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SignaTECT™ DNA-Dependent Protein Kinase Assay System

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The SignaTECT™ DNA-Dependent Protein Kinase Assay System developed at Promega accurately measures the protein kinase activity of DNA-dependent protein kinase (DNA-PK), both in purified samples and nuclear extracts. The assay can be performed quickly and gives very low background counts indicating minimal interference from endogenously phosphorylated proteins present in nuclear extracts. This new assay system provides the researcher a powerful tool with which to measure DNA-PK activity.

Introduction

Protein kinases play an important role in a variety of cellular functions including cell growth and development, and cell death. Approximately 2-3% of the genes in eukaryotic cells are predicted to encode protein kinases (1). About 200 protein kinases have been identified thus far; it is estimated that one-third of the proteins in a typical mammalian cell are phosphorylated. Protein kinases have multiple substrates *in vivo*, which may explain their diverse physiological functions. Due to the ubiquitous nature of protein kinases and their multiple roles in cellular regulation, there is a strong interest in developing an assay system that can detect the activity of specific protein kinases in crude cellular or tissue extracts, as well as in column fractions during enzyme purification.

Over the past few years, Promega has developed SignaTECT™ Protein Kinase Assay Systems for the protein kinases PKA, PKC, PTK and cdc2 (2,3). Promega is pleased to introduce the fifth member of this product family, the SignaTECT™ DNA-Dependent Protein Kinase Assay System for the detection of DNA-PK activity in purified enzyme and crude cell nuclear extract preparations. The SignaTECT™ Assay System features the SAM2™ Biotin Capture Membrane[a], a unique and proprietary streptavidin-coated membrane, and a biotinylated, p53-derived peptide substrate specific for DNA-PK. The high affinity of the biotinylated peptide substrate for the streptavidin-coated membrane (K_d of streptavidin for biotin = 10^{15}M) and the highly specific nature of the biotin-streptavidin interaction results in a very specific and sensitive detection system for DNA-PK activity.

[a]*Patent Pending.*

DNA-dependent protein kinase

DNA-dependent protein kinase (DNA-PK) is a nuclear protein belonging to the serine/threonine kinase family. The enzyme consists of multiple subunits, including a catalytic subunit (DNA-PK_{cs}) of 4,127 amino acids with an approximate molecular weight of 470kDa, and a DNA targeting component corresponding to the DNA end-binding Ku antigen. The Ku component is composed of a tightly-linked heterodimer of polypeptides of approximately 70kDa and 80kDa; it has a high affinity for DNA ends and associates with DNA-PK_{cs}, targeting it to DNA. The Ku antigen is required for the phosphorylation of several substrates. DNA-PK_{cs} can also be stimulated *in vitro* in the presence of certain oligonucleotide sequences without the need for known targeting subunits in the reaction (4,5).

Activation of DNA-PK

DNA-PK requires double-stranded DNA (dsDNA) for its enzymatic activity; the enzyme is not activated by single-stranded DNA (ssDNA), RNA or by heteroduplexes of DNA/RNA (6). Since the enzyme binds to the ends of dsDNA, it is stimulated by linear but not by supercoiled plasmid DNA. The architecture of the dsDNA ends is apparently not important, as the enzyme can be activated by dsDNA with blunt ends, 5´- or 3´-overhanging DNA ends, phosphorylated or nonphosphorylated DNA ends and even by closed DNA hairpin ends. Short double-stranded oligonucleotides (12bp) can activate the enzyme, but higher concentrations of these are required than that needed for activation by larger duplexes, (e.g., 25bp segments; 4). The binding of dsDNA to DNA-PK results in generation of the active enzyme form and brings the substrate closer to the enzyme, allowing the phosphorylation reaction to proceed.

Protein substrates and the role of DNA-PK in the cell

DNA-PK phosphorylates several intracellular proteins, including nuclear DNA-binding regulatory proteins such as tumor suppressor protein p53, the ssDNA binding protein RPA, heat shock protein hsp90, the SV40 large T antigen (TAg), a variety of transcription

factors including Fos, Jun, SRF, Myc, SP1, Oct-1, TFIID, E2F, the estrogen receptor, and the large subunit of RNA Polymerase II (4,7). The ability of DNA-PK to phosphorylate the DNA binding protein p53 suggests a role for the enzyme as a checkpoint modulator, since cell cycle progression from G_1 to S is inhibited when p53 levels are elevated (7). The ability of the enzyme to phosphorylate several transcription factors and steroid receptors argues strongly for a role in regulation of transcription. In addition, the enzyme is a crucial component in site specific VDJ recombination since the absence of the catalytic subunit of the enzyme or the Ku antigen are believed to be implicated in the development of severe combined immunodeficient (*scid*) mice and of Ataxia Telangiectasia in humans. DNA-PK was recently implicated in the early steps of apoptosis; the cleavage of the catalytic subunit of the enzyme was found to occur due to an ICE-like protease, coinciding with the onset of macrophage apoptosis (8). Furthermore, since the enzyme is activated by binding to damaged DNA, this suggests that the active enzyme assembles at sites caused by agents known to inflict DNA damage, including reactive oxygen species, UV and ionizing radiation, and several mutagenic chemicals (4,5,7).

SignaTECT™ DNA-Dependent Protein Kinase Assay System

The binding of biotin to streptavidin is unaffected by extremes in pH (2.0-10.0), temperature, organic solvents, ionic and nonionic detergents [1% SDS, 1% CHAPS, 1% Triton® X-100 (Union Carbide), 1% Tween® 20 (ICI Americas) or 1% Tween® 80], and other denaturing agents (5M guanidine HCl and 2M urea) (9). The binding capacity of the SAM2™ Membrane for the biotinylated peptide substrate is high (a minimum of 2.5nmol/square), which allows the use of high concentrations of peptide substrates, up to 1mM, in the kinase reaction. This maximizes the signal-to-noise ratio, optimizing enzyme assay results. The use of a biotinylated peptide substrate highly specific for DNA-PK in the kinase reaction results in binding of only this biotinylated, phosphorylated peptide to the membrane. The free [gamma-^{32}P]ATP and endogenously phosphorylated, nonbiotinylated proteins are not bound to the membrane, and therefore can be readily removed by a simple washing procedure. In other assays, such as the P81 filter assay, any positively charged protein that undergoes phosphorylation can bind to the filters, and provide an artificially high estimate of enzyme activity (10).

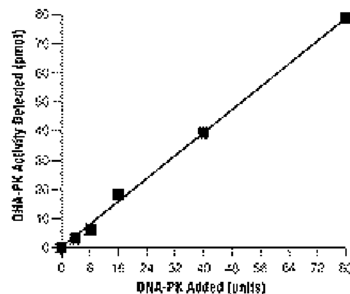

Figure 1. Effect of enzyme dilution on the DNA-PK activity in purified enzyme preparations. The linearity of enzyme activity as a function of enzyme concentration was demonstrated by diluting DNA-PK (Cat.# V5811) and assaying the activity of the indicated number of units of enzyme using the SignaTECT™ DNA-PK Assay System as described (11). It is apparent that the activity of the enzyme is linear down to 20-fold dilution of the enzyme. The activity of the enzyme in the absence of dsDNA represented less than 1-2% of its activity in the presence of dsDNA (data not shown).

The SignaTECT™ System provides for very sensitive detection of the DNA-dependent protein kinase. Figure 1 depicts DNA-PK activity quantitated in purified enzyme samples, using the SignaTECT™ DNA-PK Assay System. Note that as little as 2 units of the enzyme can be detected in a five-minute reaction. Figure 2 demonstrates DNA-PK activity in nuclear extracts of HeLa cells, where enzyme activity can be detected in a 2µl sample (0.12µg protein) diluted 10-fold; in other words DNA-PK activity can be detected in a 12 nanogram sample of crude nuclear extract. The activity measured is specific for DNA-PK, since only the biotinylated peptide substrate binds to the membrane.

To ensure that the DNA-PK activity in nuclear extracts is DNA-dependent, endogenous DNA should be removed prior to assaying for enzyme activity. This can be done by a simple and rapid column chromatography step; the DNA is bound to resin and the enzyme sample is eluted using a high salt concentration. The SignaTECT™ DNA-PK Assay System provides activation buffer containing dsDNA that will activate DNA-PK present in the nuclear extract. The assay can be completed quickly (10-15 minutes after reaction termination).

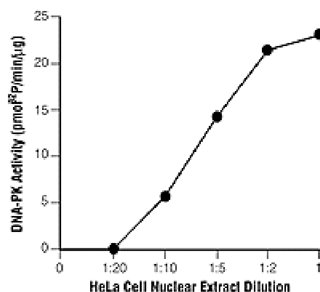

Figure 2. Effect of dilution of HeLa cell nuclear extract on the protein kinase activity of DNA-PK. A 25µl sample of HeLa Cell Nuclear Extract (Cat.# E3110) was passed over a DEAE Sepharose® Fast Flow column to remove DNA and the enzyme was eluted with 4ml of buffer containing 400mM KCl. The eluate was diluted and 2µl samples (0.12µg protein) were used to assay for DNA-PK activity using the SignaTECT™ DNA-PK Assay System. There is a linear correlation between enzyme activity and the amount of enzyme present per reaction. It is apparent that the activity of the enzyme is linear to a 10-fold dilution of the protein sample. The activity of the enzyme in the absence of dsDNA represented less than 1-2% of its activity in the presence of dsDNA (data not shown).

Summary

Promega's SignaTECT™ DNA-Dependent Protein Kinase Assay System has excellent sensitivity and linearity values for quantitation of DNA-PK activity both from purified samples and from nuclear extracts (a range of 0-80 units can be easily demonstrated). Enzyme activity has been measured in the absence and presence of the activator dsDNA. The activity of the enzyme without dsDNA only represents 1-2% of the enzyme activity detected in the presence of dsDNA (data not shown). Thus, over 98% of the activity of the enzyme measured by this system is activatable DNA-PK. In addition, due to very low nonspecific binding, the background radioactivity incorporated using this assay is less than 0.1% of input radioactivity counts. This results in a very high signal-to-noise ratio and thus a highly sensitive assay. Examples of the sensitivity of the SignaTECT™ DNA-PK Assay System include detection of DNA-PK activity with as little as 2 units of pure enzyme and as little as 12 nanograms of total protein from HeLa cell nuclear extracts; this activity can be detected in a 5 minute reaction (one unit = one pmol ^{32}P/min).

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Ordering Information		
Product	**Size**	**Cat.#**
SignaTECT™ DNA-Dependent Protein Kinase Assay System	96 reactions	V7870

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