Manual for the In Vitro SDS-K+ Precipitation Kit

☐ ***Catalog No. 1010-1*** ***100 Assays***

☐ ***Catalog No. 1010-2*** ***250 Assays***

Lot Number:

Kit Description

This kit contains all reagents for routine detection of DNA topoisomerase covalent complexes. It is based on the idea that covalent or cleavage intermediates are trapped by SDS (a protein denaturant) which binds only protein (and not DNA). After addition of KCl, a precipitate forms that traps topoisomerase and topoisomerase/DNA complexes (but not free DNA).

Storage of Reagents:

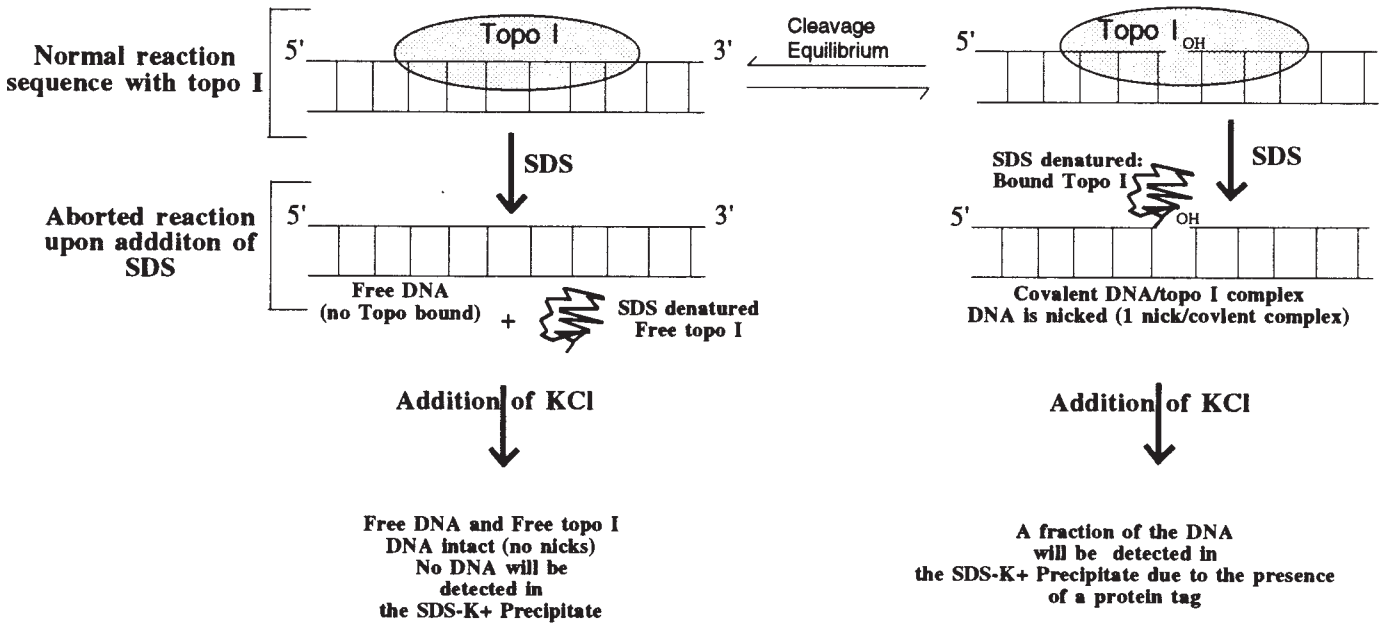
The buffers and DNAs should be stored at 4° C for short term storage and at -20° C for longer term storage. The topo II buffer must be made fresh from two components as stipulated below.

コスモ・バイオ株式会社

SDS-K+ Precipitation Kit

Introduction

Both type I and II topoisomerases can be trapped in a covalent complex with DNA by adding protein denaturants (alkali, acid or detergents) while the enzyme is actively engaged in breaking and resealing steps on DNA. It is generally accepted that capturing the covalent intermediate or "cleavable" complex is difficult because the intermediate has a relatively short life time. Thus, with normal amounts of topo I or II (i.e., sufficient enzyme to observe catalysis), detecting the covalent complex by denaturation requires the presence of topo inhibitors which tend to extend the life time of the cleavage complex. (Strictly speaking, these types of "inhibitors" are in fact better described as "poisons".) Formation of a covalent intermediate can be exploited as a means of identifying novel topo poison since the complex is a functional intermediate of the catalytic cycle of topo. Covalent topo/DNA complexes are generated by adding SDS to a reaction in which the enzyme is nicking and resealing DNA; a fraction (typically <5%) of the topo is trapped in a covalent complex with DNA. SDS binds only protein (not DNA) and non-covalently bound topo is irreversibly inactivated. Upon addition of KCl, insoluble crystals of potassium dodecyl sulfate form (we refer to this as SDS-K+) which co-precipitate free protein and only those DNAs covalently bound to topo I. If the DNA is labeled, the amount of label in the precipitate is a measure of DNA molecules with covalently bound (denatured) topo. DNA in the SDS-K+ precipitate can be recovered and shown to be all nicked (topo I reactions) or a mixture of nicks and double strand breaks (topo II), as expected from what we know of the reaction mechanism. The SDS-K+ method is particularly useful for quantifying topo DNA covalent complexes to: 1) identify topo I or II inhibitors or "poisons" and 2) to facilitate comparison of different inhibitors in their ability to increase the life time of the cleavage complex. The diagram below shows the topo I reaction mechanism and the effect of termination of the reaction with SDS, followed by selective precipitation of covalent complexes. The SDS-K+ method can be used in vitro with purified DNA targets or in vivo in a chromatin setting. TopoGEN offers kits for analysis of both (see www.topogen.com).



Kit Description:

This kit contains all the reagents for routine detection of covalent topoisomerase/DNA intermediates. The DNA target that is included that is non-radioactive and a protocol is included for end labeling. All other reagents have been pre-tested and are certified to function in the SDS-K+ precipitation method based upon the analytical method described by Trask et al. (EMBO 13: 671, 1984).

Kit Contents: (volumes given for 100 assay kit size; for 250 assay kits, multiply volumes by 2.5 fold).

1. Substrate DNA: Lambda DNA (total of 10 ug of DNA in 100 ul or 0.1 ug/ul).

2. I0x Topo I assay buffer (750 u1). Ix Buffer is 10 mM Tris-HCl pH 7.9, l mM EDTA, 0.15 M NaCI, 0.1 % BSA, 0.1 mM Spermidine, 5% glycerol).

*3. 10x Incomplete Topo II assay Buffer A (500 ul) 0.5M Tris-HCl (pH8.0), 1.2 M KCl, 0.1M MgCl2, 5 mM DTT.

*4. 10x ATP Buffer B contains 50 mM ATP in water [50 ul provided].

*FOR TOPO II ASSAY BUFFER ONLY: To prepare a fresh stock of 10x Topo II Assay buffer: Add 0.1 volume of Buffer B to 1.0 volume of Buffer A (example, if you need 50 ul of 10x Complete Buffer for a single experiment, mix 5 ul of Buffer B with 50 ul of Buffer A.. The Complete Buffer MUST be made fresh for each experiment. Prepare only the amount needed fresh each day. DO NOT FREEZE THE 10x COMPLETE ASSAY BUFFER, IT IS UNSTABLE.

•10% SDS: Cat#4060 (750 ul). SDS will precipitate at 4°C; warm briefly at 37°C before use or store at room temperature.

•l0x TKE (50 ml). Ix TKE is 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM KCI

•2.5M KCI: 1.5 ml

•Buffer Al (25 ml): I0 mM Tris-CI pH 7.5, 20 ug/ml BSA, 20 ug/ml calf thymus DNA, 1 % SDS

The following materials and reagents are not supplied with this kit but will be required:

95% and 70% Ethanol; Glass Fiber Filters (used for TCA precipitations, such as Whatman GF/C)

Filtration manifold or single Millipore Filter stack. α[32P]-dATP or [32P]-TTP; E. coli DNA polymerase (Klenow) EcoRl,

Deoxynucleoside triphosphates

Spin Columns (optional)

Scintillation vials, Scintillation fluid

III. Controls

I. Procedure for End Labeling lambda DNA

Lambda DNA is digested with EcoR1 followed by 3' end labeling using the Klenow fragment of E. coli DNA polymerase I. The Klenow and EcoR1 enzymes can be purchased from a number of different companies, such as Promega, BRL, New England Biolabs, USB and Pharmacia.

A. EcoR1 digestion of lambda DNA:

1. Restrict phage DNA:

10X Restriction Enzyme Buffer	2 ul (supplied with EcoR1 enzyme)
Distilled Water:	Adjust to 20 ul total volume
Lambda DNA (0.1 ug/ul)	1-10 ul (0.1 to 1 ug)
EcoR1 (10 U/ul)	1-2 ul (follow supplier recommedations)
	Final volume = 20 ul

2. Digest for 1-2 hrs. at 37°C. (Alternatively, digest overnight at 37°.)
3. Verify digestion by checking 2 ul on a 1% agarose gel. Stain with Ethidium Bromide (0.5 ug/ml) and compare digested and undigested phage DNA.

B. Labeling Protocol:

1. To the above reaction (on ice), add 20 to 40 uCi of [alpha-^{32}P]-dATP (400-800 Ci/mmol or 3000 Ci/mmol). The amount and specific activity of the isotope depends upon how many total counts will be required. This is the suggested range and will produce far more labeled DNA than necessary if the other reagents are in good shape. In particular the Klenow and EcoR1 enzymes need to be fresh and active.
2. Add 1 ul of 5mM mixture of dTTP and dATP (not supplied with Kit).
3. Add 1 unit of Klenow fragment and incubate for 15 min at 30°C.
4. Stop reaction by addition of 1 ul of 0.5M EDTA or by heating at 75°C for 10 min.

C. Removing unincorporated nucleotides using spin column:

(Reference: "Current Protocols in Molecular Biology" by Ausubel et al., John Wiley and Sons). Spin Columns are commercially available from a number or suppliers or you can make your own as described.

1. To make the spin column: Plug the bottom of a 1 ml plastic syringe with glass wool (silanized as described in "Current Protocols" by Ausubel et al.). Swirl the hydrated column resin (Sephadex G-50, Bio-Gel P-60 from Bio-Rad) and fill the syringe with the suspension.
2. Insert syringe into a glass tube (16x125 mm) that is suitable for table top swinging bucket centrifuge. Spin a few minutes at a low setting (in swinging bucket clinical centrifuge at a setting of 2-4...ie low speed is 1000 rpm) to pack column.
3. Dilute the labeling mixture with TE (10 mM Tris-HCL, pH75., 1 mM EDTA) to 100 ul.
4. Overlay onto syringe-column (do not let solution run down sides of syringe)
5. Place a screw-top microfuge tube (without a cap) on the end of the syringe and insert into the 16 x 125 mm glass tube and spin at 1200 rpm for 5 min.
6. Recover the column eluate from the microfuge tube (this is the end labeled DNA, free of soluble isotopes; it should be very radioactive). **Discard the column and its contents since this is radioactive waste.** Discard into an appropriate radioactive waste.
7. Count 1 ul of the eluate to determine total and CPM/ul.

D. Calculating and adjusting specific activity.

1. From the total CPM and DNA concentration determine the specific activity of the DNA in CPM/ug. Ex: 1 ug of DNA was labeled and 1.25 x 10^7 CPM was recovered. This is 1.25 x 10^7 CPM/ug.
2. Adjust the specific activity to approximately 1 x 10^6 CPM/ug by adding uncut lambda DNA from the kit. For example, if you withdraw 0.1 ug of labeled DNA , you will need to add about a 10 fold excess of non-radioactive phage DNA (i.e.. 1.0 ug) to be sure enough carrier DNA is present. It is not always necessary to adjust the specific activity; however, if labeling is high, then the Specific Activity will have to be adjusted so that for a given amount of label there is sufficient carrier to help keep background SDS-K+ precipitation (without topo).

II. Procedure for SDS-K+ using Labeled Phage DNA

The end labeled DNA (prepared in I above) should be used as target DNA. The reaction works well best with 50-250 ng of DNA per assay. Also, we recommend that each reaction contain 10,000 to 50,000 CPM of labeled DNA.

1. Set up topo I or II reactions as follows:

10x buffer (I or II) _____	2 ul
distilled Water _____	Variable to make up 20 ul total volume
[^{32}P]-DNA _____	10^4 to 5 x 10^4 CPM and 50-250 ng of DNA
Enzyme (I or II) _____	Variable (typically 1-2 ul of TopoGEN enzymes)
Inhibitor _____	Vary as appropriate

2. Incubate for 15-30 min at 37°C

3. Stop reaction with 1/10 volume of 10% SDS.
(Note, be sure to add SDS directly while reactions are at 37°C.)

4. Add 0.25 ml of buffer A1; vortex, leave at 37oC for a minute or two.

5. Add 25 ul of 2.5 M KCl, vortex briefly, place on ice 10 min at least
(Note, you can stop at this point and store reactions at 4°C)

6. Filtration step: Place glass fiber filters on manifold and pre-wet with 1x TKE buffer

7. Pour SDS-K+ ppt. onto filters with vacuum off.
(N*ote, wash out the reaction tube with a ml or two of 1x TKE and add to filter.)*

8. Apply vacuum and wash with 5ml of TKE each time for 4-5 times

9. Wash filters twice with 10ml of 95% ethanol

10. Wash filter twice with 10 ml of 70% ethanol

11. Dry filters under heat or at room temperature and count.

III. Controls: Controls should be run in duplicate.

A. It is important to include a negative control (no enzyme) to ensure that adequate washing and processing of the filters was achieved.

B. It is also a good idea to include a control inhibitor such a m-AMSA (topo II) or camptothecin (topo I). Both inhibitors are available through TopoGEN (please call). You should see elevated SDS-K+ precipitable DNA in the presence of micromolar amounts of topo I or II poisons.

C. An additional control can be performed using proteinase K: After addition of SDS to the reactions, digestion with proteinase K (50 ug/ml) should reduce the SDS-K+ signal to background levels.