Promega

Technical Bulletin

SignaTECT®
DNA-Dependent Protein Kinase Assay System

INSTRUCTIONS FOR USE OF PRODUCT V7870.

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SignaTECT® DNA-Dependent Protein Kinase Assay System

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1. **Description**..........1
2. **Product Components and Storage Conditions**..........4
3. **Quantitation of DNA-PK Activity**..........4
   A. Preparation of HeLa Cell Nuclear Extract for DNA-PK Assay..........4
   B. DNA-PK Assay Protocol..........5
   C. Calculation of the Specific Activity of [γ-^{32}P]ATP..........8
   D. Calculation of DNA-PK Enzyme Activity..........9
4. **Composition of Buffers and Solutions**..........10
5. **Related Products**..........11
6. **References**..........11
7. **SAM2® Biotin Capture Membrane Template**..........12

1. Description

The SignaTECT® DNA-Dependent Protein Kinase (DNA-PK) Assay System[a] provides an improved method to quantitate DNA-dependent protein kinase activity, both in purified enzyme preparations and cell nuclear extracts. The SignaTECT® System uses the unique SAM2® Biotin Capture Membrane[a], produced by a proprietary process that results in a high density of streptavidin on the membrane matrix. This streptavidin matrix provides rapid, quantitative capture of biotinylated substrate molecules, based on the strong affinity of biotin for streptavidin (Kd = 10^{-15}M). The SAM2® Membrane can bind biotinylated substrate at a minimum of 1.1nmol/cm^2. In addition, the membrane is optimized for low nonspecific binding.

The SignaTECT® DNA-Dependent Protein Kinase Assay System overcomes the problem of nonspecific substrate binding by using a biotinylated DNA-PK p53-derived peptide substrate in conjunction with Promega SAM2® Biotin Capture Membrane (1). The high binding capacity of the SAM2® Biotin Capture Membrane for the DNA-PK biotinylated peptide substrate and low backgrounds observed with this system maximize the signal-to-noise ratio. Following phosphorylation of the DNA-PK biotinylated peptide substrate and binding to the SAM2® Membrane, the excess free [γ-^{32}P]ATP and

1. Description (continued)

nonbiotinylated proteins are removed via a simple washing procedure. The binding of biotin to streptavidin is rapid and strong. Once formed, the biotin-streptavidin association is unaffected by extremes in pH, temperature, salt concentrations and denaturing agents. Due to the strength of this binding interaction, the ^{32}P-labeled DNA-PK Biotinylated Peptide Substrate is unlikely to be removed during the washing procedure.

The amount of ^{32}P incorporated into the DNA-PK Biotinylated Peptide Substrate is determined by liquid scintillation counting, phosphorimaging analysis or conventional autoradiography. Figure 1 provides a schematic diagram of the procedure.

DNA-PK is a nuclear serine/threonine protein kinase that requires double-stranded DNA (dsDNA) for activity (2). The binding of dsDNA to the enzyme results in formation of the active enzyme and also brings the substrate closer to the enzyme, allowing the phosphorylation reaction to proceed (3,4). 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 (4). The Ku antigen is a tightly linked heterodimer of polypeptides of approximately 70kDa and 80kDa that 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} also can be stimulated in vitro in the presence of certain oligonucleotide sequences without the need for known targeting subunits in the reaction. The enzyme is activated by dsDNA, but not by single-stranded DNA, RNA or DNA/RNA heteroduplexes. Since the enzyme binds to the ends of dsDNA, it is stimulated by linear but not by supercoiled plasmid DNA. The architecture of the DNA ends is not important since the enzyme is activated by blunt ends, 5´- or 3´-overhangs, phosphorylated or nonphosphorylated DNA ends or closed DNA hairpin ends. Short, double-stranded oligonucleotides (12bp) can activate the enzyme, but higher concentrations are needed for activation, as compared to the concentration required for activation by longer duplexes (e.g., 25bp; 5).

When activated by dsDNA, the enzyme phosphorylates several DNA-binding substrates in vitro, including tumor suppressor protein p53, single-stranded DNA binding protein RPA, heat shock protein hsp90, SV40 large T antigen (TAg of simian virus 40), a variety of transcription factors, including Fos, Jun, SRF, Myc, Sp1, Oct-1, TFIID, E2F, estrogen receptor and the large subunit of RNA polymerase II (2–5). DNA-PK recognizes serine/threonine residues located on the N-terminal side of glutamine residues. Additional acidic residues will enhance the enzyme's activity, while basic amino acid residues will inhibit it (6). The ability of the enzyme to phosphorylate the DNA-binding

protein p53 suggests a role for the enzyme as a checkpoint modulator since cell cycle progression from G1 to S is inhibited when p53 levels are elevated. In addition, the ability of the enzyme to phosphorylate several transcription factors and steroid receptors argues strongly for a role in regulating transcription. Finally, since the enzyme is strictly activated by binding to damaged DNA, this suggests that the active enzyme assembles at sites of DNA damage in vivo.

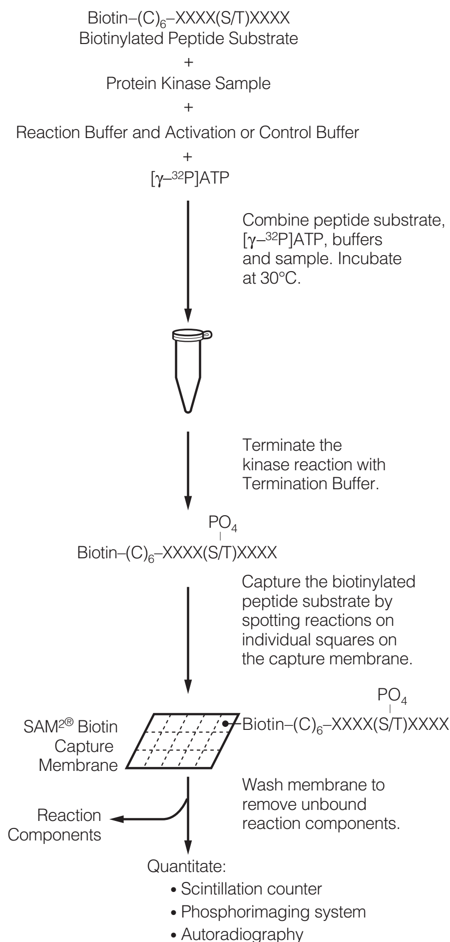

Figure 1. Schematic diagram of the SignaTECT® Protein Kinase Assay Protocol. The illustration shows an overview of the protocol steps to prepare, run and analyze the specific protein kinase activity using any of the SignaTECT® Protein Kinase Assay Systems.

2. Product Components and Storage Conditions

Product	Size	Cat.#
SignaTECT® DNA-Dependent Protein Kinase Assay System	96 reactions	V7870

Each system contains sufficient reagents for 96 kinase reactions. Includes:

- 1 SAM2® Biotin Capture Membrane
- 600µl ATP, 0.5mM
- 1,400µl Termination Buffer (2 × 700µl)
- 300µl DNA-PK Biotinylated Peptide Substrate, 4mM
- 1,000µl DNA-PK 5X Reaction Buffer
- 300µl DNA-PK Activation Buffer
- 300µl Control Buffer
- 200µl Bovine Serum Albumin (BSA, 10mg/ml)

Storage Conditions: Store the system components at –20°C where they are stable for at least six months from the date of purchase. Avoid multiple freeze-thaw cycles. For storage of <1 month, store the SAM2® Biotin Capture Membrane at 4°C (return the unused portion of the SAM2® Membrane to the resealable plastic bag). Store the Termination Buffer at room temperature (20–25°C).

3. Quantitation of DNA-PK Activity

DNA-PK activity can be quantitated in a purified enzyme sample as well as in cell nuclear extracts. The following protocol describes the assay developed by Promega to quantify DNA-PK activity. To demonstrate the specificity of DNA activation of the enzyme in nuclear extracts, endogenous DNA should be removed from the extracts prior to assaying enzyme activity. This can be accomplished by performing a simple high-salt elution.

3.A. Preparation of HeLa Cell Nuclear Extract for DNA-PK Assay

Materials to Be Supplied By the User
(Solution compositions are provided in Section 4.)

- 2ml DEAE Sepharose® Fast Flow (Pharmacia Cat.# 17-0709-01) equilibrated in buffer A
- buffer B
- nuclear cell extract (e.g., HeLaScribe® Cell Nuclear Extract in vitro Transcription Grade, Cat.# E3091, E3092)

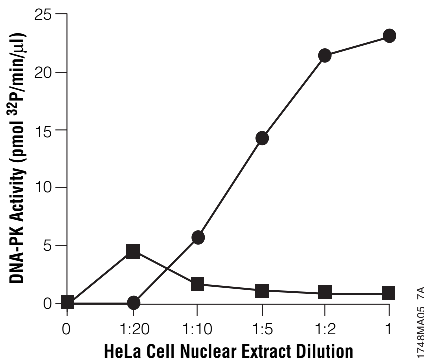

Figure 2. DNA-PK activity measured in HeLa cell nuclear extract using the SignaTECT® DNA-PK Assay System. HeLa cell nuclear extracts were prepared by a modification of the method of Dignam *et al.* (7). Endogenous DNA was removed using a DEAE Sepharose® Fast Flow column pre-equilibrated with buffer A. The Sepharose® Fast Flow was washed with buffer A, and the DNA-PK was eluted with buffer B. Enzyme activity was quantitated using the SignaTECT® DNA-PK Assay System featuring the SAM2® Biotin Capture Membrane, either in the presence (circles) or absence (squares) of activator (dsDNA).

To use the HeLa cell nuclear extract as a source of DNA-PK, it is necessary to first remove endogenous DNA from the extract. To remove endogenous DNA, apply an aliquot of the HeLa cell nuclear extract to 2ml of DEAE Sepharose® Fast Flow, pre-equilibrated in buffer A. Wash with 10ml of buffer A, then elute the enzyme with 4ml of buffer B. This procedure also can be used with nuclear extracts from other cells or tissues. The time to complete this procedure is approximately 2 hours. Figure 2 demonstrates DNA-PK activity detected in HeLa cell nuclear extracts using the SignaTECT® DNA-PK Assay System.

3.B. DNA-PK Assay Protocol

Materials to Be Supplied By the User
(Solution compositions are provided in Section 4.)

- [γ-^{32}P]ATP (3,000Ci/mmol, 10μCi/μl)
- 2M NaCl
- 2M NaCl in 1% H_3PO_4
- enzyme dilution buffer
- 30°C heating block or water bath
- scintillation counter or phosphorimaging system
- washing container (e.g., Nalgene® plastic utility box, 7.5 × 6 × 4 inches)
- deionized water
- **optional:** orbital platform shaker
- **optional:** heat lamp
- **optional:** Whatman® 3MM filter discs

3.B. DNA-PK Assay Protocol (continued)

Note: For best results, kinase assays should be run with the following controls: (i) assay without substrate and; (ii) assay with and without activator.

1. Thaw the termination buffer at 20–30°C, then vortex well. Thaw the remaining frozen components on ice. Vortex tubes before use to ensure homogeneity of the reagents.
2. Wearing gloves, cut (using scissors or a razor blade) the required number of squares from the SAM2® Biotin Capture Membrane. The squares may remain connected as a partial sheet to minimize handling. Return the unused SAM2® Membrane to the resealable plastic bag at 4°C or –20°C.

The template provided in Section 7 can be used to identify samples using the SAM2® Membrane square numbers.

3. Prepare the ATP mix as follows:

Component	Final per Reaction	20 Reactions
0.5mM ATP	5.0µl	100µl
[γ-^{32}P]ATP	0.05µl	1µl

4. Prepare the following reactions in 0.5–1.0ml microcentrifuge tubes:

Reaction in the Presence of Activator:

Component	Final per Reaction	20 Reactions
DNA-PK Activation Buffer	2.5µl	50µl
DNA-PK 5X Reaction Buffer	5.0µl	100µl
DNA-PK Biotinylated Peptide Substrate	2.5µl	50µl
BSA (10mg/ml)	0.2µl	4µl
[γ-^{32}P]ATP mix (see Step 3, above)	5.0µl	100µl

Reaction in the Absence of Activator:

Component	Final per Reaction	20 Reactions
DNA-PK Control Buffer	2.5µl	50µl
DNA-PK 5X Reaction Buffer	5.0µl	100µl
DNA-PK Biotinylated Peptide Substrate	2.5µl	50µl
BSA (10mg/ml)	0.2µl	4µl
[γ-^{32}P]ATP mix (see Step 3, above)	5.0µl	100µl

5. Mix gently and preincubate the reaction mix (Step 4, above) at 30°C for 1–5 minutes.
6. Prepare the enzyme dilution buffer as follows: Dilute the 5X Reaction Buffer to 1X with deionized water. Add BSA to a final concentration of 0.1mg/ml. Prepare appropriate dilutions of the enzyme samples in this enzyme dilution buffer. We recommend preparing and testing crude samples undiluted and serially diluted twofold to 1:16.

7. Initiate the reaction by adding the appropriate amount of enzyme sample (0–9.75µl). Adjust the reaction to a final volume of 25µl using deionized water. Incubate at 30°C for 5 minutes (other time points and temperatures may be tested if desired).

8. Terminate by adding 12.5µl of Termination Buffer to each reaction; mix well. The terminated reactions can be kept at room temperature during processing and are stable at 4°C for at least 24 hours.

9. Spot 10µl of each terminated reaction onto a prenumbered square of the SAM2® Membrane. After all samples are spotted, follow the wash procedure and rinse steps as described below. Save the reaction tubes for Step 12, below.

   Note: The 10µl spotted sample contains approximately 2.6nmol of the biotinylated substrate. This substrate concentration is optimized for the assay and does not exceed the linear binding capacity of one SAM2® Membrane square. Spotting >10µl of sample per membrane square will exceed the linear binding capacity of the SAM2® Membrane.

10. Place the SAM2® Membrane squares containing samples from Step 9, above, into a washing container. Wash using an orbital platform shaker set on low or by occasional manual shaking as follows:

Wash 1X for 30 seconds with 200ml of 2M NaCl.

Wash 3X for 2 minutes each with 200ml of 2M NaCl.

Wash 4X for 2 minutes each with 200ml of 2M NaCl in 1% H_3PO_4.

Wash 2X for 30 seconds each with 100ml of deionized water.

Total wash time <20 minutes.

Notes:

Dispose of the radioactive wash solution in accordance with the regulations of your institution.

More or less washing may be appropriate to achieve acceptably low background counts; this should be determined empirically.

For rapid drying, a final 15-second wash in 95% ethanol (100ml) can be used. Longer washes with ethanol may cause the markings on the membrane to run slightly.

3.B. DNA-PK Assay Protocol (continued)

11. Dry the SAM2® Membrane on a piece of aluminum foil under a heat lamp for 5–10 minutes or air-dry at room temperature for 30–60 minutes. (If the SAM2® Membrane has been washed with ethanol, shorten the drying time to 2–5 minutes under a heat lamp or 10–15 minutes at room temperature.)
12. Determine total counts for calculation of the specific activity of [γ-^{32}P]ATP as follows:

    Remove 5µl aliquots from any two reaction tubes from Step 9, above, and spot onto individual SAM2® Membrane squares or Whatman® 3mm filter discs. For this step dry the SAM2® Membrane squares or filter discs without washing. After analysis, use these results to calculate the specific activity of [γ-^{32}P]ATP in Section 3.C.

    Note: If 5µl is not available from a single tube, combine the contents of several tubes for Step 12.

    Analysis by Scintillation Counting: If still connected, separate the SAM2® Membrane squares (from Steps 11 and 12, above) using forceps, scissors or a razor blade, and place the squares or 3mm filter discs into individual scintillation vials. Add scintillation fluid to the vials and count.

    Analysis by Phosphorimaging: Alternatively, the SAM2® Membrane squares may remain intact and the intact membrane or 3mm filter discs may be analyzed using a phosphorimaging system.

3.C. Calculation of the Specific Activity of [γ-^{32}P]ATP

$$\text{The specific activity of } [\gamma\text{-}^{32}\text{P}]\text{ATP in cpm/pmol} = \frac{[(37.5 \div 5)\ X]}{2{,}500}$$

where:

37.5 is the sum of the reaction volume (25µl) and Termination Buffer volume (12.5µl).

5 is the volume in microliters of sample used from Section 3.B, Step 12.

X is the average counts/minute of the 5µl samples from Section 3.B, Step 12.

2,500 is the number of picomoles of ATP in the reaction.

3.D. Calculation of DNA-PK Enzyme Activity

The enzymatic activity of DNA-PK can be determined by subtracting the activity of the enzyme in the absence of activator (control buffer) from that of the enzyme in the presence of activator (activation buffer).

Enzyme specific activity in pmol ATP/minute/µg of protein =

$$\frac{(\text{cpm}_{\text{reaction with activator}} - \text{cpm}_{\text{reaction without activator}}) \times (37.5)}{(10) \times (\text{time}_{\text{min}}) \times (\mu\text{g of protein in reaction}) \times (\text{specific activity of } [\gamma\text{-}^{32}\text{P}]\text{ATP})}$$

where:

37.5 is the sum of the reaction volume (25µl) + the Termination Buffer volume (12.5µl).

10 is the volume in microliters of the sample from Section 3.B, Step 9.

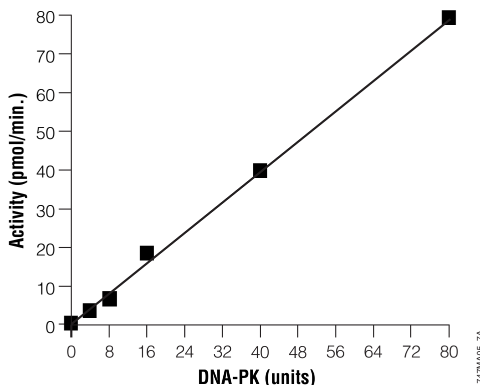

Figure 3. Sensitivity and linearity of SignaTECT® DNA-Dependent Protein Kinase Assay System. DNA-Dependent PK activity was quantitated over a range of enzyme dilutions using the SignaTECT® DNA-PK Assay System, as described in Section 3.B.

4. Composition of Buffers and Solutions

buffer A

100mM	KCl
50mM	HEPES (KOH, pH 7.5)
0.2mM	EGTA
0.1mM	EDTA
1.0mM	DTT

buffer B

400mM	KCl
50mM	HEPES (KOH, pH 7.5)
0.2mM	EGTA
0.1mM	EDTA
1.0mM	DTT

Control Buffer (provided)

10mM	Tris-HCl (pH 7.4)
1mM	EDTA (pH 8.0)

DNA-PK 5X Reaction Buffer (provided)

250mM	HEPES (KOH, pH 7.5)
500mM	KCl
50mM	$MgCl_2$
1mM	EGTA
0.5mM	EDTA
5mM	DTT

DNA-PK Activation Buffer (provided)

100µg	calf thymus DNA in TE buffer (Control Buffer)

enzyme dilution buffer

1X	DNA-PK Reaction Buffer
0.1mg/ml	BSA

Termination Buffer (provided)

7.5M	guanidine hydrochloride buffer (Control Buffer)

2M NaCl

116.9g/L	NaCl

2M NaCl in 1% H_3PO_4

116.9g/L	NaCl
11.8ml/L	85% H_3PO_4

5. Related Products

Product	Size	Cat.#
DNA-Dependent Protein Kinase	2,500 units	V5811
DNA-Dependent Protein Kinase Peptide Substrate	1mg	V5671
HeLaScribe® Nuclear Extract in vitro Transcription System	40 reactions	E3110
HeLaScribe® Nuclear Extract in vitro Transcription Grade	40 reactions	E3091
	160 reactions	E3092
SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System	96 reactions	V7480
SignaTECT® Protein Kinase C (PKC) Assay System	96 reactions	V7470
SignaTECT® cdc2 Protein Kinase Assay System	96 reactions	V6430
SignaTECT® Protein Tyrosine Kinase (PTK) Assay System	96 reactions	V6480
SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System	96 reactions	V8161
Kinase-Glo® Luminescent Kinase Assay*	10ml	V6711
Kinase-Glo® Plus Luminescent Kinase Assay*	10ml	V3771
Kinase-Glo® Max Luminescent Kinase Assay*	10ml	V6071
ProFluor® PKA Assay	4 plate	V1240
	8 plate	V1241

*Available in additional sizes.

6. References

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7. SAM2® Biotin Capture Membrane Template

Promega					SAM2® Biotin Capture Membrane						
1	9	17	25	33	41	49	57	65	73	81	89
2	10	18	26	34	42	50	58	66	74	82	90
3	11	19	27	35	43	51	59	67	75	83	91
4	12	20	28	36	44	52	60	68	76	84	92
5	13	21	29	37	45	53	61	69	77	85	93
6	14	22	30	38	46	54	62	70	78	86	94
7	15	23	31	39	47	55	63	71	79	87	95
8	16	24	32	40	48	56	64	72	80	88	96

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[a]U.S. Pat. Nos. 6,066,462, 6,348,310 and 6,753,157.

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