E. coli Topoisomerase IV Drug Screening Kit User Manual

Shipping and Storage of Reagents

The kit may be shipped at ambient temperature or on ice (dry ice or wet ice). The DNAs should be stored at 4°C and the buffers stored at -20°C upon receipt. Avoid frequent freeze/thaw cycles with the plasmid as this may contribute to DNA breakage.

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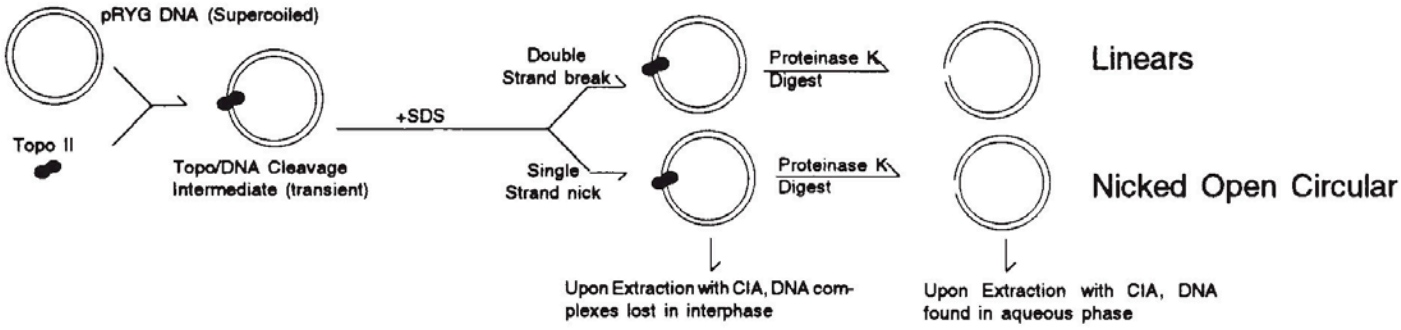
A. Introduction

The TopoGEN Topoisomerase IV Drug Screening Kit contains reagents necessary to identify compounds that affect enzyme activity. **Note that this kit does not include purified enyzme**, which is available from TopoGEN (TG2000EC).
Thiskitwillallowdetectionoftwokindsoftopoisomeraseinhibitors:thosethatstimulateformationof cleavable complexes and those that antagonize topoisomerase (topo) IV action on the DNA. Since topo IV is a type II enzyme, we may refer to it as either topo IV or topo II. A known topo II poison is included as a control. The assay system is based upon incubating supercoiled plasmid (pHOT1) with Topo IV and evaluating the formation of DNA cleavage products which may be nicked, open circular DNA or linear DNA. The products are then resolved by ethidium bromide gel electrophoresis as described in the kit (refer to diagram below).



B. Kit Contents (100 assay kit size)

1. pHOT1 DNA, supercoiled (25 ug in 100 ul) = 0.25 ug/ul

2. Marker DNA, Linear pHOT1; 0.05 ug/ul in 1x gel loading buffer (25 ul, load 2 ul as marker).

*3. Incomplete Buffer A contains the following: 200 mM HEPES-KOH [pH7.6], 500 mM potassium glutamate, 50 mM Magnesium Acetate, 50 mM Dithiothreitol, 250 ug BSA/ml. (500 ul).

*4. ATP Buffer B contains 100 mM ATP (50 ul).

**These two buffers must be mixed before use to make the final 5x Complete Topo IV Assay Buffer (A+B) as follows: add 0.1 volume of Buffer B to 1.0 volume Buffer A. Prepare only as much of the Complete Topo IV Assay Buffer (A+B) as needed for each day or set of experiments. Note that this buffer should be kept on ice and used the same day, then discarded.*

5. Ciprofloxacin control drug (Cat# 4180 for replacement) is provided as a 10x stock (10 ug/ml, total of 50 ul) 6. 10% sodium dodecyl sulfate (SDS) (300 ul): to terminate reactions, use 1/10 volume.

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7. 10x gel loading buffer (300 ul): 0.25% bromophenol blue, 50% glycerol: Use 0.1 vol.

8. Proteinase K (500 ul) at 0.5 mg/ml. This is a 10x stock of proteinase K (replacement Cat# TG4039)

As noted above, purified Topoisomerase enzyme is not included in this kit but is available on line at www.topogen.com (Cat No. TG2000EC).

C. Kit Protocol

Reaction volumes should be 20-30 ul final volume (limited by the volume that can be loaded into the wells of the agarose gel). The reactions should be assembled on ice in microcentrifuge tubes (water, buffer, and DNA, test compound and enzyme last). After adding enzyme, the tubes should be transferred to a heating block to initiate the reaction. Reactions should be incubated 30 min (37°C), terminated by rapid addition of 1/10 volume of 10% SDS followed by digestion with proteinase K (50 ug/ml final) prior to loading the gel. SDS is added to reactions at 37° to facilitate trapping the enzyme in a cleavage complex. If the reactions are heated, cooled or treated with high salt prior to SDS, the topo IV breakage and resealing equilibrium may be altered and breaks can reseal. A sample reaction is shown below.

For Reaction Mixture of 20 ul:

H2O	Variable, make up to volume (20 ul in this case)
5x Complete Buffer	4 ul (prepared fresh as described above)
DNA	1 ul (200-500 ng is sufficient)
Test Compound	Variable. (Limited by solvent effects, see controls below) Inhibitor control should be used at a final concentration of 1 ug/ml.
Topoisomerase IV	**Variable. Enzyme is supplied at 2 to 20 units/ul (depends on lot#). Usually, 2 ul of stock enzyme is enough to detect cleavages; however, up to 4 ul can be tested in a 20 ul reaction but not more (due to salts in the enzyme preparation). *Note that the amount of cleavage will be low, but still detectable. You should not expect to see 100% conversion of substrate to linear DNA (see comments below).***

•Incubate 30 minutes at 37°C and stop by addition of 2 ul 10% SDS.

•Add proteinase K to 50 ug/ml, (incubate 37°C for 15 min.); add 0.1 vol. loading buffer. Samples may be loaded directly onto the agarose gel at this point. Alternatively, the samples can be cleaned up by extraction and then loaded: Add equal volume (20 ul) of Chloroform: isoamyl Alcohol or CIA (24:1), vortex briefly; spin in a microfuge for 5 sec. Withdraw blue

colored upper aqueous phase and load onto agarose gel. Note that CIA extraction is optional but usually can improve the cosmetic quality of the agarose gel separation.

•Run 1% agarose until the dye front is at the bottom of the gel. Stain with 0.5 ug/ml ethidium bromide and photodocument results. This is a "non-ethidium bromide" gel separation which is optimal for resolving relaxed and supercoiled DNAs (see gel data, page 8). We recommend that each researcher use this method (non-EB gel assays) to verify enzyme activity, PRIOR to testing new compounds. Once enzyme activity is demonstrated, the researcher may wish to run gels containing 0.5 ug/ml ethidium bromide. The buffer and gel should contain ethidium bromide DURING the electrophoretic separation (followed by extensive destaining with water prior to photodocumentation). The ethidium bromide gel separation method allows one to clearly resolve cleavage products (open circular, linear DNA) from the closed DNA forms (relaxed circular and supercoiled circular DNA). Note that with EB gels, separation of SC and REL DNA is very subtle and difficult to resolve; thus, it is generally a good idea to split reactions in half and run one EB gel and one NON-EB gel to compare the results.

Marker DNAs and controls (see Fig. 1) are extremely important. Any nuclease-free agarose of reasonable quality can be used. Agarose gels (1%) and running buffers can be any standard nondenaturing electrophoresis buffer (example, 1 x TAE buffer, 50x stock is 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA) and ethidium bromide to a final concentration of 0.5 ug/ml (5 ul of a 10 mg/ml ethidium bromide (EB) stock per 100 ml of running buffer). Include EB in the gel and running buffer. Run gels at 1.5-2 V/cm (measured between electrodes) until the dye front has traveled about 80% of total gel length. The DNA can be monitored during electrophoresis with a handheld ultraviolet light. After running, gels should be destained (30 min) in water or buffer and photographed using a standard photodyne system. As noted above, we recommend EB gels to allow you to clearly resolve linear DNA (see below) from the topoisomers. If, however, you wish to determine whether your test compound is a catalytic inhibitor, you should run the gels without ethidium to resolve the topoisomers. The non-EB gels are also ideal for documenting relaxation activity of topo IV. As noted above, we recommend strongly that you run both non-EB and EB gels for each experiment.

D. Helpful Hints and Interpretation

Solvent controls are especially important to ensure that the drug solvent (DMSO or methanol for example) are not interfering with topo activity. Also, a quick CIA extraction prior to loading the gels is a good idea since the gels will be much more cosmetic; however, the samples must first be treated with proteinase K to digest the bound protein. CIA extractions may also be required if the test compound affects the mobility of the DNA or fluorescent detection (intercalators, strong DNA binding agents, etc.). Note also, to confirm the formation of covalent complexes, you can run the following control (refer to diagram on the cover page above). Two reactions are carried out with the inhibitor; both are stopped with SDS. One is digested with proteinase K and the other is undigested. The covalent topo/DNA complexes in the reaction that was not digested with proteinase K will be lost by CIA extraction (they will partition to the interphase) and you should lose the linears or possibly open circular DNA complexes. In contrast, the proteinase K digested sample should contain expected cleavage products (linear and nicked DNAs). Fig 1 shows a typical Gel (EB) result with the markers and controls:

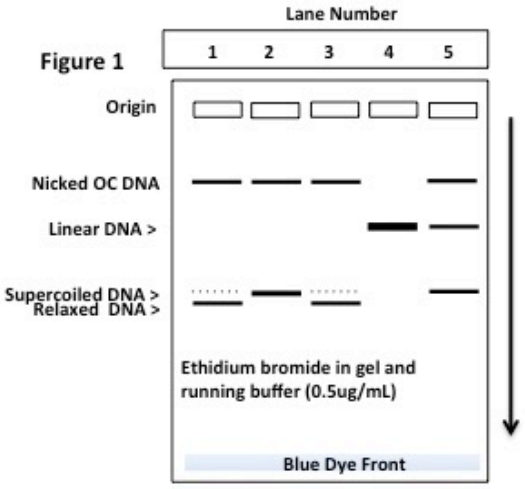


Figure 1. Representative Plasmid DNA Products from Topo IV on Agarose – Ethidium Bromide Gel.

Lane 1: Control, shows that incubating plasmid DNA with topo IV gives relaxed DNA product. Note that resolution between relaxed and supercoiled DNA is not always ideal (proper conditions must be worked out empirically for each lab's gel unit system). We suggest running gels until blue dye is at the end using a voltage that will migrate the dye over a 2h period (room temperature). Since a positive control of enzyme activity is critical, we suggest running both EB and non-EB gels (splitting the sample in half and loading the two different gels with equal amounts of reaction).
Lane 2: A marker of pHOT1 DNA. Note that a small amount of nicked OC (open circular) DNA is always present in plasmid preps.
Lane 3 shows the solvent control that demonstrates that the solvent alone is not affecting topo IV activity.
Lane 4: Linear DNA marker (essential to mark position of cleavage product). Marker DNAs are critical.
Lane 5 shows cleavage in presence of a control poison, like Ciprofloxacin. Note that not all supercoiled DNA is converted to linears. As discussed below, the cleavage event is a low yield process in most assay screens of the type.

E. Important Controls and Considerations (see Fig. 1)

1. Reaction containing enzyme without test drug or solvent: To detect enzymatic activity in the absence of test compound. This reaction should be carried out in assay buffer to detect relaxation.

2. Reaction containing solvent alone without enzyme: To ensure that solvent (that the test compound is dissolved in) does not affect mobility of DNA species in the assay. Also to make sure that DNA nicking independent of enzyme is not occurring. (Often DMSO is used; test a concentration of solvent at the highest concentration of compound being tested, see example in next point.)

3. Reaction containing enzyme plus solvent: To ensure that solvent (ex. DMSO or methanol) is not affecting the activity of purified enzyme. This control should be carried out with assay buffer to allow you to detect relaxation activity (run a non-EB gel). Test the concentration of solvent corresponding to the highest concentration of test compound (example if DMSO is solvent and the test drug is dissolved in 50% DMSO and a 1:100 dilution of stock drug is tested, one must show that 0.5% DMSO is not causing inhibition of enzyme activity). DMSO concentrations in this range can (depending on the DMSO source) affect topo II activity; this must be pre-determined to ensure that a range of drug concentration is used that does not cause a solvent inhibition artifact. As noted above, solvents may also cause nicking of the DNA substrate. This can create serious complications, obviously.

4. Marker DNAs: Linear DNA should be included as a reference marker. Also, we strongly recommend that super- coiled DNA be incubated in cleavage buffer without enzyme at 37° for 30 min to make sure that nicking is not occurring in the absence of topo II enzyme.

5. Control reactions with known topo IV poisons are not required with every gel, but should be performed initially to reconstruct the inhibition one should expect. As noted above, a drug control will yield linear DNA cleavage complexes provided sufficient enzyme is used.

6. The cleavage reaction is a low yield event. That is, the amount of cleavage product will generally be only weakly detected, even with ciprofloxacin control. This is because the cleavage event actively consumes the enzyme leading to stoichiometric complexes between topo

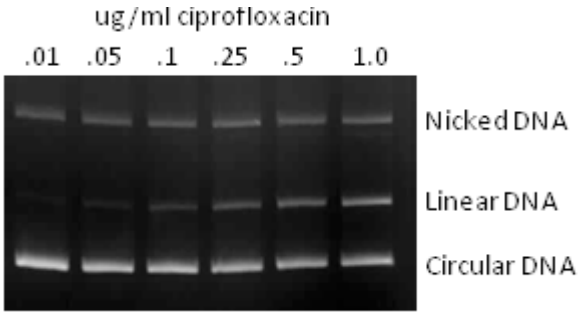
IV/DNA; thus, the cleavage conversion uses up the enzyme. You should expect to see some detectable level of linear DNA but it is rare to see complete conversion of supercoiled DNA into linear DNA cleavage products. It is more likely that you will see 5-25% conversion of substrate to linears. If higher cleavage conversion is desired, one can convert to a radioactive assay format using 32P-end labeled DNA and/or use high levels of topo IV in order to improve sensitivity.

References

1. Kato et al. (1990) Cell 63:393-404. 2. Peng,H.,and Marians,K.(1999).DNA Topoisomerase Protocols Volume I; page 163 (available from TopoGEN, www.topogen.com)

Topo IV Drug Screening Results

Reactions indicated above each lane were carried out with topo IV (4 units, based on relaxation activity or 20 units based on decatenation assay) and 250 ng plasmid DNA. Reactions were incubated for 30 min at 37 deg C, stopped with 0.1 vol of 10% SDS, digested with proteinase K and CIA extracted as described above. A 1% agarose gel containing 0.5ug/ml EB was run until the dye front was at the bottom of the gel (2 v/cm). In this experiment, we titrated increasing amounts of Ciprofloxacin as a control drug. In this case, linear DNA formation (primary cleavage product) is readily detected under these conditions with as little 0.1 ug/ml of Ciprofloxacin. Note that in this gel, the supercoiled and relaxed DNAs did not resolve (but since both are circular DNAs, we label them as such).



Analysis of topo II relaxation activity using agarose gels without ethidium bromide (EB)

Notice that the above gel (contains EB in gel and running buffer during electro- phoresis) is optimal for resolving cleavage products (linear, nicked or OC DNA); however it is not ideal for resolving form I supercoiled DNA (substrate) from relaxed DNA (product). In other words, clear demonstration of topo IV relax- ation activity is sometimes difficult with an EB gel; however, it is important that you demon strate enzyme activity as a control. Non-ethidium bromide gels (ie, no EB in gel or buffer DURING electrophoresis) are ideal for this purpose. Gels are run and stained with EB after electrophoresis is completed. The gel to the left shows a typical result. It is easy to see that Supercoiled DNA is relaxed to form a series of topoisomers ("Relaxed" bands in the gel). Since it is essential to have good enzyme activity in order to detect cleavage products, this analysis is important to verify that the enyzme is active. In general, it makes sense to run both EB and non-EB gels for each experiment. Simply divide the reactions in half and run one EB and one non-EB gel.