® Biotin Capture Membrane offers many novel features that make it superior to other assay systems and to other commercially available avidin-coated 96 well plates. Due to the specificity of interaction between the phosphorylated, biotinylated peptide substrate and SAM²® Membrane, the amount of bound radioactivity represents a true estimate of the phosphorylated product, and therefore of the amount of enzyme present (Figure 2). Phosphorylated, endogenous proteins do not bind to SAM²® Membrane. Because of the minimal nonspecific binding of interfering compounds such as $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and endogenous substrates, the background counts are extremely low, making it the assay of choice for measuring kinase enzyme activities present in low concentrations in the sample. This extremely high sensitivity enables one to determine the kinase activity from nanogram amounts of samples containing enzymes such as PKA, PKC, cdc2PK, PTK, DNA-PK and CaM KII. (Promega provides highly purified enzymes for use in screening protein libraries for enzyme-specific inhibitory molecules.)

Another advantage of the strong, selective binding is that the SAM²® Membrane can withstand extreme reaction conditions (9), resulting in minimal loss of bound phosphorylated, biotinylated peptide. This feature is critical for assaying enzyme activity in cellular or tissue extracts as well as for the assay of in vitro-expressed enzymes. The presence of other protein kinases and protein substrates can pose a formidable challenge to accurate assessment of enzyme activity. In addition, the assay can be performed in less than 10 minutes after reaction termination (8).

A paramount feature of the assay system is that the binding of the biotinylated peptide substrate to the SAM²® Membrane is independent of the amino acid sequence of the substrate. Thus, several peptides and protein substrates can be assayed simultaneously and their kinetic properties compared with confidence. In assays based on electrostatic interaction, binding is dependent on the sequence of the substrate. In these systems a wide range of values for enzyme activities will be obtained when using several substrates for a particular enzyme, making it difficult to assess the appropriateness of the substrates for the enzyme under investigation (10). No accurate estimation of the amount of bound peptide substrate can be obtained. Furthermore, in methods based on ionic interaction, such as P81 detection, there is a requirement for modification of the consensus sequence of the peptide substrate for it to bind to the matrix. This may result in a change in the specificity of the substrate toward the enzyme, making it appear to be a preferred substrate for other enzymes. For example, the addition of arginine residues to the peptide substrate for the CDK kinase makes it a good substrate for PKA as well (11).

PROTEIN KINASE INHIBITORS

Chemical inhibitors, either in the form of organic molecules or peptide inhibitors, have been widely used to complement the use of dominant-negative mutants of enzymes, overexpression of natural protein inhibitors, temperature-sensitive mutant cell lines, or microinjection of kinase-selective antibodies, as a means of inhibiting protein kinases (12,13). The early findings that PKA and PKC play important roles in metabolic regulation, inflammatory responses, abnormal cell growth and apoptosis paved the way to intense investigations on regulation of these enzymes. These studies led to the discovery of one of the most potent natural kinase inhibitors for PKA, cAMP-Dependent Protein Kinase Peptide Inhibitor (PKI; Cat.# V5681). Similar studies led to the discovery of a peptide inhibitor of PKC, Myristoylated PKC Peptide Inhibitor (Cat.# V5691), based on the pseudo-substrate region of the enzyme. These inhibitors are highly selective for their corresponding kinases, much more so than the cell-permeable organic molecule inhibitors H7 or H8 (14).

The search for ideal cell-permeable inhibitors for protein kinase targets has been accelerated due to i) progress in the field of small molecule library generation using combinatorial chemistry methods coupled to high-throughput screening, and ii) the ability to characterize in detail ligand-protein interaction sites that can be exploited for ligand design, via structural methods. The coupling of these two technologies could lead to greater numbers of kinase-based drugs (15). This approach has been elegantly demonstrated in several studies. For example, the use of ATP binding site-directed drugs and the exploitation of structural features of an adjacent site, which is unique to an individual kinase, resulted in the development of unique inhibitors for p38 protein kinase that have lower potency towards other MAP kinases (16). Similarly, by taking advantage

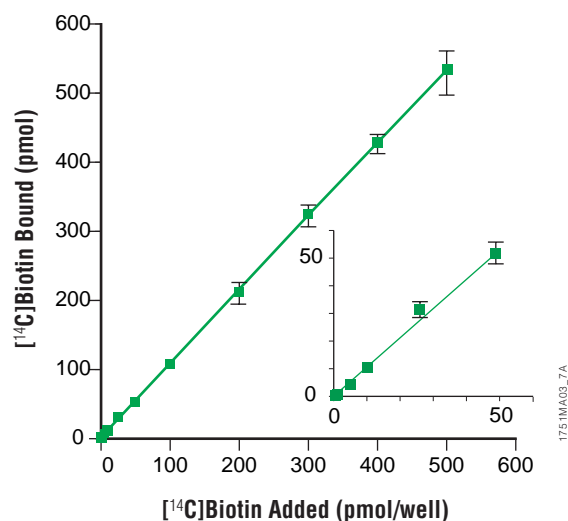


Figure 1. Linearity of binding of [¹⁴C]biotin to the SAM²® 96 Biotin Capture Plate. Samples (5μl) of various concentrations of [¹⁴C]biotin were added to individual wells of a SAM²® 96 Biotin Capture Plate. The plate was washed, dried and counted as described in Table 1. The inset is an enlargement of the 0–50pmol portion of the graph.

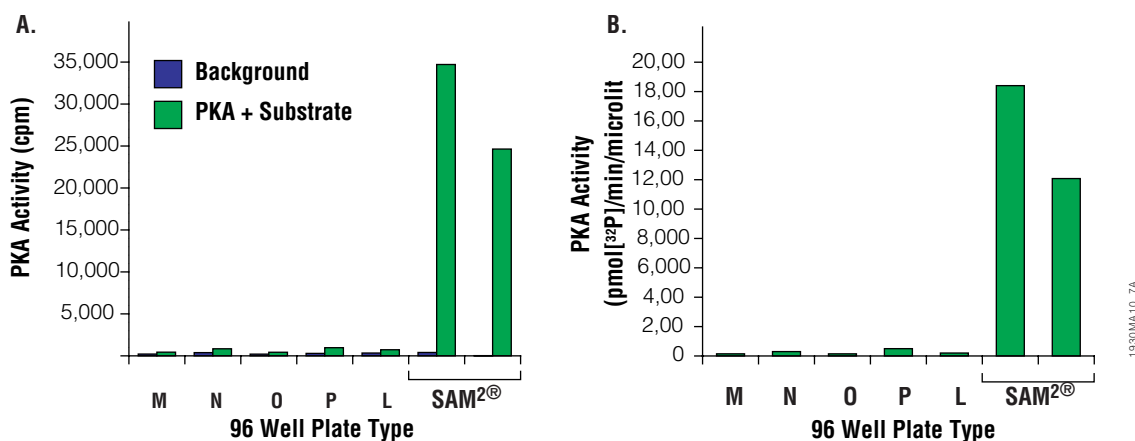


Figure 2. PKA activity assayed with the SAM²® 96 Biotin Capture Plate and several other commercially available streptavidin and neutravidin plates. Samples (5µl) were incubated in each plate type (L, M, N, O, P and SAM²®) for 5, 30, 60 and 120 minutes (recommended incubation for SAM²® Plates is 5 minutes, for other manufacturers' plates is 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:** Counts per minute for background and PKA activity for each type of plate tested. **Panel B:** PKA activity minus background counts for each plate tested. **Note:** 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 more accurate results.

of the structural features of olomoucine, an inhibitor selective for CDK family of protein kinases, and by screening a chemical library based on olomoucine as the parent compound, it was possible to generate a highly selective, highly potent inhibitor. The later studies have resulted in one of the most potent inhibitors of CDK2-cyclin A (IC₅₀ = 6nM), which is currently in human clinical trials.

Because of the enormous number of studies implicating the MAPK pathway in tumorigenesis and apoptosis, there is a strong interest in finding the ideal inhibitor of MAPK for use in human clinical trials (17–20). Several of the inhibitors that targeted the ERK pathway resulted in three new inhibitors, namely PD098059 (21), U0126 (22) and PD184352 (23). We have confirmed the specificity of U0126 in inhibiting the activation of ERK1/2 by MEK1/2 in several cell lines and under several conditions of ERK activation (24). MEK Inhibitor U0126 (Cat.# V1121) is available from Promega.

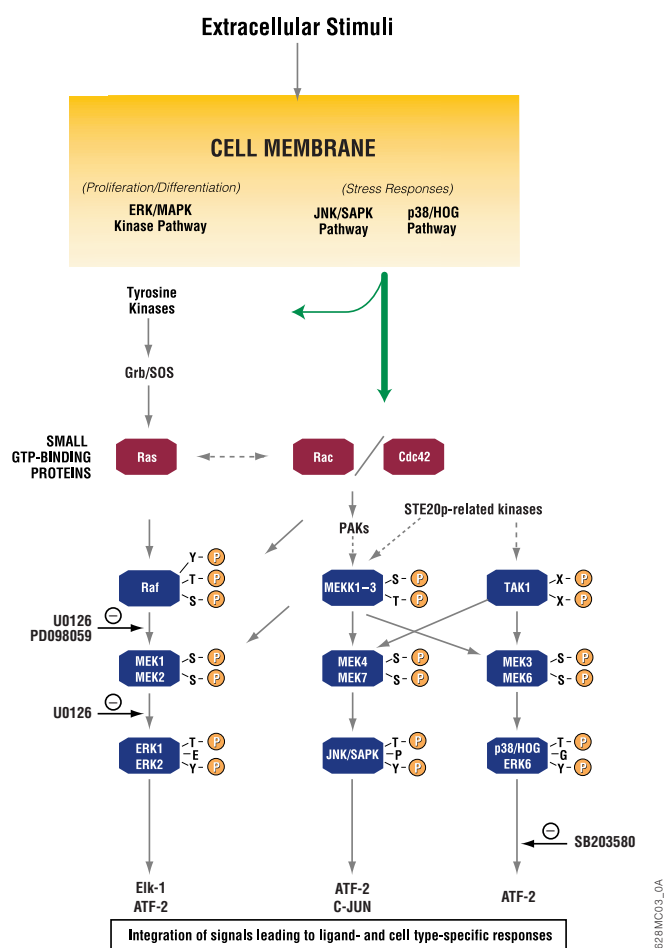
Recent studies have shown that molecular recognition between biomolecules plays a significant role in signal specificity within the cell in response to many extracellular stimuli. Thus protein-protein interaction via select groups of motifs/modules has been the focus of intense investigation (24).

We have been interested in disrupting signals that are PKA anchor-mediated, using cell-permeable peptides modeled on the PKA binding recognition sequence in anchoring proteins (25). Promega has introduced two such reagents useful in studies on the role of PKA anchoring. These reagents can be used to investigate several functions that are dependent on PKA anchoring in the cell. The InCELLect™ AKAP St-Ht31 Inhibitor Peptide^(a) (PKA anchoring peptide inhibitor; Cat.# V8211) and its control peptide, InCELLect™ St-Ht31P Control Peptide^(a) (Cat.# V8221), have been successfully used to study the role of PKA in sperm motility, aquaporin-2 pump translocation to plasma membrane and progesterone-mediated acrosome reaction in human sperm (26).

PHOSPHOSPECIFIC ANTI-ACTIVE® ANTIBODIES TO MAP KINASES

After the discovery of a new gene and its expressed product, it is desirable to generate a protein-specific antibody to detect the protein of interest amongst many thousands of other proteins in cell extract. Kinase-specific antibodies have been highly useful in determining the presence and the expression levels of such proteins under various conditions. In addition, when antibodies are fluorescently labeled, they can be used to study alterations in the activation as well as the localization of the enzyme in vivo in a time-dependent manner. Several protein kinases have been shown to be modulated by phosphorylation; their activation may be regulated by a single or by multiple phosphorylations. This is best illustrated by the MAP kinases, whose dual phosphorylation by upstream protein kinases results in their activation (17). These kinase cascades are found in all eukaryotic organisms and consist of a three-kinase module. A canonical MAPK module consists of three protein kinases: a MAPK kinase kinase (MEKK) that phosphorylates and activates a MAPK kinase (MEK), which in turn, phosphorylates and activates a MAPK/ERK enzyme (17,27). There are at least three main modules in mammalian cells that fall under this category, and these are conserved across all eukaryotes indicating that they are involved in mediating a diverse array of extracellular signals. The three modules contain the Extracellular signal Regulated Kinase (ERK), c-Jun N-Terminal Kinase (JNK), and p38 MAPK (p38) pathways (Figure 3).

The first enzyme in the module, MEKK, represents a group of enzymes that are serine/threonine protein kinases and phosphorylate MEK enzymes on two serine or threonine residues within a Ser-X-X-Ser/Thr motif. The latter enzymes in turn phosphorylate the MAPK on Thr and Tyr residues within the Thr-X-Tyr consensus sequence. An important feature



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Figure 3. Activation of different ERK/MAPK signaling cascades by different extracellular stimuli. PD098059 acts by inhibiting the activation of MEK1/2 by Raf kinase, whereas MEK Inhibitor U0126 acts directly upon MEK1/2. Another inhibitor, SB203580, acts by inhibiting the kinase activity of p38.

of the MAPK superfamily of enzymes is that they are activated upon dual phosphorylation of the TXY motif present in the activation loop of the catalytic domain. The middle amino acid varies among the three MAPKs, glutamate in ERKs, proline in JNKs and glycine for p38. A single phosphorylation on Thr or Tyr is not sufficient to activate MAPKs. Dual phosphorylation is required for their activation, and the phosphorylation on tyrosine usually but not invariably precedes that of threonine in a two-step reaction (28).

Assessment of MAPK activation has traditionally been carried out by either of two methods: i) immunoprecipitation of the enzyme by a MAPK-specific antibody and determination of the activity of the active enzyme in the precipitate using myelin basic protein and radioactive ATP as substrates or ii) by band shift of activated MAPKs in gels. The former is a slow procedure requiring multiple steps, none of which can be precisely controlled for accurate and reproducible measurement of enzyme activity; immunoprecipitation also requires the use of radioactivity. The latter (gel shift assay) does not usually result in accurate assessment of the active enzyme and results are difficult to interpret when other protein bands with similar molecular weights appear in the vicinity of the MAPKs. Thus the need for a simple method to accurately determine enzyme activation.

Toward this goal, we developed a new class of antibodies to selectively detect only the dually phosphorylated form of each MAPK (ERK, JNK or p38). This is of paramount importance since the three MAPK signaling modules act in concert with other cell signaling systems. These novel Anti-ACTIVE® antibodies provide the selective tools required to study the role of each of the MAP kinase signaling pathways in response to extracellular stimuli in an accurate and time-dependent fashion. In addition to detection of the dually phosphorylated (active) form of MAPK, in situ localization of MAPKs using these novel antibodies enables scientists to determine the subcellular localization of each MAPK before and after activation (29). Furthermore, when fluorescently labeled with a fluorophore and used in combination with anti-nonphospho kinase antibodies conjugated to a different fluorophore, these antibodies can be utilized to determine the location of the subcellular site for the phospho- and nonphospho-enzyme using Fluorescence Resonance Energy Transfer (FRET) analysis in a cell-based assay (30). Similar to the anti-phosphotyrosine antibodies, the dual phospho specific antibodies have proven to be valuable reagents in the pursuit of selective inhibitors, such as U0126, of these enzymes (22).

SUMMARY

The study of kinases and phosphatases and their role in cell regulation continues to expand as the human genome is sequenced and new kinases are identified as expression products of newly discovered genes. Reagents and assay systems that allow for high-throughput analysis of kinases and phosphatases will enhance both discovery and characterization of these products, and thus will speed the identification of appropriate targets and the development of novel therapeutics.

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SAID GOUELI

Ordering Information

Product	Size	Cat. #
SAM ² [®] Biotin Capture Membranes	96 samples	V2861
	7.6 × 10.9cm	V7861
SAM ² [®] 96 Biotin Capture Plates	96 wells	V7541
	5 × 96 wells	V7542

Related Products

Product	Size	Cat. #
MEK Inhibitor U0126	5mg (5 × 1mg)	V1121
Anti-ACTIVE [®] MAPK pAb, Rabbit, (pTEpY)	40µl	V8031
Anti-ACTIVE [®] ERK1/2 pAb, Rabbit	40µl	V1141
Anti-pT ¹⁸³ MAPK pAb, Rabbit	50µl	V8081
Anti-ACTIVE [®] JNK pAb, Rabbit, (pTPpY)	40µl	V7931
	120µl	V7932
Anti-ACTIVE [®] p38 pAb, Rabbit, (pTGpY)	100µl	V1211
PPase-2A Catalytic Subunit	25 units	V6311
PC12 Cell Extracts, Western Controls	Sorbitol/Untreated	10 blots V8100
	NGF/Untreated	10 blots V8110
Donkey Anti-Rabbit, IgG, (H+L) HRP	60µl	V7951
Donkey Anti-Rabbit, IgG, (H+L), AP	60µl	V7971
Protein Kinase C (Rat Brain)	1µg	V5621
SignaTECT [®] Protein Kinase C Assay System ^(a)	96 reactions	V7470
PepTag [®] Non-Radioactive Protein Kinase C Assay ^(b)	120 assays	V5330
Myristoylated PKC Peptide Inhibitor	1mg	V5691
InCELLect [™] AKAP St-Ht31 Inhibitor Peptide	150µl	V8211
InCELLect [™] St-Ht31P Control Peptide	150µl	V8221
cAMP-Dependent Protein Kinase, Catalytic Subunit Type II (Bovine Heart)	2,500u	V5161
SignaTECT [®] cAMP-Dependent Protein Kinase Assay System	96 reactions	V7480
PepTag [®] Non-Radioactive cAMP-Dependent Protein Kinase Assay ^(b)	120 assays	V5340
cAMP-Dependent Protein Kinase Peptide Inhibitor	1mg	V5681
cAMP, 1mM	500µl	V5611

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^(a)Patent Pending.

^(b)U.S. Pat. No. 5,580,747.