

High-throughput assays for DNA gyrase and other topoisomerases

Anthony Maxwell*, Nicolas P. Burton¹ and Natasha O'Hagan

Department of Biological Chemistry and ¹John Innes Enterprises Ltd, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK

Received March 24, 2006; Revised May 5, 2006; Accepted June 30, 2006

ABSTRACT

We have developed high-throughput microtitre plate-based assays for DNA gyrase and other DNA topoisomerases. These assays exploit the fact that negatively supercoiled plasmids form intermolecular triplexes more efficiently than when they are relaxed. Two assays are presented, one using capture of a plasmid containing a single triplex-forming sequence by an oligonucleotide tethered to the surface of a microtitre plate and subsequent detection by staining with a DNA-specific fluorescent dye. The other uses capture of a plasmid containing two triplex-forming sequences by an oligonucleotide tethered to the surface of a microtitre plate and subsequent detection by a second oligonucleotide that is radiolabelled. The assays are shown to be appropriate for assaying DNA supercoiling by *Escherichia coli* DNA gyrase and DNA relaxation by eukaryotic topoisomerases I and II, and *E.coli* topoisomerase IV. The assays are readily adaptable to other enzymes that change DNA supercoiling (e.g. restriction enzymes) and are suitable for use in a high-throughput format.

INTRODUCTION

DNA topoisomerases are essential enzymes that control the topological state of DNA in cells (1,2). In prokaryotes these enzymes are targets of antibacterial agents, in eukaryotes they are anti-tumour drug targets and potential herbicide targets (3–5). All topoisomerases can relax supercoiled DNA, and DNA gyrase, present in bacteria, can also introduce supercoils into DNA. Despite being the target of some of the key antimicrobials and anti-cancer drugs in use today (e.g. ciprofloxacin and camptothecins), their basic reaction, the inter-conversion of relaxed and supercoiled DNA, is not readily monitored. The standard assay is gel-based

(see e.g. Figure 3), and suffers from the drawback of being slow and, due to the electrophoresis step, requires a lot of sample handling. There is a pressing need to develop higher-throughput assays, which would greatly facilitate work on topoisomerases (and other enzymes), and specifically would potentiate the use of combinatorial chemical libraries to screen for novel lead compounds (antimicrobials, anti-tumour drugs and herbicides). To this end we have developed topoisomerase assays based on DNA triplex formation; the underlying principle being the greater efficiency of triplex formation in negatively supercoiled DNA compared with the relaxed form. Using this principle, we have developed assays where the signal is either radioactivity or fluorescence.

DNA triplexes are alternative structures to the DNA double helix. In these structures a DNA duplex associates with another single strand in either a parallel or antiparallel orientation to form the triple-stranded structure (6–9). Triplexes can be intra- or intermolecular and generally consist of a polypyrimidine or polypurine strand lying in the major groove of a DNA duplex. Triplexes are of two basic types: one purine and two pyrimidine strands (YR*Y), or one pyrimidine and two purine strands (YR*R), stabilized by Hoogsteen base pairing. A protonated C forms two hydrogen bonds to the N7 and O6 of G, or a T forms hydrogen bonds to the N7 and 6-NH₂ groups of A. YR*Y triplexes have a pyrimidine third strand bound parallel to the duplex purine strand (involving T.AT and C⁺.GC triplets); YR*R triplexes have a purine third strand bound antiparallel to the duplex purine strand (involving G.GC, A.AT and T.AT triplets).

Triplex formation has been used in a variety of applications, including therapeutic targeting of oligos to specific DNA sequences (8). More recently triplexes have been used as a basis for assays for DNA translocation by type I restriction enzymes (10,11); the principle of these assays is the displacement of a fluorescently-labelled triplex-forming oligo (TFO) by the translocating enzyme. In other work it has been shown that triplex formation inhibits DNA gyrase activity, presumably by blocking access to the DNA duplex (12).

The aspect of DNA triplex formation that we have sought to exploit is the observation that triplex formation is favoured

*To whom correspondence should be addressed. Tel: +44 1603 450771; Fax: +44 1603 450018; Email: tony.maxwell@bbsrc.ac.uk
Present addresses:

Natasha O'Hagan, School of Biological Sciences, University of Edinburgh, Mayfield Road, Edinburgh, EH9 3JR, UK
Nicolas Burton, Inspiralis Ltd, Norwich Biocubator, Norwich Research Park, Colney, Norwich, NR4 7UH, UK

by negative supercoiling (13,14); so far this has only been reported for intramolecular triplexes, but it is likely to affect intermolecular triplexes as well. Previously immobilized biotinylated TFOs have been shown to be able to capture supercoiled plasmid DNAs (15). Following on from this work, we have now developed methods for assaying topoisomerases and other enzymes based on the differential capture of negatively supercoiled versus relaxed plasmids by immobilized TFOs.

MATERIALS AND METHODS

Enzymes, DNA and drugs

Escherichia coli DNA gyrase and DNA topoisomerase (topo) IV were from John Innes Enterprises Ltd (gifts of Mrs A.J. Howells); DNA topoisomerase I (wheat germ) was purchased from Promega, human topoisomerase I and II were from Topogen. Restriction enzymes were purchased from New England BioLabs (AvaI and AatII) and Invitrogen (EcoRI). TFOs were purchased from Sigma Genosys and are listed in Table 1. Plasmid pBR322* [a high copy number mutant form of plasmid pBR322 (16)] was from John Innes Enterprises Ltd (gift of Mrs A.J. Howells). To construct plasmid pNO1, oligos TFO1W and TFO1C (Table 1) were annealed and ligated into the AvaI site of pBR322*. To construct plasmid pNO11, oligos TFO2W and TFO2C (Table 1) were annealed and ligated into the AatII site of pNO1. The sequences of pNO1 and pNO11 were verified by DNA sequencing. Supercoiled forms of plasmids were prepared by transforming them into Top10 competent cells (Invitrogen) and growing in Luria-Bertani (LB) broth containing ampicillin (Sigma), and preparing the DNA using Qiagen mini and midi prep kits. Relaxed plasmids were prepared by incubating the supercoiled forms with topo I [~ 40 – 50 μ g plasmid, 200 U topo I, in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl and 0.1 mM EDTA], for 1 h at 37°C, and were purified by phenol/chloroform extraction and ethanol precipitation. Plasmids with specific linking difference (σ) in the range 0–0.11 were made by relaxing the supercoiled form with topo I in the presence of different concentrations of ethidium bromide [100 μ g plasmid, 400 U topo I, in 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.25 mM EDTA, 5% glycerol plus 0.01 μ g/ μ l BSA], for 1.5 h at 37°C, and subsequent purification by two phenol/chloroform extractions, one chloroform extraction and precipitation with ethanol. Plasmids were resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. TFO2 was radiolabelled using polynucleotide kinase and [γ - 32 P]dATP. Radiolabelled oligos were separated from unlabelled material using Microspin G-25 columns (Amersham Biosciences). Ciprofloxacin and novobiocin were purchased from Sigma

and Fluka, respectively; SYBR Gold nucleic acid stain was purchased from Invitrogen; camptothecin was purchased from Sigma.

Surface plasmon resonance (SPR)

SPR was carried out using a Biacore X instrument. Streptavidin-coated chips (SA chip; Biacore International SA) had ~ 5 μ l 100 nM biotinylated oligo (TFO1 or TFO2) in HBS-EP Buffer (Biacore International SA) immobilized on to flow cell 2 (giving a response of ~ 250 RU). Plasmids in TF Buffer [50 mM sodium acetate (pH 5.0), 50 mM NaCl and 50 mM MgCl₂] were injected at a concentration of 4 nM. The SA chip was regenerated using 50 μ l 1 M NaCl in 50 mM NaOH.

Enzyme assays

DNA gyrase supercoiling assays, using gel electrophoresis, were carried out based on published procedures (17) as follows. Reactions (30 μ l) contained 1 μ g relaxed plasmid DNA, in 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM MgCl₂, 2 mM DTT, 1.8 mM spermidine, 1 mM ATP, 6.5% (w/v) glycerol, 0.1 mg/ml albumin (John Innes Enterprises) and were incubated at 37°C for 30 min. Samples were analysed either using microplate assays (below) or by electrophoresis on 1% agarose gels; results from gel assays were quantitated using the intensity of the ethidium fluorescence of the supercoiled DNA band using a Syngene GelDoc system. Where indicated, ciprofloxacin and novobiocin were also added to assays. Topo I, topo II and topo IV assays were carried out according to the manufacturer's instructions (Promega, Topogen and John Innes Enterprises Ltd) using 1 μ g supercoiled plasmid DNA as substrate.

Microplate DNA gyrase supercoiling assay based on one triplex formation

Black streptavidin-coated 96-well microplates (Pierce) were re-hydrated using 3 \times 200 μ l Pierce wash buffer. A total of 100 μ l 500 nM biotinylated oligo (TFO1) in Pierce wash buffer was immobilized onto the wells. Excess oligo was washed off using 3 \times 200 μ l Pierce wash buffer. Enzyme assays (30 μ l) were carried out as described above using 1 μ g relaxed pNO1 or pNO11 as the substrate. Reactions were incubated at 37°C for 30 min, and then TF buffer (100 μ l) was added to the wells and incubated at room temperature for 30 min to allow triplex formation. Any unbound plasmid was washed off with 3 \times 200 μ l TF buffer, then 200 μ l 1X SYBR Gold (Invitrogen) in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA was added and allowed to stain for 20 min. After mixing, fluorescence was read using a SPECTRAMax Gemini fluorimeter and Softmax Pro Software. In some experiments 10 μ l samples were removed from wells for gel assays (see above).

Microplate DNA gyrase supercoiling assay based on two triplex formations

Clear streptavidin-coated 96-well microplates were purchased from Pierce or BD Biosciences and re-hydrated using the manufacturer's recommended procedures. DNA gyrase supercoiling assays were performed as described above except, after removing any unbound plasmid with 3 \times 200 μ l TF

Table 1. Oligonucleotides used in this study

Name	Sequence (5'–3')	5' Modification
TFO1	TCTCTCTCTCTCTC	Biotin
TFO2	TTCTTCTTCTTCTTCT	Biotin or 32 P
TFO1W	TCGGAGAGAGAGAGAGAG	
TFO1C	CCGACTCTCTCTCTCTCT	
TFO2W	AAGAAGAAGAAGAACGT	
TFO2C	TCTTCTTCTTCTTCTTACGT	

buffer, 100 μ l 32 P-labelled TFO2 in TF Buffer was added to the wells and incubated at room temperature for 30 min. Any unbound radiolabelled oligo was washed off using $5 \times 200 \mu$ l TF buffer. The microplate was then placed on a Fuji imaging plate in a cassette and left overnight. Radioactivity was estimated using Fuji Bas 1000 and its software; samples for gel analysis were taken as above.

RESULTS

Construction of triplex plasmids

To enable specific capture of plasmids by triplex formation with immobilized oligos, plasmids containing triplex-forming sequences were constructed (see Materials and Methods). Plasmid pNO1 is a modified form of pBR322* containing a 20 bp insert with triplex-forming potential; pNO11 is a modified form of pNO1 containing a second 20 bp insert with triplex-forming potential. The first triplex-forming insertion, in both pNO1 and pNO11, should allow them to be captured by biotinylated oligo TFO1 (Table 1). The second triplex-forming insertion, in pNO11, should allow a second triplex formation with TFO2, which is end-labelled for quantitation. The pyrimidine-rich TFO1 and TFO2 oligos should form triplexes in the major groove of the inserted sequences in pNO1 and pNO11, binding parallel to the purine strand, forming C⁺.GC and T.AT triplets. In control experiments, we showed that both plasmids (pNO1 and pNO11) can be relaxed by topo I, and that the relaxed form can be supercoiled by DNA gyrase (data not shown).

SPR

We used SPR to demonstrate plasmid capture by TFOs and to optimize conditions for triplex formation. The TFO was first immobilized onto one of two flow cells using a streptavidin-coated chip and a biotinylated oligo. The plasmid was then injected over the flow cells. Any bound plasmid was subsequently washed off to regenerate the chip. In control experiments we found that plasmids with multiple triplex-forming inserts were captured less efficiently than those with single inserts (data not shown). When the solution conditions were varied, we found that a metal ion was required ($MgCl_2$), salt was required (either NaCl or KCl) and that low pH is optimal (pH \sim 5); the best buffer for triplex formation was found to be: 50 mM sodium acetate (pH 5.0), 50 mM NaCl and 50 mM $MgCl_2$ (= TF Buffer). Under these conditions we evaluated the capture of negatively supercoiled, relaxed and partially supercoiled pNO1 by TFO1 (Figure 1A and B). These experiments showed that supercoiled pNO1 was efficiently captured by comparison with its relaxed form; partially supercoiled pNO1 (specific linking difference \sim 0.03) gave a response approximately mid-way between the other two forms (see below). In control experiments, we found that supercoiled pBR322*, which has no triplex-forming inserts, was not captured (data not shown). We also showed that pNO1 could not be efficiently captured by TFO2 (data not shown).

The second plasmid, pNO11, has two triplex-forming inserts, which could potentially form triplexes with TFO1 and TFO2; we found in SPR experiments that pNO11 could

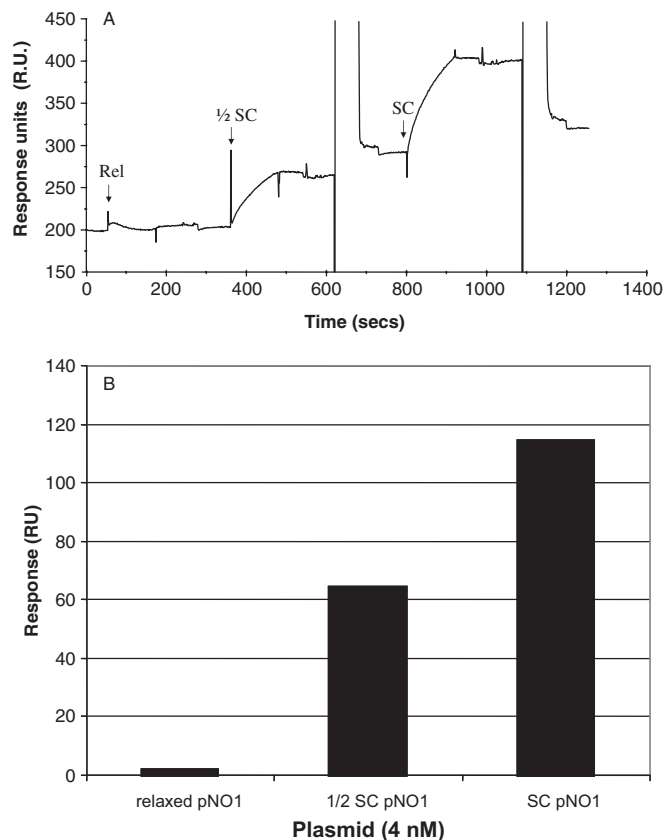


Figure 1. Capture of plasmids by TFOs using detection by SPR. Biotinylated oligos were immobilized on a streptavidin chip in a Biacore X instrument, and plasmids (4 nM) were flowed over the chip surface. (A) Sensorgram showing capture of different forms of pNO1 by immobilized TFO1: Rel = relaxed, 1/2 SC = partially supercoiled, SC = supercoiled. (B) Histogram of response for each of the forms of pNO1 in (A).

be captured by either oligo, although the efficiency of capture by TFO2 was somewhat less than by TFO1 (data not shown). In both cases the supercoiled form was captured preferentially to the relaxed form. From these data we were able to conclude that both plasmids, when in a negatively supercoiled form, could be captured by an oligonucleotide immobilized on a chip; the relaxed forms of the plasmids were not efficiently captured. These observations form the basis of the assays for topoisomerases, and other enzymes, based on DNA triplex formation.

Microplate DNA gyrase supercoiling assay based on single triplex formation

Having observed plasmid capture by triplex formation using SPR, we transferred these principles to a microplate format. Biotinylated TFO1 was bound to the streptavidin-coated surface of microtitre plates to which plasmids were applied in TF Buffer. Any unbound plasmids were subsequently washed off using the same buffer. The wells were then stained with the nucleic acid stain SYBR Gold and any fluorescence detected using a microplate fluorescence spectrometer. Figure 2A shows the results of such an experiment using pNO1 and pNO11. As before, the supercoiled form of the plasmids is

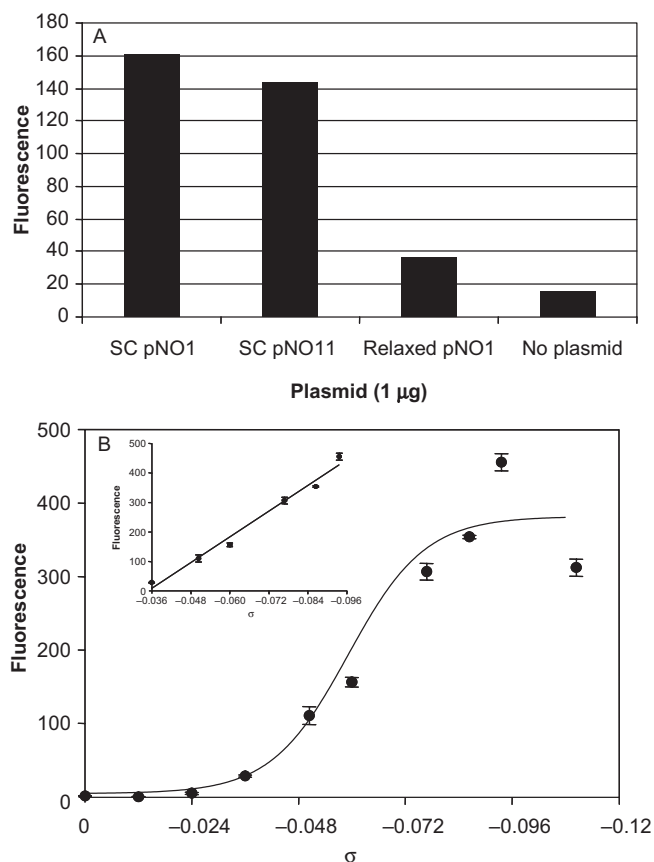


Figure 2. Capture of plasmids by TFOs using fluorescence detection. (A) Plasmids captured by TFO1 in the microplate assay as detected by SYBR staining. (B) Dependence of fluorescence signal from the microplate assay on specific linking difference (σ) of plasmid pNO1.

preferentially captured; control experiments showed that linear pNO1 gave little or no fluorescence signal in the microplate assay and did not interfere with the fluorescence signal from supercoiled pNO1 (Supplementary Data). Using pNO1 prepared at a range of specific linking differences ($\sigma = 0$ to -0.11), we found that the fluorescence response in the microplate assay was approximately linearly proportional to σ in the range -0.035 – -0.10 (Figure 2B). A similar relationship was found in samples containing mixtures of relaxed and supercoiled DNA (Supplementary Data). In addition, we found that the binding of SYBR Gold to DNA did not depend on σ (Supplementary Data), i.e. the fluorescence signal in the assay reflects the efficiency of triplex formation on the microplate.

To investigate the utility of this assay for DNA topoisomerases, a DNA gyrase supercoiling assay was carried out in a microplate format using a range of gyrase concentrations; samples were removed from the wells after incubation with gyrase for analysis by gel electrophoresis (Figure 3A). This experiment shows that the conversion of the relaxed DNA substrate to the supercoiled product is readily detected by the fluorescence assay and that the fluorescence results parallel those in the gel assay (Figure 3B and C). In control experiments we showed that the presence of the oligo TFO1 had no effect on the gyrase supercoiling assay monitored by

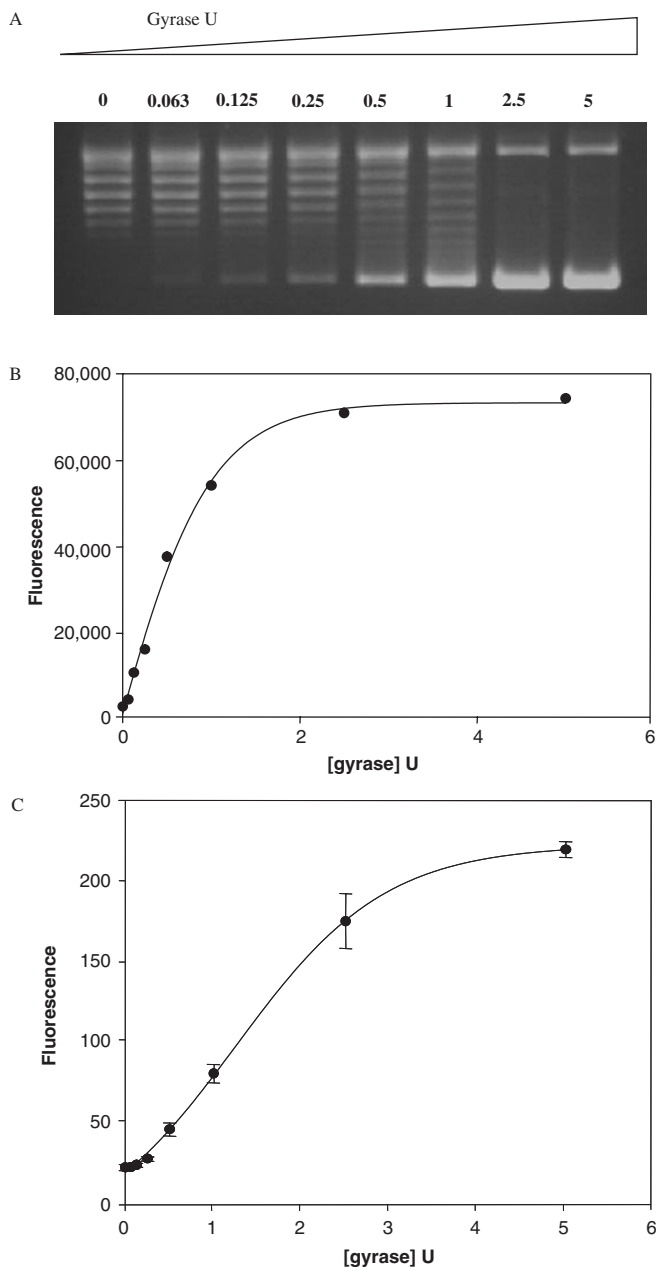


Figure 3. DNA gyrase supercoiling assay using fluorescence and gel-based assays. Gyrase assay with relaxed pNO1 as a substrate using the indicated amounts of enzyme in units. Samples were analysed by gel electrophoresis (A and B) and by SYBR fluorescence using the microplate assay (C).

gel electrophoresis (Supplementary Data). We also showed that the presence of TF Buffer prevents the gyrase reaction, i.e. addition of TF Buffer stops the enzyme reaction (Supplementary Data).

A key feature of a topoisomerase assay is that it can be utilized to screen for inhibitors. To illustrate this we carried out gyrase supercoiling assays in the presence of varying concentrations of the drugs ciprofloxacin and novobiocin (3); again samples for analysis by gel electrophoresis were taken in parallel (Figure 4; data for novobiocin not shown). The data show that the fluorescence assay mirrors the results in the gel assay and correctly reflects the degree

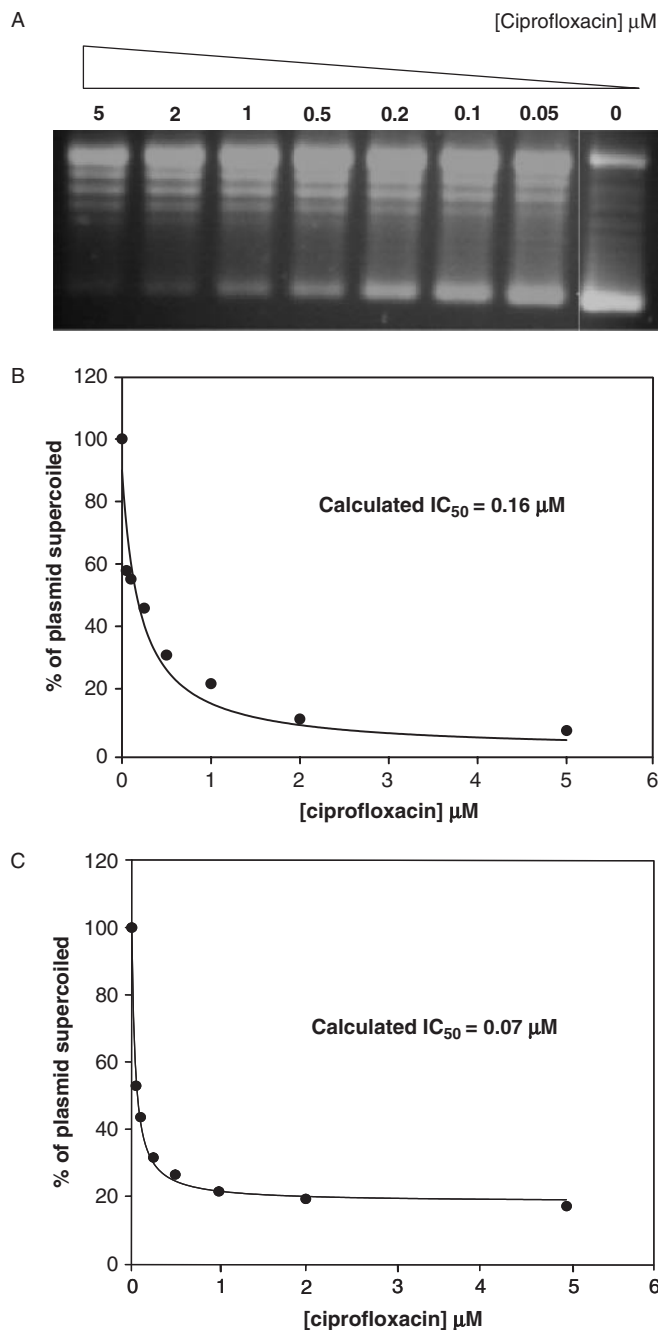


Figure 4. Effect of ciprofloxacin on DNA gyrase detected by fluorescence and gel-based assays. Gyrase assay (using 5 U of enzyme) with relaxed pNO1 as a substrate in the presence of the indicated amounts of ciprofloxacin. Samples were analysed by gel electrophoresis (A and B) and SYBR fluorescence (C).

of inhibition by the drugs. The IC_{50} values for the two drugs differ between the gel assay and the microplate assay; e.g.: ciprofloxacin, gel assay, $0.16 \mu\text{M}$; microplate assay, $0.07 \mu\text{M}$; novobiocin, gel assay $0.06 \mu\text{M}$; microplate assay, $0.003 \mu\text{M}$. This can be explained as follows. In the gel assay the apparent IC_{50} value is determined as the drug concentration when the intensity of the supercoiled band is $\sim 50\%$. However, as the gel is unable to resolve supercoiled

species with σ greater than ~ -0.03 , significant inhibition will have occurred before any change is apparent on the gel. Hence the gel assay for inhibition of gyrase supercoiling will tend to give a higher value than the true IC_{50} . In contrast, the microplate assay shows the full range of supercoiling (Figure 2B) and so a more accurate IC_{50} value is determined, i.e. the microplate IC_{50} values will be lower in this case.

We also carried out relaxation assays using wheat germ and human topo I, human topo II and *E. coli* topo IV; in all cases, we found that the microplate assay was applicable. Figure 5 shows data for relaxation by topo IV and inhibition of human topo I by camptothecin. In this case the substrate (supercoiled DNA) shows high fluorescence and the product (relaxed DNA), low fluorescence. Again the fluorescence assays (Figure 5B and D) mirror the results in the gel assays (Figure 5A and C); the difference in the IC_{50} values for camptothecin (Figure 5C and D) can be rationalized as above, i.e. it would be expected that the gel assay would give a lower value for the IC_{50} as the intensity of the supercoiled band would reach 50% before the reaction was inhibited by half. Note that with all the inhibitors used in this paper, we found no significant effect on triplex formation at concentrations $\geq \text{IC}_{50}$ s, as judged by observed variations in fluorescence, which were generally $< 10\%$ compared with controls (Supplementary Data).

It is likely that this assay can be adapted for any DNA topoisomerase; indeed any enzyme that changes the supercoiling of DNA can be assayed. For example, Figure 6 shows microplate fluorescence assays monitoring the cleavage of pNO1 by restriction enzymes. This plasmid contains sites for EcoRI and AatII but not AvaI; this is reflected by a loss of fluorescence with EcoRI and AatII, but not with AvaI.

As the most expensive element of this assay is the microplates, we performed control experiments to determine whether wells can be re-used. Following capture of pNO1 by bound biotinylated TFO1, wells were extensively washed with Pierce wash buffer and then re-used for triplex capture up to four times without loss of signal strength (data not shown).

Microplate DNA gyrase supercoiling assay based on two triplex formations

The microplate assay based on two triplex formations has the same principles as described above but requires the formation of a second triplex to give the signal that there is a captured plasmid. In this case, we immobilized biotinylated TFO1 onto streptavidin-coated microplate wells and monitored the capture of pNO11. Following capture, the presence of bound plasmid was detected using radiolabelled TFO2. In control experiments we showed that supercoiled pNO11 was captured more efficiently than its relaxed form (Figure 7A); partially supercoiled pNO11 gave an intermediate signal. We also showed that triplex formation between TFO2 and pNO11 is specific, as supercoiled pNO1 (which does not contain the second triplex sequence) did not give significant signal (data not shown). A DNA gyrase supercoiling assay, similar to that in Figure 3, was performed in the microplate using this method (Figure 7B and C). Again the radioactive

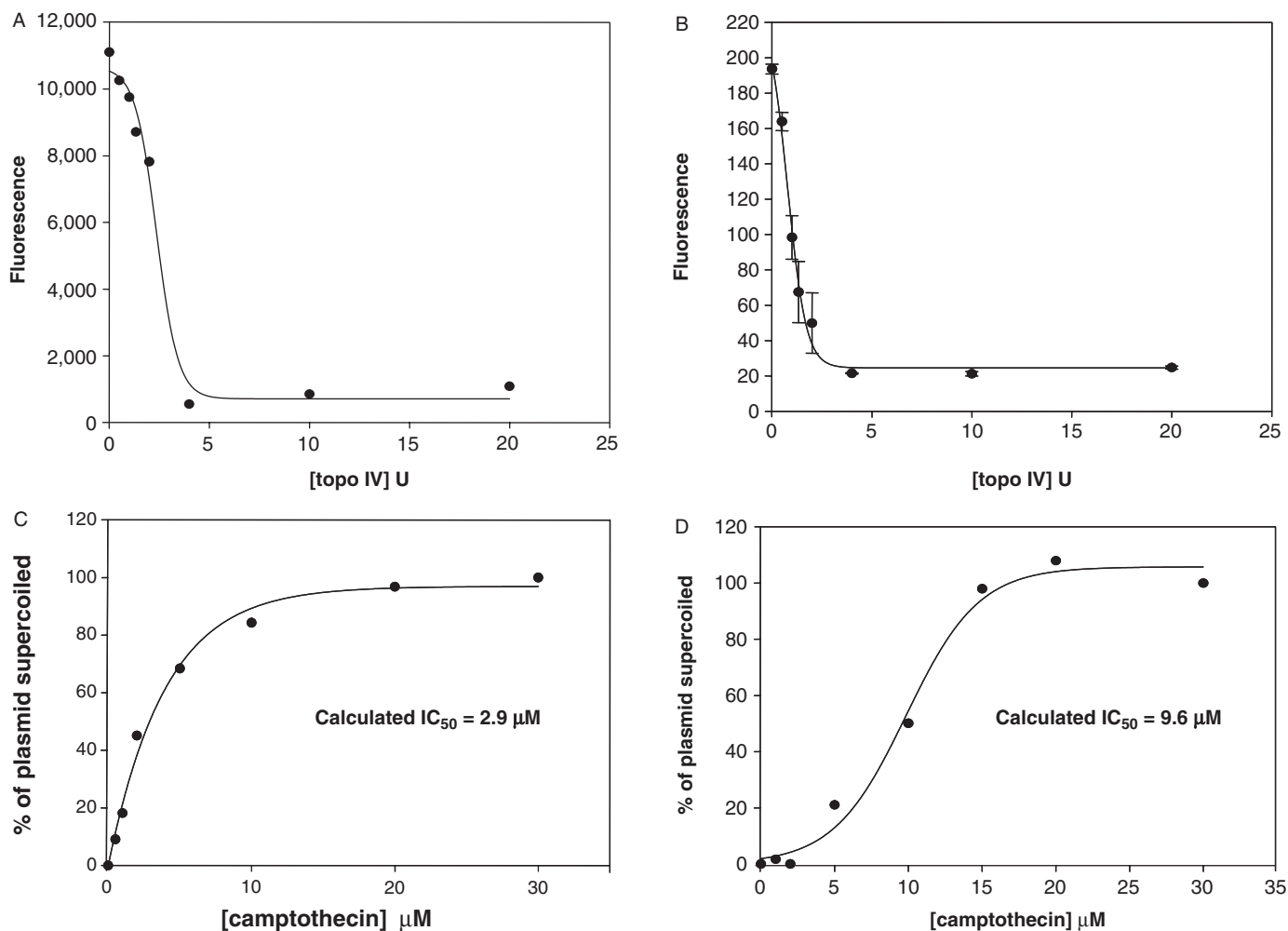


Figure 5. Topo I and topo IV assays using fluorescence and gel-based assays. Relaxation assay with supercoiled pNO1 as a substrate in the presence of the indicated amounts of topo IV (A and B) or topo I (C and D) in the presence of the range of camptothecin concentrations indicated. Samples were analysed by gel electrophoresis (A and C) and SYBR fluorescence (B and D).

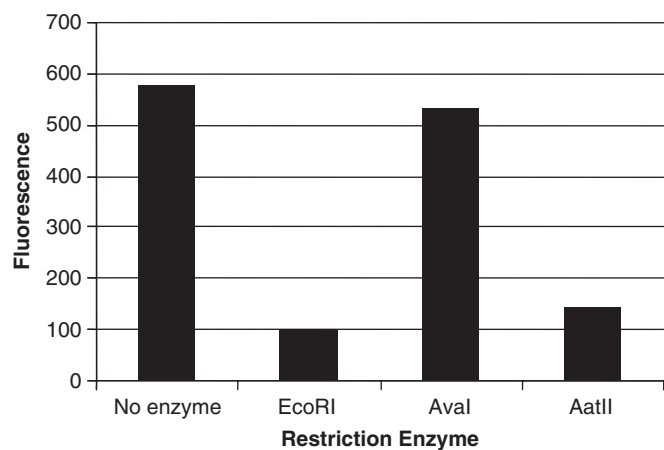


Figure 6. Restriction enzyme cleavage assayed using fluorescence. Supercoiled plasmid pNO1 was cleaved with the indicated restriction enzymes and samples were analysed by SYBR fluorescence in the microplate assay.

signal detected reflected the results seen in the gel-based assay.

DISCUSSION

DNA topoisomerases are excellent targets for a range of anti-microbial and anti-cancer agents; a major reason for this is that they form a cleavage complex with DNA, which constitutes a particular vulnerability for cells. However, gel-based assays for topoisomerase activity *in vitro* suffer from the drawback of being work-intensive and intrinsically low-throughput. We have described two versions of a microplate-based topoisomerase assay that exploit the differential ability of negatively supercoiled and relaxed plasmid DNAs to form intermolecular triplexes. These assays are relatively inexpensive and are straightforward to perform and will be of use to those working in the drug-discovery area and those interested in topoisomerase enzymology. We have found that for drug-screening purposes, the single triplex

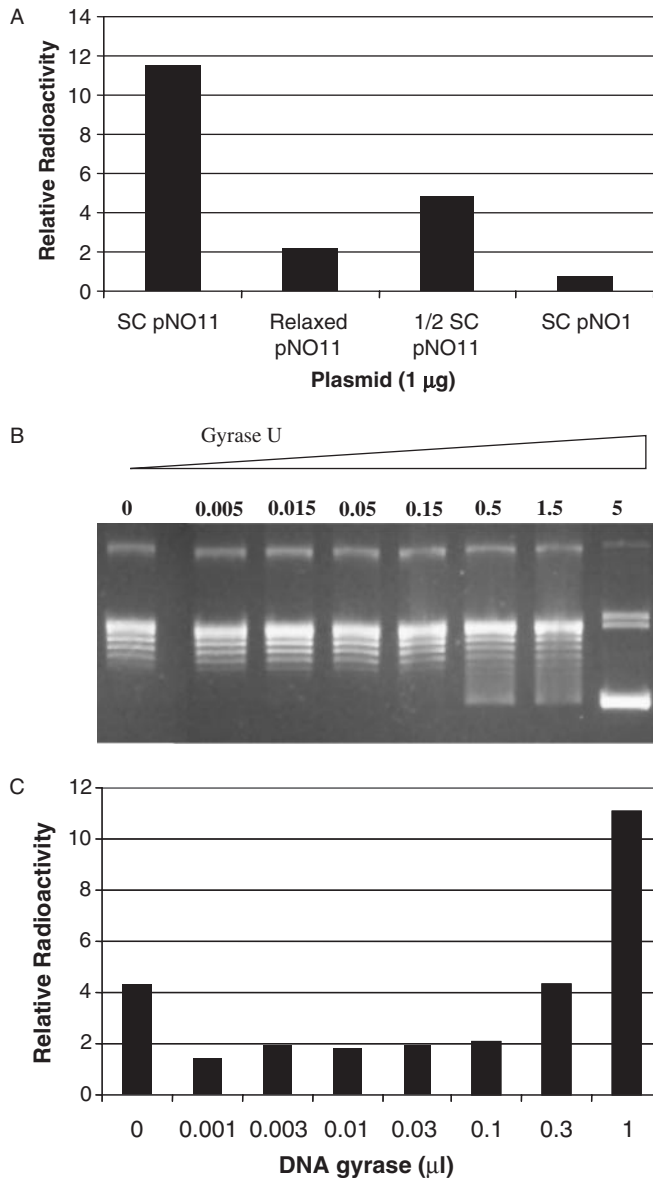


Figure 7. Plasmid capture and supercoiling assay using the two triplex method. (A) Plasmids captured by TFO1 in the microplate assay and detected by radiolabelled TFO2. (B and C) Gyrase assay with relaxed pNO11 as the substrate using the indicated amounts of enzyme. Samples were analysed by gel electrophoresis (B) and binding of radiolabelled TFO2 (C).

assay is the most convenient as it uses non-radioactive detection and the formation of a single triplex is more efficient than the formation of two triplexes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

ACKNOWLEDGEMENTS

This work was supported by BBSRC (UK) and Plant Biosciences Ltd. The authors thank Joe Stringfellow and Alison Howells for their contributions to the experiments, and Keith Fox for assistance with the design of triplex-forming oligos. The triplex-based microplate assays are protected by patent application GB0424953.8. Funding to pay the Open Access publication charges for this article was provided by BBSRC.

Conflict of interest statement. None declared.

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