TopoGEN
Products and Services for **Cancer Research.**

Application Manual

Gyr-Drug Screen Kit™

Purified *E. coli* DNA Gyrase Drug Screening Kit (plasmid based).

- ☐ **Catalog Number TG2001G-1KIT 100 Reaction Set [50 ug DNA]**
- ☐ **Catalog Number TG2001G-3KIT 500 Reaction Set [250 ug DNA]**
- ☐ **Catalog Number TG2001G-5KIT 1000 Reaction Set [500 ug DNA]**
- ☐ **Catalog Number TG2001G-7KIT 2000 Reaction Set [1 mg DNA]**

Overview:

DNA Gyrase is an important target for anti-bacterial drugs and new antibiotics are constantly required to deal with drug resistant strains of pathogens. This Kit is designed to identify agents (small molecules, natural products, biological material or synthetics) that directly target DNA Gyrase from E. coli. It will identify catalytic inhibitors, that block enzyme action, as well as poisons, that induce DNA cleavages.

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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I. Introduction (see TopoGEN YouTube Channel as well).

A. Summary

This kit contains purified bacterial (*E. coli*) DNA Gyrase purified to homogeneity (>98% based on SDS-PAGE). The Gyrase is prepared from overexpressing strains and is supplied as purified holoenzyme in an A_2B_2 complex. The enzyme is supplied at the unit concentration given on the quality control data provided with the kit. The enzyme is stored in a stabilization buffer [50 mM Tris-Cl pH 7.5, 100 mM KCl, 2 mM dithiotreitol, 1 mM EDTA, 50% glycerol]. Also included is substrate DNA supplied in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Concentration of the relaxed DNA is provided on the label of the tube.

This kit is designed to screen for novel gyrase targeting drugs, either as interfacial poisons (or IFPs that induce cleavage of DNA) or as catalytic inhibitory compounds (CICs that block gyrase action by any number of mechanisms). It is a straight forward gel assay kit that is easily adopted.

The Assay: This drug screening Kit is based on gyrase supercoiling action on a relaxed plasmid DNA substrate provided (pHOT1, which is a derivative of pBR322) and roughly 2.7 KB. One unit of gyrase will supercoil 500 ng (0.5ug) in 1 hr at 37°C under conditions defined below. The reaction mechanism is describe below in Fig. 1. The enzyme introduces negative supercoils into a relaxed pHOT1 plasmid substrate via a sign inversion model. An energy co-factor (ATP) is required for the complete supercoiling reaction. The inset gel image in Fig. 1 represents

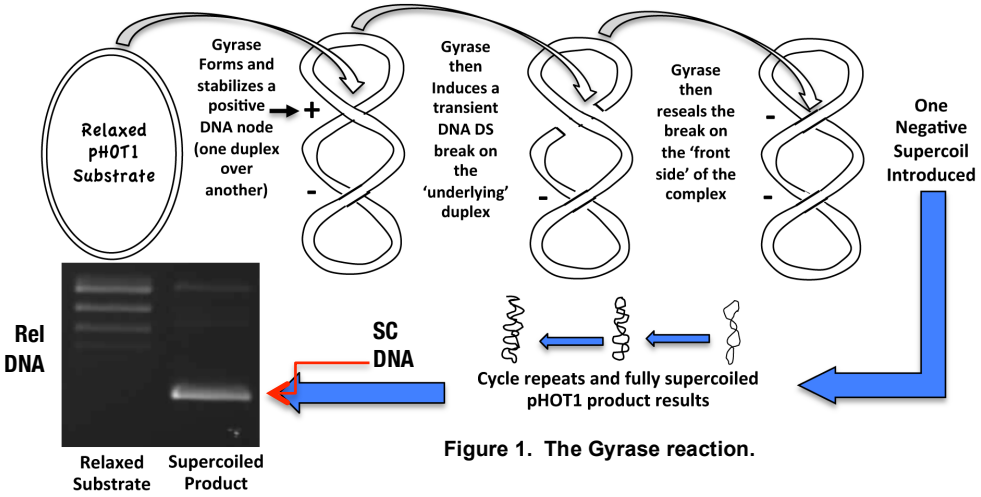

Figure 1. The Gyrase reaction.

a typical substrate and product (supercoiled DNA) result based on an agarose gel that lacks ethidium bromide. These non-EB gels are ideal for detecting gyrase activity as attested by the excellent resolution between supercoiled and relaxed DNA forms.

Drug Screening Assay. This kit is designed to identify IFP and CIC (poisons and inhibitors, respectively) using the assay described in Fig. 1. A typical IFP (certain fluoroquinolones) will induce double strand DNA breaks while a prototypical CIC will simply block the enyzme from acting on relaxed DNA (novobiocin, some intercalators for example). Thus, an IFP (ciprofloxacin) will result in formation of a linear DNA product while a CIC will block supercoiling activity. These events can easily be detected with this kit.

C. DNA Gyrase Quality Control.

1. A test for nuclease contamination was carried out by assaying for the formation of linear Kinetoplast DNA (kDNA) and linear plasmid DNA. Incubations of 1 µg of catenated kDNA or supercoiled pUC19 DNA (4 hrs. at 37°C in the presence of 10 mM $MgCl_2$) were performed. Linear DNA or breakdown products were not generated under these conditions.
2. The A and B subunits are >98% pure based upon SDS-PAGE and certified to be endonuclease free.
3. Note: with high input levels of DNA gyrase, a small amount of linear DNA may be detected due to spontaneously aborted reactions. This is normal.

D. Dilution Buffer

Dilution of purified DNA gyrase should be performed in 50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 2 mM dithiothreitol, 1 mM EDTA and 50% glycerol.

E. Supercoiling Assay Conditions

One unit of gyrase is typically incubated with 0.5 ug of relaxed plasmid DNA in a reaction volume of 20-30 ul for 1 hr. at 37°C in assay buffer[a].

[a]Assay buffer (1x recipe is shown below; kit includes a 5x stock based on this formula):
35 mM Tris-Cl pH 7.5
24 mM KCl
4 mM $MgCl_2$
2 mM dithiothreitol
1.8 mM spermidine
1 mM ATP
6.5% glycerol
0.1 mg BSA/ml

F. Relaxed DNA Quality Control Tests:

1. Purity was evaluated spectrophotometrically using A_{260}:A_{280} readings.
2. Incubations with gyrase buffer alone at 37°C for 60 min did not result in formation of nicked or linear DNA species.
3. Relaxation of supercoiled DNA was carried out using TopoGEN's purified DNA topoisomerase I (TG2005H-RC1). Greater than 95% of the plasmid is relaxed under these conditions.

G. Kit Contents.

Volumes are given for the TG2001G-1KIT size. (For the larger kits size, multiply volumes as appropriate, see page 1 for Kit sizes).

1. Relaxed pHOT1 DNA (50 ug total) DNA concentration is specified on page 1.
2. Supercoiled pHOT1 DNA (25 ul in gel loading buffer). Load 2 ul as a marker.
3. Linearized pHOT1 DNA (25 ul in gel loading buffer). Load 2 ul as a marker for cleavage (DS DNA breaks).
4. 5x Gyrase assay buffer (600 ul) 1x buffer contains recipe is given above.
5. Dilution buffer (600 ul). Dilute gyrase with this buffer. Recipe for dilution buffer given above.
6. 10x gel loading buffer (300 ul): 0.25% bromophenol blue, 50% glycerol, use 0.1 volume in reactions.
7. 10x Proteinase K (100 ul) at 0.5 mg/ml (10x stock of proteinase K and should be diluted to a final 1x to degrade proteins after reaction termination; i.e. use at 50 ug/mL final).
8. 10% SDS (300 ul). To be added to reaction mix to give a final concentration of 1% to terminate the relaxation reactions.
9. Purified DNA gyrase; 100 units (unit definition given on page 1).
10. Ciprofoxacin 10ug/ml: 50 ul (10x Stock; dilute 1:10 in final reaction mix to give 1 ug/ml final).

H. Protocol for a typical reaction mixture (final volume of 20 ul)

Protocol Overview: Reaction volumes should be 20-30 ul final volume (generally limited by volume that can be loaded onto the gel). Reactions are assembled in this order (on ice) in microfuge tubes: water, buffer, relaxed DNA, and test drugs (or Ciprofoxacin control) as appropriate. The gyrase should be added last (while on ice) and the reactions moved to 37^{o}C for 30-60 min. (or longer as appropriate) then terminated by addition of 0.1 vol 10%SDS (1% final). Next, proteinase K is added, followed by organic extraction and loaded onto an agarose gel. Typically, one runs a non-EB (ethidium bromide) containing gel; however In some cases, you may wish to run an EB gel (0.5ug/ml EB IN GEL AND BUFFER) in order to resolve nicked and linear DNA from circular substrate (relaxed pHOT1) and supercoiled DNA products. If you are unsure which to run, we recommend that you try running both gel systems and divide your samples into two parts. Usually there is sufficient DNA in the reaction to run two gel systems.

I. An Example (20 ul, order of addition is shown):

1. Sterile Distilled H_20: Vary as needed to bring volume up to 20 ul
2. 5x Assay Buffer: 4 ul
3. pHOT1 Relaxed DNA: 1 ul (vary as needed)*
    i. **The amount of relaxed DNA depends on sensitivity and detection methods. With routine staining using ethidium bromide, about 150-250 ng of DNA is usually sufficient; however, less may be used with more senstitive stains.*
4. Test agent or Ciprofloxacin control Drug (2ul of 10ug/ml stock). Beware that solvents (methanol or DMSO) in excess of 0.5% can affect the gyrase. Ciprofloxacin final concentration to be 1ug/ml.
5. Gyrase: 1 ul (always add enzyme last on ice and transfer all tubes to heat block to initiate reaction sequence).

Procedure for a non-EB gel analysis of DNA Gyrase Supercoiling:

1. Incubate 30- 60 min at 37°C.
2. Add 0.1 vol of 10% SDS (2ul to a 20ul Reaction Mixture)
3. Add proteinase K to 50 ug/ml and digest for 30 min at 37°C; then add 0.1vol of 10x loading dye (bromophenol blue).
4. Organic Extraction: Add 20 ul of Chloroform:isoamyl alcohol (24:1 mixture), vortex briefly, withdraw blue, aqueous phase.
5. Load blue phase onto a 1% agarose (50x TAE buffer: 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5M EDTA). Be sure to include markers (linear and supercoiled DNA; 2 ul is sufficient)
6. Electrophorese at 50-100 v. until dye travels 60% down the gel.
7. Stain with 0.5 ug/ml ethidium bromide for 30 min (caution, EB is a mutagen, WEAR GLOVES).
8. Destain (distilled water) for 10 min room temperature (handle gel with gloves). NOTE: be sure to process the gel for image capture as soon as possible. (Do not let gels sit more than 15' after destaining to minimize band diffusion).
9. Photo-document using image capture software or a simple UV transilluminator.

J. Analysis of reaction products by electrophoresis: For each gel, run a negative control (relaxed DNA lacking gyrase plus and minus drug being tested). The negative control should look very different from the gyrase reaction products (which will be faster migrating, supercoiled DNA forms; see Fig. 2, below).

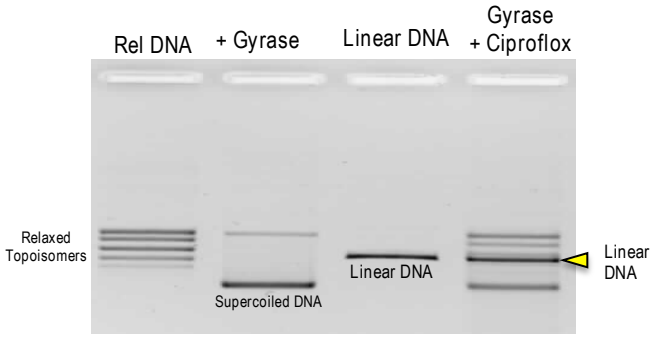

Figure 2. Gyrase Products Resolved on a Non-EB Gel system

Gyrase reactions were carried out in a final volume of 20 ul (see above protocol). Reactions were terminated with 1% SDS, digested with proteinase K and extracted with CIA. The Gel were run at 50v for 45-50 min and stained with EB (non-EB gel) per protocols given above. The data show the positions of relaxed DNA as well as gyrase reaction products (supercoiled DNA). The reaction with a fluoroquinolone (Ciprofloxacin control at 1ug/ml final concentration in reaction mixture) yields a linear DNA cleavage product in the last lane on the right (marked with a small triangle). For full interpretation of gel data, it is important to include relaxed DNA, linear DNA markers as well as a key controls (gyrase and gyrase + drug), as shown here. In this way, negative results are still meaningful.

K. Frequently asked questions.

What are the critical controls to allow me to clearly identify drug that is affecting DNA gyrase using this assay?

- Marker DNAs (supercoiled, linear DNAs) are extremely important.
- Be sure to run a positive DRUG control (like a fluoroquinolone) to demonstrate good cleavage activity. You should see an increase in linear DNA.
- Include a negative control (either no drug or a topo II drug such as etoposide). VP16 or etoposide is not supplied but we have it available.
- Be sure to check solvent effects. Solvents like DMSO or methanol are used to dissolve some test drugs. Test with a control reaction lacking drug but with solvent (e.g. 1% DMSO).

What kind of agarose should I buy?

Any nuclease-free agarose of reasonable quality from any number of sources can be used (Sigma-Aldrich works).

What is the best gel buffer to use?

Agarose gels (1%) and running buffers can be any standard nondenaturing electrophoresis buffer (example, to prepare a 50x of TAE Gel Buffer: 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA). Dilute to 1x to use for gel separations. Be sure that the gel also has 1x TAE buffer.

Should I run EB or Non-EB gels and how do I run the EB gels?

- In general, 1% gels in the absence of Ethidium Bromide (EB), can be used (these gels are ideal for testing enzyme activity); however, the nicked open circular DNA product cannot be clearly identified in this gel system. As noted above, an EB containing gel (0.5 ug/ml, EB in gel and buffer) will improve the resolution of cleavage products (nicked open circular and linear DNA). Be sure to destain with water for 15 min prior to photodocumenting your data.
- In some cases, depending on how the gel is run, the topoisomer distribution can interfere with your ability to see the cleavage products; however, EB gels remove this complication.
- *IMPORTANT*: If unsure about whether to run an EB or Non-EB gel, we suggest that you run both. Simply divide your reactions into equal parts and run two gels at the same time. In this way, you will be sure to see all reaction products and enhance your interpretation of the experiment. With all markers in the gel system (supercoiled, relaxed, linear for example) you will obtain very clear and unambiguous results.

What are the running conditions in terms of time and voltage?

- Run gels at 1.5-2 V/cm (measured between electrodes) until the dye front has traveled about 80%
- After running, non-EB gels should be stained with EB (0.5 ug/ml) for 15-30 min and then destained in water or buffer for 15 min prior to photodocumentation.
- EB gels are run in the presence of 0.5 ug/ml (in gel and running buffer), then destained with water for 15 min prior to photodocumentation.
- IMPORTANT: Try not to run the gels overnight but keep your electrophoresis times to less than 1-2 hrs. Long run times cause band diffusion and degrade the quality of your gel results.

What reaction volumes do you recommend for these assays?
- 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 microfuge tubes (water, buffer, and DNA, test compound and enzyme, which should be added last).
- After adding enzyme, the tubes should be transferred to a heating block to initiate the reaction.

Does it matter whether I run a large format gel or a minigel?
- Either will work. Minigels are very convenient and give reasonably fast run times.
- The actual shape and size of the well slot is a factor in how well your bands resolve. It is best to use a comb that gives a long and thin rectangular 'well'. Avoid combs that make 'boxy' wells. Do NOT let gels sit around after electrophoresis. Photo-document as soon as possible after completion of the electrophoretic run, staining and destaining gels.

Are the termination conditions critical for detecting cleavages?
- Yes. Reactions should be incubated 30 min (37°C), terminated by rapid addition of 1/10 volume of 10% SDS followed by digestion with 50 ug/ml proteinase K prior to loading the gel. SDS is added to reactions at 37° to facilitate trapping the enzyme in a cleavage complex.
- Also, if the reactions are heated, cooled or treated with high salt prior to adding SDS, the topo breakage and resealing equilibrium may be altered and breaks can reseal.

Why is proteinase K required?
- Drugs that trap topo/DNA complexes will induce covalent complexes between DNA and protein (topo) and this protein must be removed (degraded). Failure to do so will prevent detection of the cleavage products.
- If the reactions are heated, cooled or treated with high salt prior to SDS, the topo DNA breakage and resealing equilibrium may be altered and breaks can reseal.

Can you help us with data interpretation?
- Yes, we can definitely help! The best way to proceed is to send us your data (support@topogen.com) with a full description of the experiment. We will get back to you quickly with feedback.

Can you show us some real gel data and discuss the results?
- Yes, we can. The results and a helpful discussion is shown in Fig. 2 (see legend).

Any further questions or comments, please feel free to contact us:
support@topogen.com