DNA Unwinding Kit Catalog# TG1025
USER MANUAL

Overview:
This kit is designed to determine whether a particular compound is a DNA intercalator. The kit includes everything except for DNA topoisomerase I. Purified topoisomerase I is available from TopoGEN and may be ordered on-line at http://www.topogen.com (TG2005H-RC1).

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. The *m*-AMSA should be stored at ambient temperature (-20°C). Avoid frequent freeze/thaw cycles with the plasmid as this may contribute to DNA breakage.

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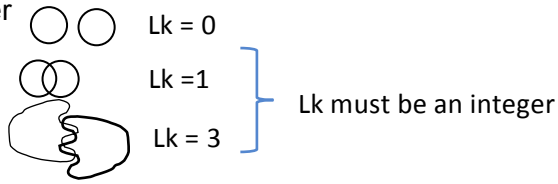
Introduction

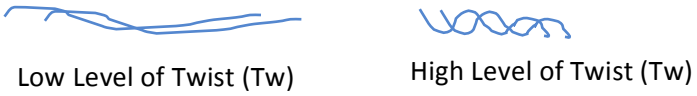
Unwinding of the double strands of the DNA helix is a hallmark feature of intercalating drugs, such as chloroquine, ethidium bromide and *m*-AMSA. As part of characterization of novel agents, it is often important to know whether a novel compound is a DNA intercalator. This can easily be tested influence of a compound on the distribution of topoisomers (generated by relaxation of closed circular DNA with topo I) will reveal the presence of a DNA intercalator. For example, intercalators induce a change in the average DNA linking number of a population of topoisomerase generated by topo I (compared to reactions lacking the test compound). Stated differently, DNA intercalation induces unwinding of the DNA template and thereby alters a parameter called Twist (Tw). Defining the details of DNA supercoiling is beyond the scope of this introduction; however, excellent reviews on the subject can be found in the literature (1-4). Briefly, the relation between strand twists, linking and writhe can be expressed by the simple equation:

Lk = Tw + Wr Where

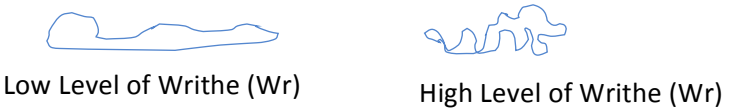
- Lk is linking number: An integer describing the number of times one strand is linked with the other



- Tw is the arrangement in space of one strand twisting about the other and is continuously variable



- Wr is the writhing numberwhich describes deformation of the helical axis in 3D space.



Wr is also a continuously variable parameter. Lk is fixed property of a circular plasmid DNA and cannot change no matter how Tw and Wr fluctuate; Lk can only change by breaking/resealing and subsequent strand passing (topoisomerase I does this for example). By extension, when a DNA molecule is fully relaxed by topo I, Wr approached zero and Lw = Tw. For fully ***relaxed*** DNA, Lk roughly equals the number of a base pairs in the plasmid divided by 10 (since each strand of the duplex makes a full 360° rotation every 10 bp or so). The products of relaxation by topo I strongly influenced by conditions that are exist at the time of strand closure: as a population of DNAs one sees a gaussian distribution of topoisomerase (refer to Fig. 1). Each topoisomer will differ from its nearest neighbor by a Lk difference of 1. DNA intercalators are known to locally unwind the DNA (5). Upon intercalation, Tw is reduced and since topo I can sense this change and adjust Lk, the equilibrium distribution od topoisomers is subsequently adjusted; therefore, the Lk now reflects the Tw parameter of the intercalated form. After phenol extraction removes the topoisomerase (and any excess drug), any alteration in the population

distribution of topoisomers is revealed by running an agarose gel. The gels are run to high resolution to display topoisomers; thus, gels are generally run without ethidium bromide and typically at a low voltage to maximize separation of the Gaussian distribution of topoisomers. These DNA unwinding experiments are informative and reveal useful and subtle information about drug/DNA interaction. Comparison of DNA unwinding with well known DNA intercalators (such as *m*-AMSA) give the investigator some idea of the potency of DNA binding. One important caveat, however is that the investigator must be sure that the test drug is not inhibiting topo I activity. Proper controls will eliminate this potential complication.

References:
1. "DNA Replication" By Kornberg. WH Freeman and Company, section 1-7
2. Bauer Ann.Rev. Biophys Bioeng. 7: 287-313 (1978)
3. Bauer et al. Science 243:118-133 (1980)
4. Shure and Vinograd Cell 8:215-266 (1976)
5. Waring, Ann. Rev. Biochem 50:159-192 (1981)

Kit Description

The purpose of this kit is to determine if a compound induces DNA unwinding characteristic of DNA intercalators. The procedure requires catalytic amounts of topo I (not supplied with kit, order TG2005H-RC1 see www.topogen.com). Sufficient materials are provided for at least 50 independent determinations.

Kit Contents

1. Substrate DNA: pHOTI DNA (total of 12.5 ug of supercoiled DNA in 50 ul) Final concentration of DNA is typically 0.25 ug/ul however, please refer to the tube label for verification.

2. Relaxed DNA Marker: 0.05 ug/ul in lx gel loading buffer. A total of 50 ul of marker is provided in the kit; use 2-4 ul per lane on each gel as marker.

3. l0x Topo I assay buffer, TG4010 (750 ul). 1x TGS is 10 mM Tris-HCI, pH 7.9, 5% glycerol, 0.1 mM Spermidine, 1 mM EDTA, 0.15M NaCI, 0.1% BSA.

4. 10x Gel Loading buffer, Cat# TG4038 (750 ul).

5. 10% SDS, Cat# TG4060 (750 ul).

6. *m*-AMSA, Cat# TG4150 (250ul) inDMSO(10mMStock).

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

- Agarose and related electrophoretic supplies and equipment transilluminator/photodocumentation system
- DNA topoisomerase I (available at www.topogen.com specify TG2005H- RC1)
- DNA extraction and purification related reagents: Proteinase K, Choloroform: Isolamyl alcohol (24:1)

Procedure

Step 1. Set up topo I reactions as follows (assume a 20 ul reaction volume).

Component:	Volume:	Notes:
10x Topo I buffer	**2 ul**	**For a final reaction volume of 20 ul**
Sterile Water	**Variable**	**Bring to final of 20 ul**
pHOT1 DNA	**1 ul**	**150-250 ng of DNA is sufficient**
Topoisomerase I	**Variable**	**Typically 1-2 ul or 2-4 units of purified topo I**

Step 2. Incubate for 30 min at 37°C

Step 3. Add variable amounts of the test drug (or m-AMSA control). For m-AMSA add 2 ul of the following dilutions (prepare from stock): 100 uM, 200 uM, 500 uM, 1000 um.

Step 4. Continue the incubation for another 30 min. at 37°C.

Important control: Be sure to test relaxation of pHOT1 in the presence of the highest concentration of test compound. You must verify that the drug does not inhibit topo I activity. Also verify that the solvent (DMSO, Methanol) alone does not inhibit topo I by running suitable control reactions.

Step 5. Terminate reactions by addition of SDS to 1%. Add proteinase K to ca. 50 ug/ml and digest for 15-20 min at 56°C.

Step 6. Add 0.1 Vol of l0X gel loading buffer.

Step 7. Extract 1-2 times with an equal volume of CIA (Chloroform:isoamyl Alcohol; 24:1) as follows: Add CIA, vortex briefly and spin in microfuge 1 min and recover the blue, aqueous phase; load directly onto a 1% agarose gel.

Step 8. Run an agarose gel to maximize resolution of topoisomers under the following conditions: Cast gel in TPE buffer (36 mM Tris-HCI, pH 7.8, 1 mM EDTA, 30 mM NaH2P04) and 0.2 ug/ml Chloroquine. Run the gel in TPE buffer at room temperature at 15 volts for 15 hrs. Destain in water for 15 min, stain in 0.5 ug/ml ethidium bromide and destain for 30-45 min in water. These conditions should give excellent separation of the topoisomers. ALTERNATIVELY: Run standard TAE gels without any dye at 12-25v until dye front is roughly 80-90% down the gel (bromophenol blue).

Controls and Important Considerations.

A. Markers:
It is important to run a relaxed DNA marker to mark the position of this DNA in the gel (included with kit).

B. Drug effects:
This assay is based upon the ability of topo I to act catalytically in the presence of the test compound; clearly if the latter inhibits topo I, then the results will be meaningless. For this reason, the analysis should include the following reactions:

1. First, start with supercoiled pHOT, relax with topo I (30min reaction) then add the drug and continue the incubation for an additional 30 min. This is described in “Steps 1-4” above.
2. Second, it is also important to perform the same basic reaction except add the test compound at the same time as addition of topo I. This will verify that topo I is active in the presence of all drug concentrations being tested.
3. Third, you should incubate pHOT1 DNA with the drug in the absence of topo I to ensure there is no topo I independent effects on the DNA (such as DNA nicking

Variations:

Concentrations of test drug should cover a fairly wide range within the constraints if the assay (i.e. to ensure that topo I activity is not inhibited), As noted above, it is also important to verify that the drug itself does not cause any DNA nicking independent of topo I.

Fig 1: Outline of DNA Unwinding Assay for Intercalators

This diagram shows a relaxation reaction (step1) and reaction products (see schematic of agarose gel in the bottom panel) that result when topo I is allowed to adjust DNA linking number in the presence of compounds that influence the DNA unwinding angle (Step 2). The two outcomes of this experiment (after step 2) are illustrated: 1) intercalators will shift the gaussian distribution of topoisomers as shown; 2) Non-intercalators will not affect the final equilibrium distribution of topoisomers from that seen with relaxed DNA (step 1 products)