Single-Molecule Detection of Human Topoisomerase I Cleavage—Ligation Activity

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ABSTRACT In the present study, we demonstrate the conversion of a single human topoisomerase I mediated DNA cleavage—ligation event happening within nanometer dimensions to a micrometer-sized DNA molecule, readily detectable using standard fluorescence microscopy. This conversion is achieved by topoisomerase I mediated closure of a nicked DNA dumbbell structure, followed by rolling circle amplification. The resulting product consists of multiple tandem repeats of the DNA dumbbell and can subsequently be visualized by annealing to fluorescently labeled probes. Since amplification involves no thermal cycling, each fluorescent rolling circle product, which gives rise to an individual signal upon microscopic analysis, will correspond to a single human topoisomerase I mediated cleavage—ligation event. Regarding sensitivity, speed, and ease of performance, the presented activity assay based on single-molecule product detection is superior to current state of the art assays using supercoiled plasmids or radiolabeled oligonucleotides as the substrate for topoisomerase I activity. Moreover, inherent in the experimental design is the easy adaptation to multiplexed and/or high-throughput systems. Human topoisomerase I is the cellular target of clinically important anticancer drugs, and the effect of such drugs corresponds directly to the intracellular topoisomerase I cleavage—ligation activity level. We therefore believe that the presented setup, measuring directly the number of cleavage—ligation events in a given sample, has great diagnostic potential, adding considerably to the possibilities of accurate prognosis before treatment with topoisomerase I directed chemotherapeutics.

KEYWORDS: single-molecule detection · human topoisomerase I · cancer diagnostics · rolling circle amplification · cleavage—ligation activity

The demand for fast, highly sensitive and high-throughput drug and disease screening systems without the need for complex instrumentation has increased dramatically during recent years and is likely to increase even further in the years to come. Especially, the realization of the complexity and diversity of many human diseases, including most cancer forms, emphasizes the need for a thorough molecular characterization of the individual disease to enable treatment specifically designed to improve prognosis for the individual patient.[1]

Motivated by these considerations, novel assay and readout formats for the detection of nucleotide sequences or proteins at the nanoscale or even at the single-molecule levels are currently being developed.[2−6] The activity of DNA modifying or interacting proteins, such as DNA topoisomerases, restriction enzymes, polymerases, and others, has been detected at the single-molecule level using magnetic tweezers, optical trapping, or other specialized setups to manipulate DNA.[6−10] Such methods have proven very powerful for the detection and analysis of protein functions within nanosecond timeframes on single DNA molecules and require only trace amounts of proteins. However, a concern of many established single-molecule methods is the relatively low-throughput and limited possibilities for multiplexing, that is, detecting numerous targets at the same time.[6] Such aspects are crucial for the future advancement of single-molecule assays toward routine applications in genomic analysis, disease, and drug screening. At the micrometer scale, high-throughput and multiplexed assays have successfully been developed using specialized microdevices. These miniaturized devices enable rapid standardized processing of large amounts of data, and although still in its infancy, the combination of such microdevices with single-molecule detection techniques poses great potential. Examples of such strategies are single-molecule assays using nanochannels, polyacrylamide gel-based microchambers, microfluidic channels, or chip technologies.[3,7,11−14]

One of the promising single-molecule detection techniques, which has successfully been applied to various high-throughput microdevices,[13−16] is the use of circularizing oligonucleotide probes (the so-called padlock-probes), in combination with enzymatic signal amplification by a rolling circle amplification (RCA)

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mechanism. Padlock-probes are synthetic single-stranded DNA molecules that can be circularized by enzymatic ligation upon annealing to a specific target DNA sequence[17] or as a result of a specific protein recognition reaction using a modified version of the proximity probe ligation technique.[18] A subsequent RCA results in the creation of one rolling circle product (RCP) for each circularized probe.[19,20] Each RCP consists of multiple (up to 10^3) tandem copies of the padlock-probe and can consequently be detected optically at the single-molecule level by hybridization to fluorescently labeled probes followed by microscopic[20,21] or microfluidic analysis[13] or as shown more recently by volume-amplified magnetic nanobeads,[22] allowing enumeration of closed probes at the single-molecule level. An important inherent property of the setup is the easy adaptability to multiplexing simply by using differently labeled probes for the detection of various RCPs. These possibilities are continuously increasing with the development of new optical nanoparticles.[2]

We have previously reported RCA of self-folding probes for production of long 5′-phosphorylated oligonucleotides[23] and for detection of non-polyadenylated RNA species.[24] In the current study, we present a self-folding oligonucleotide substrate system allowing a single-cleavage ligation event mediated by the clinically important human enzyme, topoisomerase I (hTopoI) to be detected in terms of a single-molecule RCP. This is, to our knowledge, the first example of a RCA-based method designed for detection of enzyme activity.

The cellular function of hTopoI is to regulate DNA topology during essential DNA metabolic processes, such as replication and transcription. This is achieved by the enzyme introducing transient single-strand breaks in double-stranded DNA using a mechanism that involves a cleavage intermediate stage characterized by the covalent attachment of the enzyme to the 3′-DNA end *via* a phosphotyrosyl linkage. The formation of a cleavage intermediate allows DNA topology to be altered by rotation of the generated 5′-hydroxyl end around the uncut strand before the ensuing DNA ligation step, which restores the integrity of the DNA backbone.[25] Besides its important biological functions, hTopoI has gained much clinical interest being the cellular target of clinically important anticancer drugs of the camptothecin (CPT) family, which are presently used in the systemic treatment of ovarian, colon, and small-cell lung cancer.[26] CPTs act by turning hTopoI activity into a cell poison by prolonging the half-life of the covalent cleavage complexes, which by colliding replication forks leads to genome fragmentation and cell death.[26] Consequently, the antitumor efficiency of CPTs is directly proportional with the hTopoI cleavage−ligation activity level in the treated cancer cells, and a common mechanism for the development of CPT-resistant cancer cells is down regulation of the intracellular hTopoI activity level.[26−29]

Clinical diagnosis often relies on very small tissue or cell samples, which is why qPCR presently offers the only realistic way of measuring the presence of hTopoI in individual cancers. However, qPCR detects hTopoI at the mRNA level and provides no information regarding the actual activity of the hTopoI, which can be down regulated by chemical modifications, mutations, and/or proteolytic degradation.[26,30,31] Therefore, besides the obvious advantages for basic science, a fast and technically simple setup for highly sensitive detection of hTopoI activity possesses great clinical potential, enabling the response of individual cancer patients toward CPT treatment to be predicted. The detection of hTopoI activity in terms of single-molecule RCP presented in this study fulfills the requirements for simplicity, speed, and sensitivity and presents the additional advantage of being suitable for multiplexed high-throughput systems provided that interfering signals from potential background enzyme activities can be avoided as will be discussed. Moreover, as opposed to already available single-molecule hTopoI activity detection techniques measuring strand rotation dynamics,[8,32,33] the presented setup measures selectively hTopoI mediated cleavage−ligation, hence, allowing a new step of catalysis to be investigated at the single-molecule level and being of particular relevance for clinical purposes.

RESULTS AND DISCUSSION

Design of Single-Molecule Detection Assay for hTopoI Activity. The experimental setup for single-molecule detection of the cleavage−ligation activity of hTopoI relies on a DNA substrate (referred to as "dumbbell-substrate" in the following) composed of a short single-stranded oligonucleotide, which spontaneously folds into a double-looped dumbbell-shaped structure, as shown in Figure 1A. The sequence of the double-stranded stem of the dumbbell-substrate is designed to match a preferred interaction site for hTopoI[34] and has the properties of a standard substrate for the detection of hTopoI cleavage−ligation activity.[35−37] Cleavage of the substrate by hTopoI occurs three bases upstream to the oligonucleotide 3′-end, resulting in the temporary covalent attachment of the enzyme and dissociation of the released three nucleotide (nt) fragment. This allows the 5′-overhang of the substrate, designed to match the noncleaved strand of the stem, to fill in the generated gap and bring the 5′-hydroxyl group in position for hTopoI mediated ligation (Figure 1B). As a result, hTopoI cleavage−ligation activity on the dumbbell-substrate will generate a closed single-stranded DNA circle (Figure 1C). To minimize nonspecific circularization events by DNA ligases, the 3′-end of the dumbbell-

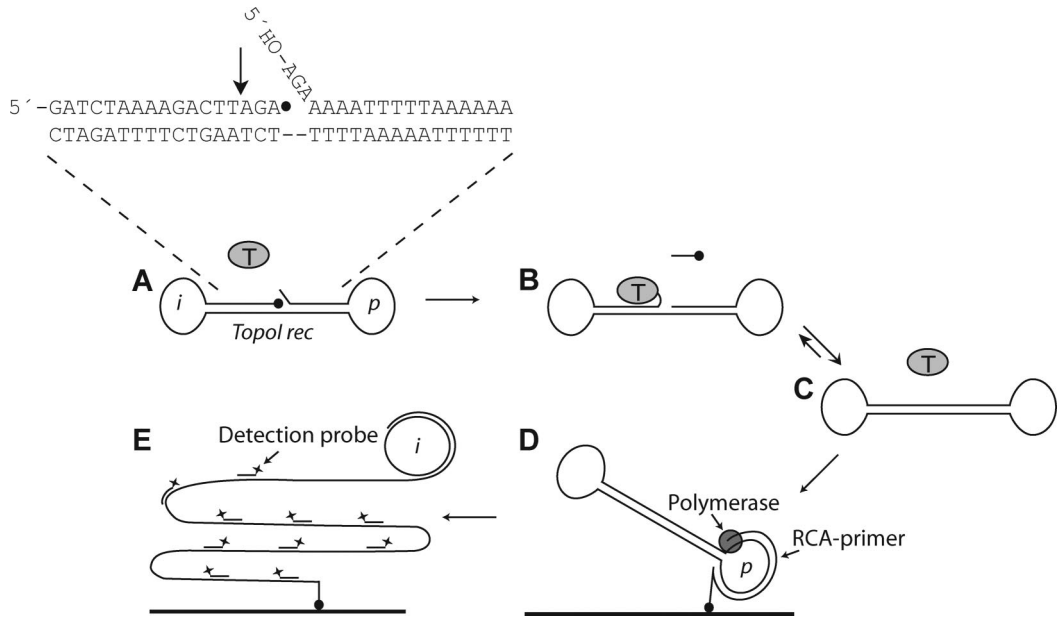


Figure 1. Schematic representation of the solid support RCA-based Topo I activity detection assay. (A) The hTopoI substrate folds into a double-looped dumbbell-shaped structure, with one loop containing an identifier element (marked *i*), the other loop containing the primer hybridization sequence (marked *p*), and the double-stranded stem region having a preferred hTopoI recognition sequence (marked *Topol rec*). The preferred hTopoI cleavage site is situated three bases upstream to the 3′-end of the dumbbell structure forming oligonucleotide (marked with an arrow in the blowup of the stem region), and the 5′-end of the oligonucleotide contains three bases overhang matching the noncleaved region of the stem. Note also that the oligonucleotide has a 5′-hydroxyl end, making it suitable for hTopoI mediated ligation. (B) Following hTopoI mediated cleavage at the preferred cleavage site, the generated three nt fragment diffuses away and the 5′-three nt overhang anneals to the noncleavage strand, positioning itself for hTopoI mediated ligation. (C) The hTopo I seals the nick in the substrate by ligating the 5′-hydroxyl end and dissociates from the substrate. This reaction transforms the open dumbbell-substrate into a closed circle. Since the reaction equilibrium of hTopoI is shifted toward ligation, the formation of the closed circular product is favored. (D) A 5′-amine linked primer attached to a glass surface is hybridized to the *p* region of the dumbbell-substrate, allowing polymerase-assisted RCA of the circularized dumbbell-substrates (on unreacted open dumbbell-substrates, the replication reaction is terminated at the strand interruption). (E) Visualization is performed by hybridization of fluorescently labeled oligonucleotide probes to the RCP region corresponding to the *i* element of the dumbbell-substrate. Light gray ellipse marked T, hTopoI; dark gray circle, Phi29 polymerase; asterisk, fluorophore; black circle, amine residue.

substrate is blocked with a 3′-amine group, which does not prevent the hTopoI catalysis (see Figure 3). The hTopoI mediated circularization of the dumbbell-substrates is detected using a solid support assay comprising rolling circle DNA synthesis and fluorescent labeling, essentially as described previously by Lizardi *et al.*[21] The RCA is initiated from a primer (RCA-primer) attached to a glass surface to ensure anchoring of the generated RCPs (Figure 1D), which are subsequently detected at the single-molecule level by annealing to specific fluorescent labeled probes (detection probes) and microscopic analysis (Figure 1E). It was previously shown that each microscopically detected fluorescent spot represents one RCP, which in turn represents one closed circle product.[21] Hence, since each cleavage−ligation reaction by hTopoI generates one closed circle product, the presented setup allows hTopoI activity to be detected at the level of single cleavage−ligation events.

Detection of hTopoI Activity on Dumbbell-Substrates. The feasibility of the described single-molecule detection system as an hTopoI activity assay was initially tested using recombinant hTopoI expressed in and purified to homogeneity from yeast *Saccharomyces cerevisiae* (see Figure 2).

To verify that the dumbbell-substrate supported hTopoI mediated cleavage−ligation, a gel-based assay was employed. The hTopoI was incubated with the substrate in hTopoI reaction buffer, and the resulting prod-

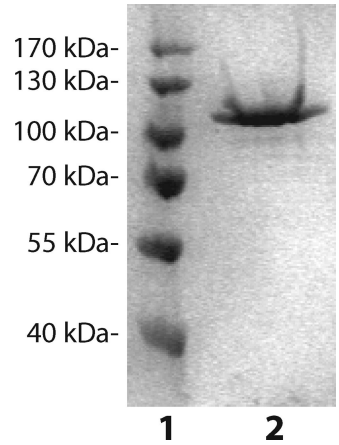


Figure 2. Purified hTopoI fraction. Coomassie stain of proteins separated in a 1% SDS polyacrylamide gel. Lane 1, size marker; lane 2, 4 μg of purified hTopoI.

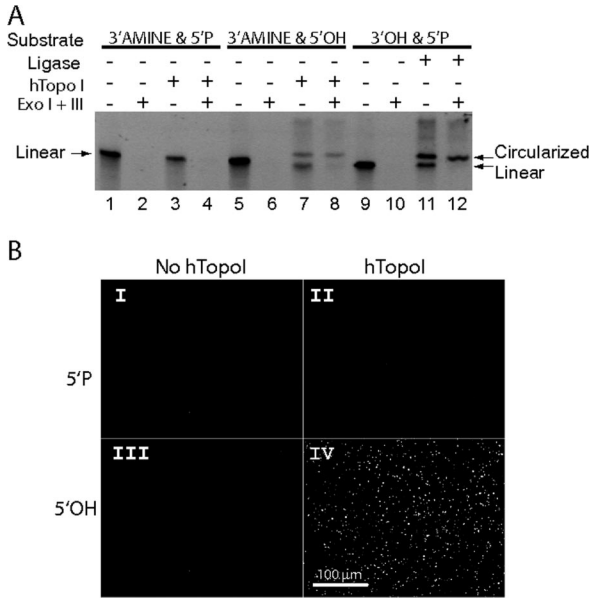


Figure 3. hTopoI reaction on the dumbbell-substrate. (A) Polyacrylamide gel-based analysis of the hTopoI or T4 ligase reaction with the dumbbell or the control ligase substrate, respectively. Lanes 1−4, a 3′-amine and 5′-phosphate containing dumbbell-substrate was incubated in the absence or presence of hTopoI as indicated in top of the figure; lanes 5−8, a 3′-amine and 5′-hydroxyl containing dumbbell-substrate was used as substrate for hTopoI. Lanes 9−12, the 3′-hydroxyl and 5′-phosphate containing control ligase substrate was incubated in the absence or presence of T4 ligase as indicated. The sequence of the oligonucleotide forming the control ligase substrate was similar to the sequence of dumbbell-substrate forming oligonucleotide, except that it lacked the three nt 5′-overhang and had a different identifier element. Exonucleases I and III were added to the samples as indicated at the top of the figure. The mobilities of linear or circularized substrates are indicated at the left or right of the figure. Ligase, T4 DNA ligase; Exo I+III, exonucleases I and III. The reaction products were visualized by SYBR Gold staining. For clarity, colors of the gel picture were inverted during image processing. (B) The hTopoI activity was tested using the solid support RCA-based detection assay. Panels I and II, a 5′-phosphorylated dumbbell-substrate was incubated without and with 1000 fmol of hTopoI, respectively, prior to RCA and detection of RCP by fluorescent labeling. Panels III and IV, as panels I and II except that a 5′-hydroxyl containing dumbbell-substrate was used. Each image shows a representative section of the sample slide. Scale bar, 100 µm.

ucts were separated in a 12% denaturing polyacrylamide gel and visualized by subsequent staining with SYBR Gold. As evident from Figure 3A, hTopoI converted the dumbbell-substrate to a product resistant to exonuclease digestion, indicating the lack of free DNA ends (lane 8). Moreover, the product had a gel electrophoretic mobility corresponding to the mobility of the circularized control ligase substrate having a sequence similar to the dumbbell-substrate but without the 5′-three nt overhang and containing a 3′-hydroxyl and a 5′-phosphate group to allow circularization by standard ligation using T4 DNA ligase (compare lanes 7 and 8 with lanes 11 and 12). Phosphorylation of the 5′-end of the dumbbell-substrate made it resistant toward ligation during incubation with the purified hTopoI fraction, resulting in degradation by exonuclease treatment (compare lanes 3 and 4). This result is consistent with the specific utilization of 5′-hydroxyl ends in hTopoI mediated DNA ligation and confirms that the observed circularization of the dumbbell-substrate can be ascribed to hTopoI activity.

To address whether the hTopoI-dependent products observed in Figure 3A represent closed circles suitable for single-molecule detection, the solid support assay comprising RCA and fluorescent labeling as illustrated in Figure 1 was carried out. Products corresponding to the ones shown in Figure 3A, lanes 1, 3, 5, and 7, were hybridized to a surface coated with the 5′-amine-coupled RCA primer and DNA synthesis initiated by addition of Phi29 polymerase at appropriate buffer conditions. Subsequently, the RCPs were hybridized to fluorescent detection probes and visualized by fluorescent microscopy. As evident in Figure 3B, fluorescent signals representing RCPs appeared only in samples preincubated with hTopoI (compare panels I and III with panels II and IV). Also, when comparing the results shown in Figure 3A and B, there seems to be a correlation between the appearance of fluorescent signals and the production of circularized dumbbell-substrate (compare panels III and IV of Figure 3B with lanes 5 and 7 of Figure 3A). In the single-molecule detection setup, however, trace amounts of signals could be observed after incubation of hTopoI with the 5′-phosphorylated substrate (Figure 3B, panel II), whereas no activity on this substrate was observed in the gel-based assay (Figure 3A, lanes 3 and 4). This result is likely caused by incomplete 5′-phosphorylation (a well-known phenomenon of the T4 kinase reaction) of the dumbbell-substrate giving rise to circularized products at amounts too low to be detected by SYBR Gold staining of bulk substrate sample but sufficient to be detected at the single-molecule level following RCA.

As opposed to PCR-based methods, RCA involves no thermal cycling, which is why each circularized dumbbell-substrate (produced by a single hTopoI cleavage−ligation event) is being represented by a single RCP in the presented setup. Each RCP, in turn, contains a sufficient number of tandem repeats of the target sequence to allow its optical detection upon hybridization to fluorescent probes at the single-molecule level.[21] Hence, the presented RCA-based assay allows the detection of single cleavage−ligation events by hTopoI. It is therefore not surprising that the RCA-based assay shows a much higher sensitivity that the bulk gel-based assay (Figure 3a) and reveals even a slightly incomplete 5′-phosphorylation of the dumbbell-substrate, as demonstrated in Figure 3B. Amplification of products resulting from hTopoI mediated cleavage−ligation could also be envisioned using PCR-based methods which presumably would result in a highly sensitive detection of hTopoI activity. However, since PCR relies on thermal cycling, signals obtained using such technology would not reflect single

cleavage−ligation events *per se* but rather represent the bulk amount of products in a given reaction sample.

Comparison of the RCA-Based Single-Molecule Detection Assay with Current State of the Art Assays for hTopoI Activity Detection. The sensitivity of the RCA-based single-molecule detection of hTopoI activity was compared to that of standard bulk cleavage−ligation[36] and DNA relaxation hTopoI activity assays.[37,38] As a substrate for the bulk cleavage−ligation assay, we used a nicked double-stranded DNA fragment (referred to as dsDNA substrate) composed of three short oligonucleotides (cleavage strand (cl-str), ligator strand (lig-str), and noncleaved strand (noncl-str)) designed in such a way that the substrate upon annealing of the oligonucleotides was identical in sequence, length, and structure to the stem of the dumbbell-substrate (compare right panel of Figure 4C with Figure 1). The hTopoI will cleave and ligate this substrate essentially as described for the dumbbell-substrate (see also Figure 4C, right panel). Visualization of reaction products was ensured by 5′-radiolabeling of the cl-str. In the experimental setup, hTopoI was incubated with the radiolabeled substrate and the resulting products were analyzed in a denaturing polyacrylamide gel subsequent to tryptic removal of enzyme from the sample. Products were visualized by standard PhosphoImaging. DNA relaxation was assayed using purified supercoiled (SC) plasmid (pBR322) as a substrate. Following incubation with hTopoI, the conversion of supercoiled plasmids to relaxed forms was detected by separating reaction products in a 1% agarose gel prior to visualization by EtBr staining.

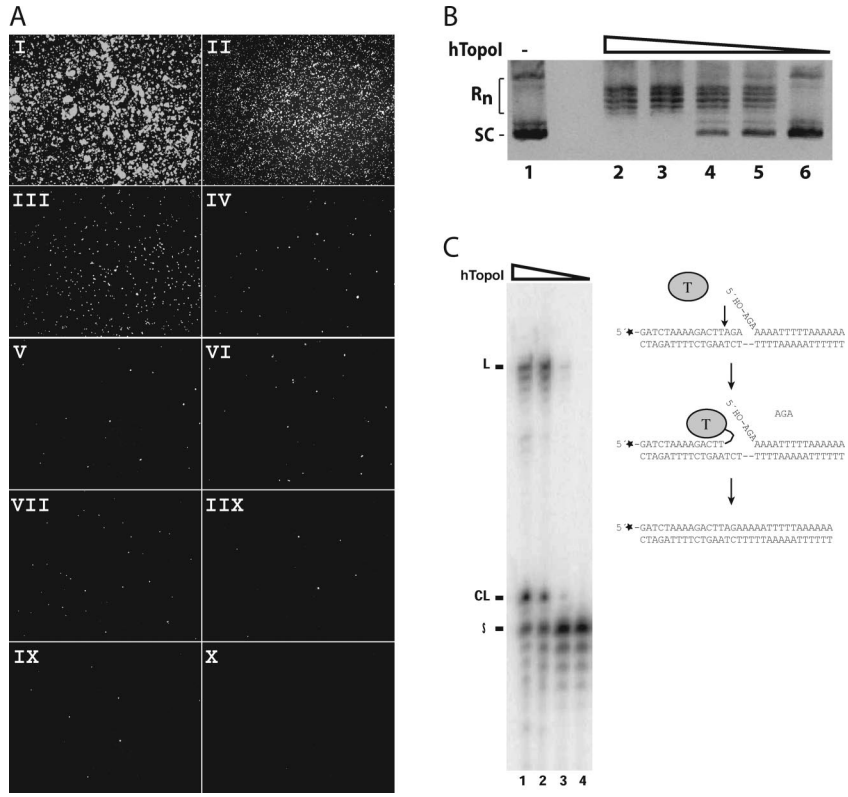
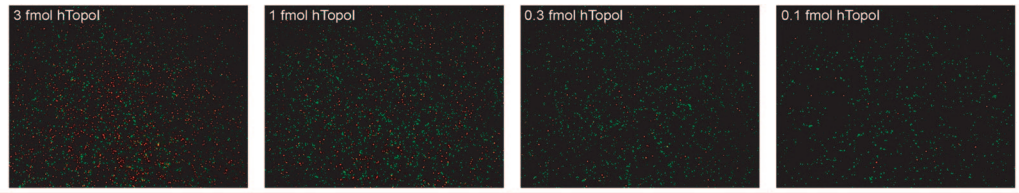


Figure 4. Comparison of the solid support RCA-based hTopoI activity detection assay with standard DNA relaxation and DNA cleavage−ligation assays. (A) Solid support RCA-based hTopoI activity assay. Panels I−IX, the dumbbell-substrate was incubated with 1000, 100, 10, 1, 0.5, 0.25, 0.17, 0.13, or 0.1 fmol of hTopo1 in a standard hTopoI reaction buffer prior to RCA and detection of RCP by fluorescence microscopy. Panel X, control experiment performed without the addition hTopoI to the dumbbell-substrate. Each image shows a representative section of the sample slide. (B) Detection of hTopoI activity using a DNA relaxation assay followed by gel electrophoresis and visualization of reaction products by EtBr staining. Lane 1, DNA plasmid incubated without hTopoI; lanes 2−6, DNA plasmid incubated with 1000, 100, 10, 1, or 0.1 fmol of hTopoI in standard relaxation buffer. SC, supercoiled plasmid; R_n, relaxed species of the plasmid. For clarity, colors of the gel picture were inverted during image processing. (C) Detection of hTopoI using the radiolabeled dsDNA substrate followed by gel electrophoretic analysis and visualization of reaction products by PhosphoImaging. The utilized substrate was similar in length and sequence to the stem region of the dumbbell-substrate, and the reaction scheme is schematically illustrated to the right of the figure. Lanes 1−4, the radiolabeled substrate was incubated with 1000, 100, 10, or 1 fmol of hTopoI at reaction conditions similar to the ones described in (A). S, substrate; CL, cleavage product; L, ligation product; asterisk, ^{32}P-labeling; T enclosed in light gray ellipse, hTopoI; arrow, indicate preferred hTopo cleavage site.

For the comparative experiments, each of the three substrates (the dumbbell-substrate, the radiolabeled dsDNA substrate, or the SC plasmid) was incubated with identical dilution series of the same purified hTopoI fraction in hTopoI reaction buffer before analyzing the products in the three different setups. As evident from Figure 4A, the RCA-based assay allows easy detection of down to 0.1 fmol of hTopoI in the current setup (Figure 4A, panel IX). Individual RCPs could be identified as separate fluorescent spots except at high concentrations of added hTopoI, where signals from several RCPs were merging, presumably due to high concentrations of circularized products (Figure 4A, panels I and II). DNA relaxation was approximately 10 times less sensitive than the RCA-based assay, with 1 fmol of hTopoI being the detection limit as measured by the conversion of the fast migrating SC plasmid to the relaxed (marked R_n) forms (Figure 4B, lane 5). SYBR Gold staining is in principle more sensitive than EtBr staining. However, staining the relaxation products with SYBR Gold did not improve the sensitivity of the relaxation assay (data not shown). The hTopoI activity measured by the conversion of the dsDNA substrate into cleavage or ligation products could be detected with no less than 10 fmol of purified enzyme (Figure 4C, compare lanes 3 and 4), corresponding to an approximate 100-fold decreased sensitivity compared to the RCA-based assay. Note, that in this latter assay the cleavage product (marked by CL) shows slightly slower gel electrophoretic mobility than the substrate (marked S), al-

A



	3 fmol hTopol			1 fmol hTopol			0.3 fmol hTopol			0.1 fmol hTopol		
	R	G	R/G	R	G	R/G	R	G	R/G	R	G	R/G
Picture 1	2552	2456	1,039	751	1764	0,426	276	2102	0,131	60	1676	0,036
Picture 2	2600	2360	1,102	776	1732	0,448	273	1834	0,149	52	1539	0,034
Picture 3	2412	2302	1,048	799	1747	0,457	258	1763	0,146	51	1445	0,035
Picture 4	2221	2087	1,064	768	1701	0,451	256	1793	0,143	49	1287	0,038
Picture 5	2249	1909	1,178	855	2261	0,378	263	1825	0,144	55	1381	0,040
Picture 6	2371	1910	1,241	855	2150	0,398	259	1906	0,136	38	1230	0,031
Picture 7	2141	1536	1,394	772	1724	0,448	237	1729	0,137	29	1056	0,027
Picture 8	2045	1532	1,335	788	1651	0,477	223	1704	0,131	29	1047	0,028
Picture 9	1967	1591	1,236	552	1276	0,432	243	1924	0,126	34	1062	0,032
Picture 10	2145	2048	1,047	549	1202	0,457	272	1928	0,141	28	934	0,030
	Average R/G ratio		1,168	Average R/G ratio		0,437	Average R/G ratio		0,138	Average R/G ratio		0,033
	Standard deviation		0,129	Standard deviation		0,030	Standard deviation		0.007	Standard deviation		0,004

B

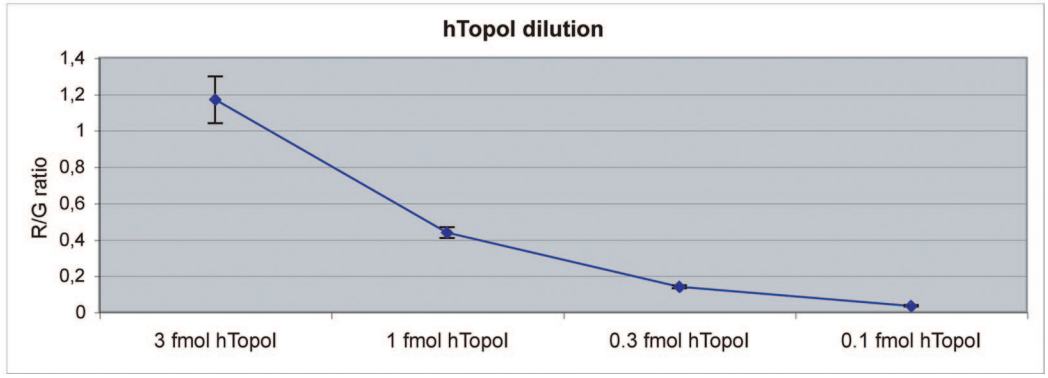


Figure 5. Quantification of RCPs obtained from hTopoI circularized dumbbell-substrates relative to RCPs obtained from a control circle. The dumbbell-substrate was incubated with 3, 1, 0.3, or 0.1 fmol of hTopoI in a standard reaction buffer. Circularized substrates were amplified by RCA, and the resulting RCPs were visualized by hybridization to the red fluorescent labeled probe. Prior to loading the reaction samples on microscopic slides, 5 fmol of the control circle was added to the reaction mixtures. The control circles were subjected to RCA in parallel with the hTopoI products, and the RCPs were visualized with a green fluorescent labeled probe. (A) Raw data obtained from the experiment. One example randomly picked out of 10 individual microscopic images of each reaction sample is shown in the top panel. In the lower panel, the number of red (R) and green (G) spots present on each of the individual images is stated together with the ratio between the numbers of red and green spots (R/G) present in each image. (B) Graphical representation of the data presented in (A).

though three nt are removed from the radiolabeled cl-str during cleavage. This apparent discrepancy is caused by the covalent attachment of a short trypsin-resistant hTopoI-derived peptide with a mobility corresponding to approximately 5 nt in the utilized gel as described previously.[39] The ligation product, which is not attached by hTopoI, runs with a mobility corresponding to the expected 32 nt resulting from the joining of the cleaved cl-str (17 nt to 3 nt) and the lig-str (18 nt).

As evident from Figure 4, even in the current preliminary setup, the RCA-based hTopoI activity assay shows a considerably increased sensitivity compared to bulk cleavage−ligation and relaxation assays. Moreover, the RCA-based assay possesses the advantage of being completed within a 2−3 h timeframe without the use of complex instrumentation. In comparison, the relaxation assay could be completed in no less than 5 h, while the bulk cleavage−ligation assay required at least 24 h for completion, and both these methods required specialized gel electrophoretic and visualization equipments.

Quantification of Signals Obtained by RCA-Based Detection of hTopoI Activity. The microscopic images shown in Figure 4A cover a randomly selected area of only approximately 0.1 mm^2 out of a reaction surface of 3 × 3 mm^2

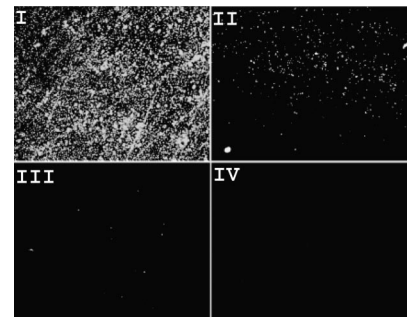


Figure 6. Solid support RCA-based hTopoI activity detection in a divalent cation depleted environment. Solid support RCA-based hTopoI activity assay essentially as the one shown in Figure 4, except that the standard hTopoI reaction buffer was replaced with a buffer depleted of divalent cations. Panels I−III, the dumbbell-substrate was incubated with 1000, 100, or 10 fmol of hTopo1. Panel IV, control experiment without the addition of hTopoI. Each image shows a representative section of the sample slide.

total. Since the concentration of closed circles captured on the surface may vary to a certain extent from one area to another of the microscopic slide, individual images may not give a very precise quantitative estimate of the hTopoI activity present in a given sample.

In order to develop more quantitative assay conditions, an experiment similar to the one described in Figure 4A was performed except that a fixed amount (5 fmol) of a gel purified closed control circle was added to each reaction mixture before loading on the microscopic slide. This circle was of the same size as the hTopoI product and had the same base sequence—except for the identifier element (marked *i* in Figure 1). Following RCA, the control circle was visualized by hybridization to a green fluorescent probe (specific for the identifier element of the control circle), while the circularized hTopoI dumbbell-substrate was hybridized to a red fluorescent probe (specific for the identifier element of this substrate) as described above.

Since the RC primer hybridization sequence (marked *p* in Figure 1) was identical in the control circle and the dumbbell-substrate, it seems reasonable to assume that the relative frequency by which each of two DNA species are captured on the individual areas of the microscopic slide was also identical. Hence, the ratio between the number of red spots (representing hTopoI reacted dumbbell-substrates) and the number of green spots (representing the control circles) within a randomly picked microscopic image should provide a fairly precise quantitative measure of hTopoI activity in a given sample.

To test this methodology, the number of red or green spots observed in 10 randomly selected microscopic images resulting from assaying the activity of 3, 1, 0.3, or 0.1 fmol of purified hTopoI were counted, and the ratio between the number of red and green spots was calculated. A representative example of the microscopic images obtained for each sample is shown at the top panel of Figure 5A. No cross reaction between the dumbbell-substrate and the green fluorescent probe or between the control circle and the red fluorescent probe was observed (data not shown).

As demonstrated both from the raw data (Figure 5A) and the graphical representation of the results (Figure 5B), the ratios between the number of red and green spots were very reproducible for a given concentration of hTopoI, while the absolute number of red spots (and green spots) varied up to a factor of 2 between the individual images of a given sample. Hence, the addition of the control circle appears to make a precise quantification of the hTopoI activity possible even from single microscopic images, while counting of red spots representing closed dumbbell-substrates alone within a single image gives rise to an error of up to a factor of 2.

The absence of yellow spots (representing a merge of the green and red fluorescent colors) of the microscopic images obtained in Figure 5A (except for a single yellow spot in the image representing the reaction performed with 0.3 fmol hTopoI) lends further credence to previously published results showing that each spot represents a singe RCP.[21]

RCA-Based Single-Molecule Detection of hTopoI in Cell Extract. The relatively high sensitivity and fast completion of the RCA-based hTopoI activity assay together with the numerous possibilities of multiplexing inherent in the technique make it appealing for the future detection of hTopoI activity for diagnostic purposes. For such an application, it is imperative that the RCA-based assay performs well in unpurified extracts of biological material since diagnosis typically relies on small cell or tissue samples, which do not allow purification of protein components. Crude extracts of biological material challenge the assay by containing a diversity of DNA modifying enzymes in addition to hTopoI. Such enzyme activities, including nucleases, ligases, and repair enzymes, are potentially capable of circularizing the dumbbell-substrate, causing false positives in the RCA-based assay.

The possibility of such false positives obviously represents a challenge especially to the development of multiplexed detection systems since it may be necessary to specifically adjust assay conditions or substrates to make them selective for the enzyme(s) of interest. Such adjustments are likely to differ for each enzyme of interest and may, hence, at least for some enzyme activities, require substantial optimizations of assay conditions. However, when the aim is to detect only hTopoI, as in the present study, it seems likely that false positive signals caused by other enzyme activities present in a crude biological extract may be avoided simply by adding surplus of the divalent cation chelating agent, EDTA, to the reaction mixtures since most DNA modifying enzymes require divalent cations for activity[40,41] while hTopoI performs well in the absence of any cofactors.[42]

First, the performance of the RCA-based hTopoI activity assay was tested in a divalent cation-free environment using purified enzyme. No attempts were made to make a precise quantification of hTopoI activity in this experiment, as it merely aimed at testing if topoisomerase I activity could be detected in the absence of divalent cations with our assay. The experiment was performed essentially as described above (see under Figure 4) except that the hTopoI reaction buffer was replaced with a divalent cation, depleting buffer containing 10 mM EDTA. As evident from Figure 6, hTopoI activity could readily be detected in the RCA-based assay under these conditions although the sensitivity of detection was decreased compared to the experiment performed using optimal buffer conditions for hTopoI (compare Figure 6, panel III, with Figure 4A, panel III). The decreased hTopoI activity detection in the divalent cation-free environment was not specific to the RCA-based assay since the detection limits of hTopoI activ-

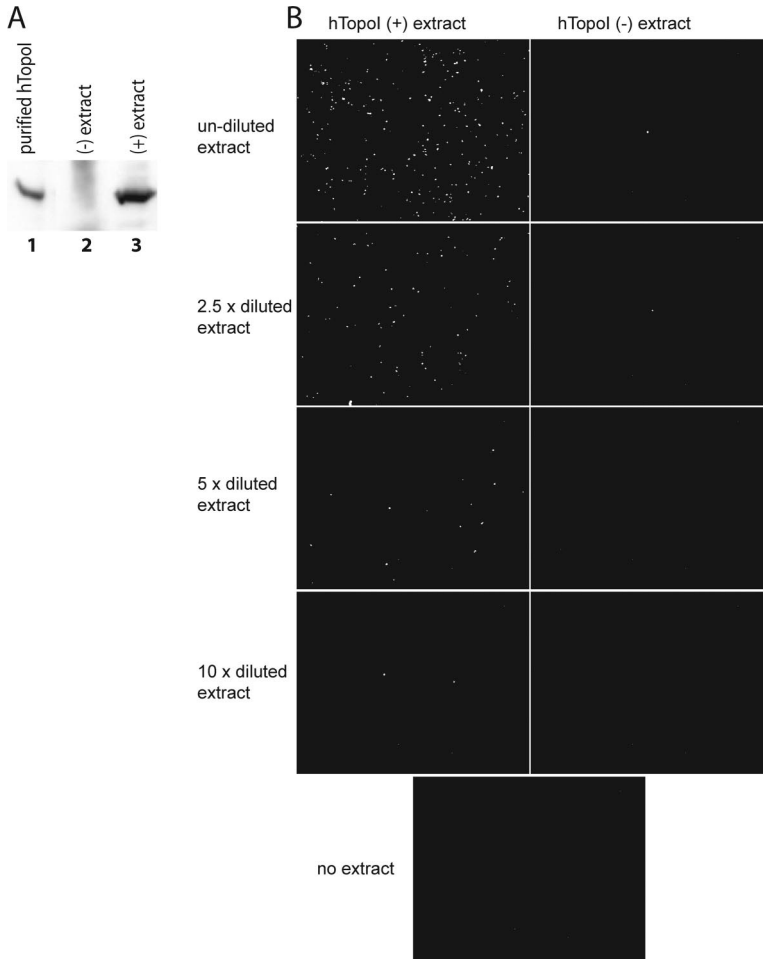


Figure 7. Detection of recombinant hTopoI in cell extract using the RCA-based activity assay. Recombinant hTopoI was expressed in yeast and extract from the resulting cells (hTopoI positive extract) tested for hTopoI activity using the solid support RCA-based detection assay. As a negative control, extract was made from cells in which hTopoI expression was not induced (hTopoI-negative extract). (A) Result of a Western blot analysis of hTopoI-positive ((+)extract) or hTopoI negative ((−)extract), lanes 2 and 3, respectively. Lane 1, a positive control containing purified hTopoI. (B) RCA-based detection of hTopoI activity in cell extracts. Undiluted or diluted extracts (as indicated to the left of the figure) from the hTopoI positive or negative cells were incubated with the dumbbell-substrate prior to RCA and detection of RCPs using fluorescent microscopy. Left row shows the result of incubation with hTopoI-positive extracts. Right row shows the result of incubation with hTopoI-negative extracts. The lower panel shows the result of replacing the cell extract with the extraction buffer used for the cell extract preparation. Each image shows a representative section of the sample slide.

ity were decreased approximately 10-fold in all utilized activity assays when replacing the hTopoI reaction buffer with an EDTA containing buffer in a comparative experiment like the one shown in Figure 4 (data not shown). Hence, we concluded that also, in an EDTA containing reaction buffer, the detection limit of the RCA-based technology was lower than that of the bulk hTopoI activity assays.

The next step was to test the performance of the RCA-based hTopoI activity assay in crude cell extracts. As in the experiment presented in Figure 6, no attempts were made to quantify the hTopoI activity, as the aim of the experiment merely was to test if specific detection of hTopoI in cell extracts is possible with our assay. For this purpose, we used extracts from yeast *S. cerevisiae* as the test material. Yeast poses the advantage of being able to survive without the endogenous gene encoding TopoI and, hence, this gene can be deleted resulting in a topI-null strain. In the presented experiment, a yeast topI-null strain was transformed with an episomal vector containing a recombinant hTopoI gene under the control of a GAL promoter. In this setup, hTopoI expression was induced by the addition of galactose to the growth medium giving rise to hTopoI-positive cells. The addition of glucose to the growth media largely prevents expression from the GAL promoter, and cells grown under these conditions were therefore expected to be hTopoI-negative. The absence or presence of hTopoI in crude extracts from cells grown in glucose- or galactose-containing media was confirmed by standard Western blotting analysis (Figure 7A). Extract from hTopoI-positive or -negative cells was tested in the RCA-based hTopoI activity assay in a divalent cation-free environment essentially as described for the experiments shown in Figure 6. As demonstrated in Figure 7B, left panel, decreasing numbers of fluorescent signals were observed upon RCA on dumbbell-substrates incubated with decreasing amounts of the hTopoI-positive cell extracts. In contrast, no or only trace amounts of fluorescent signals could be observed when the hTopoI-negative extract was subjected to analysis. The few signals observed when assaying undiluted hTopoI-negative cell extracts most probably reflect a very weak hTopoI expression in the uninduced cells, which is a well-known phenomenon of genes under control of the utilized GAL promoter.[38] The pronounced difference in the number of fluorescent signals observed upon incubation of the dumbbell-substrate with hTopoI-positive or -negative cell extracts, respectively, demonstrates that at the condition used the RCA-based assay allows the effective and specific detection of hTopoI activity even in a very crude biological sample.

Taken together, the presented results demonstrate a highly sensitive and specific detection of hTopoI activity using the RCA-based assay both when testing purified enzyme and cell extract. The detection limit of approximately 0.1 fmol (10^7 molecules) of purified hTopoI in the current crude setup indeed holds promise for the future detection of the TopoI activity level in even very small clinical tissue or cell samples, the content of TopoI in mammalian cells being estimated to at least 10^5 molecules.[42] The single-molecule detection of RCPs allowing the visualization of a single hTopoI

cleavage−ligation event opens up for even more sensitive measurement of hTopoI activity, in principle, down to the detection of cleavage−ligation events mediated by a single-enzyme molecule over time. Such a technique would present obvious advantages for both basic and applied science, allowing subtle variations between individual enzymes in a large population, for instance, within a single cell, to be recognized. The presented experiments serve as proof of principle for the application of the RCA-based method for specific detection of hTopoI activity at the single cleavage−ligation event level. The major drawback of the current setup is the spotting of the RCA-primer, which was performed with a hand-held pipet spreading the signals over an approximate area of 3 × 3 mm, orders of magnitude larger than the field of view in the microscope, thus decreasing the sensitivity of the assay by a simple dilution effect. It is our expectation that concentration of the signals to a nanometer scale area using automated spotting of primers onto an appropriate MicroArray chip will improve the sensitivity of the assay considerably. Such an approach presents a second advantage by allowing automated readouts of the results simply by using customized equipment for MicroArray analysis, which although at the cost of single-molecule detection may pave the road to high-throughput analysis of the hTopoI activity level in small biological samples or even in single cells. To improve sensitivity without compromising the single-molecule detection scheme, counting RCPs using a microfluidic channel[13] or using volume-amplified magnetic nanobeads,[22] which are both methods that hold great promise in the detection of single RCPs, may be an even better approach. Applying such methods is likely not only to improve sensitivity but may also circumvent the problem of signals arising from several RCPs merging when assaying high concentrations of hTopoI observed in the current setup (Figures 4 and 6)

The possibilities for improving the sensitivity of the RCA-based hTopoI activity assay using the different methods described above are currently being investigated. Furthermore, based on the current results dumbbell- or other circularizable substrates may be designed for the specific and sensitive detection of other clinically relevant enzyme activities for the future design of multiplexed systems, allowing simultaneous screening for numerous clinically relevant factors.

CONCLUSION

The RCA-based single-molecule detection system presented here enables the measurement of single cleavage−ligation events by hTopoI. This was achieved by the conversion of each hTopoI mediated cleavage−ligation reaction on a specially designed DNA dumbbell-substrate to an RCP that could be visualized at the single-molecule level using fluorescently labeled probes. In the present setup, the RCA-based assay allowed detection of hTopoI activity with a 10−100-fold improved sensitivity compared to current state of the art methodologies. Concentration of the RCP signals or employment of already existing microfluidic counting devices[13] is likely to improve sensitivity even further, maybe even to the single-cell or single-enzyme detection levels.

Besides its potential for basic enzymatic investigations, the presented method for single hTopoI cleavage−ligation detection on a crude cell extract background holds great clinical promise. In particular, the technique is highly relevant for cancer diagnostic purposes since the efficiency of important chemotherapeutics of the CPT family has proven to correlate directly with the TopoI cleavage−ligation activity inside the cells. The diagnostic value of the presented technology is further increased by the numerous possibilities of multiplexing inherent in the RCA-based detection system as well as its easy adaptability to high-throughput systems.

METHODS

Reagents and Enzymes. T4 polynucleotide kinase, Phi29 DNA polymerase, T4 DNA ligase, exonuclease I, and exonuclease III were from MBI Fermentas, [γ^{32}P]ATP (7000 Ci/mmol) was from ICN. All oligonucleotides were purchased from DNA Technology A/S, Aarhus, Denmark. SYBR Gold was from Invitrogen, CodeLink Activated Slides were from GE Healthcare, and Vectashield was from Vector Laboratories.

Yeast Strains and Construction of hTopoI Expression Plasmids. The yeast *Saccharomyces cerevisiae* top1-null strain RS190 was a kind gift from R. Sternglanz (State University of New York, Stony Brook, NY). Plasmid pHT143, for expression of recombinant full-length hTopoI, was described previously.[35]

Expression of hTopoI and Preparation/Analysis of Cell Extracts or Purified Enzyme. The plasmid pHT143 was transformed into the yeast *S. cerevisiae* strain RS190. The protein was expressed, and crude extracts or purified enzyme was prepared as described previously.[35,38] The protein concentrations were estimated from Coomassie blue-stained SDS-polyacrylamide gels by comparison to serial dilutions of BSA. The amount of cells used for extract preparation was estimated to 10^5 cells/μL(extract) by measuring absorbance at OD_{600}. Western blotting to test the expression level of hTopoI in yeast extracts was performed essentially as described by Hede *et al.*[43]

Synthetic DNA Substrates, Probes, and Primers. Oligonucleotides for construction of the dumbbell-substrate, the control ligase substrate, the dsDNA substrate, and the utilized detection probes and RCA-primer were synthesized by DNA Technology on a model 394 DNA synthesizer from Applied Biosystems. The sequences of the oligonucleotides are as follows: dumbbell-substrate, 5′-AGAAAAATTT TTAAAAAAAC TGTGAAGATC GCTTATTTTT TTAAAAATTT TTCTAAGTCT TTTAGATCCC TCAATGCTGC TGCTGTACTA CGATCTAAAA GACTTAGA-amine-3′; control ligase substrate, 5′-AGAAAAATTT TTAAAAAAAC TGTGAAGATC GCTTATTTTT TTAAAAATTT TTCTAAGTCT TTTAGATCCC TCAATGCTGC TGCTGTACTA CGATCTAAAA GACTT-3′; dsDNA substrate, 5′-GATCTAAAAG ACTTAGA (cl-str), 5′-AGAAAAATTT TTAAAAAA (lig-str), 5′-TTTTTTAAAA ATTTTTCTAA GTCTTTTAGA TC (noncl-str); RCA-primer, 5′-amine-CCAACCAACC AACCAAATAA GC-

GATCTTCA CAGT-3′; detection probes, ID16 5′-TAMRA-CCTCAATGCT GCTGCTGTAC TAC-3′, and ID33 5′-FITC-CCTCAATGCA CATGTTTGGC TCC-3′. The oligonucleotides representing the dumbbell-substrate and the RCA-primer were modified by a 3′- and a 5′-amine group, respectively. The detection probes ID16 and ID33 were fluorescently labeled by 5′-coupling of the fluorophores TAMRA and FITC, respectively. For preparation of the control circle, the control ligase substrate was circularized by T4 ligase, and the resulting ligated circles were purified from a 8% polyacrylamide gel. The concentration of the obtained circles was determined by spectrophotometric measurement. For preparation of the radiolabeled dsDNA substrate, the purified cl-str was 5′-radiolabeled prior to hybridization by applying the T4 polynucleotide kinase reaction using [γ^{32}P]ATP as the phosphoryl donor. For assembly of the substrate by hybridization, 10 pmol of each of the three oligonucleotides, the cl-str, the lig-str, and the noncl-str, were mixed in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, heated to 85 °C, and cooled slowly to room temperature. All oligonucleotides used for substrate assembly were gel-purified as described previously.[39,44]

Detection of TopoI Activity on the Dumbbell-Substrate. Purified hTopoI (1000 fmol) was incubated with 0.2 μM dumbbell-substrate in a standard hTopoI reaction buffer containing 10 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM DTT, 0.2 μg/μL BSA for 30 min at 37 °C. As a positive control, 0.1 units of T4 DNA ligase was added to the control ligase substrate in a standard T4 ligase buffer for 30 min at 37 °C. Both the hTopoI reactions and T4 DNA ligase reactions were terminated by inactivation for 5 min at 95 °C. For exonuclease digestion, the reactions were supplemented with 7 units exonuclease I and 70 units exonuclease III and incubated for 60 min at 37 °C before inactivation for 15 min at 80 °C. Subsequently, the samples were EtOH precipitated and analyzed by denaturing 12% polyacrylamide gel electrophoresis essentially as described previously. The reaction products were visualized by SYBR Gold staining according to the manufacturers description.

Solid Support Amplification. The 5′-amine-coupled primer was linked to CodeLink Activated Slides according to the manufacturers description. The hTopoI reaction was carried out in a 10 μL reaction volume containing either a divalent cation depletion buffer (1 mM Tris-HCl, pH 7.5, and 5 mM EDTA) or a standard hTopo I reaction buffer (10 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM DTT). At both buffer conditions, the reaction mixtures were supplemented with 100 nM dumbbell-substrate. Reactions were initiated by the addition of different amounts of hTopoI as indicated in the figure legends in a total volume of 0.5 μL containing (in addition to purified hTopoI) 10 mM DTT, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 50% glycerol. Incubation was continued for 30 min at 37 °C before heat inactivating hTopoI for 5 min at 95 °C. Subsequently, hybridization to the covalently coupled primer was performed for 60 min at room temperature (22−25 °C). (For the quantitative assay, 5 fmol of control circle was added to the heat inactivated reaction mixture prior to this step.) Slides were washed for 2 min at room temperature in wash buffer 1 (0.1 M Tris-HCl, pH 7.5, 150 mM NaCl, and 0.3% SDS) and for another 2 min at room temperature in wash buffer 2 (0.1 M Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20). Finally, the slides were dehydrated in 99.9% ethanol for 1 min and air-dried. Rolling circle DNA synthesis was performed for 45 min at 37 °C in 1× Phi29 buffer supplemented with 0.2 μg/μL BSA, 250 μM dNTP, and 1 unit/μL Phi29 DNA polymerase. The reaction was stopped by washing in wash buffers 1 and 2. The RCPs were detected by hybridization to 0.17 μM of each of the detection probes ID16 and ID33 in a buffer containing 20% formamide, 2× SSC, and 5% glycerol for 30 min at 37 °C. The slides were washed in wash buffers 1 and 2, dehydrated, mounted with Vectashield, and visualized as described previously.[24]

Detection of hTopoI Activity Using a Radiolabeled Cleavage−Ligation Substrate. Standard cleavage−ligation reactions were carried out by incubating 50 fmol of the radiolabeled cleavage−ligation DNA substrate with hTopoI in a 10 μL reaction volume containing hTopoI buffer supplemented with 0.2 μg/μL BSA. Reactions were initiated by adding 0.5 μL of 10 mM DTT, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 50% glycerol containing different amounts of hTopoI (as indicated in the figure legends) and were continued for 30 min at 37 °C before termination by the addition of SDS to a final volume of 0.2% (w/v). Samples were EtOH precipitated and trypsinated prior to analysis in a 12% denaturing polyacrylamide sequencing gel as described previously.[35]

Assay for hTopoI Mediated DNA Relaxation. Relaxation reactions were carried out in a total volume of 10 μL containing a standard relaxation buffer (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 150 mM NaCl), 200 fmol of supercoiled circular plasmid DNA (pBR322), and 0.5 μL of the hTopoI preparation containing 10 mM DTT, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 50% glycerol, and different amounts of hTopoI as indicated in the figure legend. The reactions were performed for 30 min at 37 °C and stopped by addition of SDS to a final concentration of 0.2% (w/v). Subsequent to proteolytic digestion with 0.5 μg/mL of proteinase K at 37 °C for 30 min, the products were separated in a 1% agarose gel. DNA was visualized by staining of the gel with 0.5 μg/mL ethidium bromide, and the gel image was analyzed using the Bio-Rad Gel Doc-2000 system.

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REFERENCES AND NOTES

1. Belle, D. J.; Singh, H. Genetic Factors in Drug Metabolism. *Am. Fam. Physician* **2008**, *77*, 1553–1560.
2. Wang, L.; O'Donoghue, M. B.; Tan, W. Nanoparticles for Multiplex Diagnostics and Imaging. *Nanomedicine* **2006**, *1*, 413–426.
3. Zhang, D.; Wu, J.; Ye, F; Feng, T.; Lee, I.; Yin, B. Amplification of Circularizable Probes for the Detection of Target Nucleic Acids and Proteins. *Clin. Chim. Acta* **2006**, *363*, 61–70.
4. Cornish, P. V.; Ha, T. A Survey of Single-Molecule Techniques in Chemical Biology. *ACS Chem. Biol.* **2007**, *2*, 53–61.
5. Kim, S.; Blainey, P. C.; Schroeder, C. M.; Xie, X. S. Multiplexed Single-Molecule Assay for Enzymatic Activity on Flow-Stretched DNA. *Nat. Methods* **2007**, *4*, 397–399.
6. Lam, L.; Iino, R.; Tabata, K. V.; Noji, H. Highly Sensitive Restriction Enzyme Assay and Analysis: A Review. *Anal. Bioanal. Chem.* **2008**, in press.
7. Healy, K. Nanopore-Based Single-Molecule DNA Analysis. *Nanomed.* **2007**, *2*, 459–481.
8. Charvin, G.; Strick, T. R.; Bensimon, D.; Croquette, V. Tracking Topoisomerase Activity at the Single-Molecule Level. *Annu. Rev. Biophys. Biomol. Struct.* **2005**, *34*, 201–219.
9. van Mameren, J.; Peterman, E. J.; Wuite, G. J. See Me, Feel Me: Methods to Concurrently Visualize and Manipulate Single DNA Molecules and Associated Proteins. *Nucleic Acids Res.* **2008**, *36*, 4381–4389.
10. Herbert, K. M.; Greenleaf, W. J.; Block, S. M. Single-Molecule Studies of RNA Polymerase: Motoring Along. *Annu. Rev. Biochem.* **2008**, *77*, 149–176.
11. Handal, M. I.; Ugaz, V. M. DNA Mutation Detection and Analysis Using Miniaturized Microfluidic Systems. *Exp. Rev. Mol. Diagn.* **2006**, *6*, 29–38.
12. Dittrich, P. S.; Tachikawa, K.; Manz, A. Micro Total Analysis Systems. Latest Advancements and Trends. *Anal. Chem.* **2006**, *78*, 3887–3908.
13. Jarvius, J.; Melin, J.; Göransson, J.; Stenberg, J.; Fredriksson, S.; Gonzalez-Rey, C.; Bertilsson, S.; Nilsson, M. Digital Quantification Using Amplified Single-Molecule Detection. *Nat, Methods* **2006**, *3*, 725–727.
14. Melin, J.; Jarvius, J.; Göransson, J.; Nilsson, M. Homogeneous Amplified Single-Molecule Detection: Characterization of Key Parameters. *Anal. Biochem.* **2007**, *368*, 230–238.

15. Ericsson, O.; Jarvius, J.; Schallmeiner, E.; Howell, M.; Nong, R. Y.; Reuter, H.; Hahn, M.; Stenberg, J.; Nilsson, M.; Landegren, U. a Dual-Tag Microarray Platform for High-Performance Nucleic Acid and Protein Analyses. *Nucleic Acids Res.* **2008**, *36*, e45.
16. Mahmoudian, L.; Melin, J.; Mohamadi, M. R.; Yamada, K.; Ohta, M.; Kaji, N.; Tokeshi, M.; Nilsson, M.; Baba, Y. Microchip Electrophoresis for Specific Gene Detection of the Pathogenic Bacteria *V. cholerae* by Circle-to-Circle Amplification. *Anal. Sci.* **2008**, *24*, 327–332.
17. Nilsson, M.; Malmgren, H.; Samiotaki, M.; Kwiatkowski, M.; Chowdhary, B. P.; Landegren, U. Padlock Probes: Circularizing Oligonucleotides for Localized DNA Detection. *Science* **1994**, *265*, 2085–2088.
18. Söderberg, O.; Gullberg, M.; Jarvius, M.; Ridderstråle, K.; Leuchowius, K. J.; Jarvius, J.; Wester, K.; Hydbring, P.; Bahram, F.; Larsson, L. G.; Landegren, U. Direct Observation of Individual Endogenous Protein Complexes *In Situ* by Proximity Ligation. *Nat. Methods* **2006**, *3*, 995–1000.
19. Fire, A.; Xu, S. Q. Rolling Replication of Short DNA Circles. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 4641–4645.
20. Banér, J.; Nilsson, M.; Mendel-Hartvig, M.; Landegren, U. Signal Amplification of Padlock Probes by Rolling Circle Replication. *Nucleic Acids Res.* **1998**, *26*, 5073–5078.
21. Lizardi, P. M.; Huang, X.; Zhu, Z.; Bray-Ward, P.; Thomas, D. C.; Ward, D. C. Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification. *Nat. Genet.* **1998**, *9*, 225–232.
22. Strömberg, M.; Göransson, J.; Gunnarsson, K.; Nilsson, M.; Svedlindh, P.; Strømme, M. Sensitive Molecular Diagnostics Using Volume-Amplified Magnetic Nanobeads. *Nano Lett.* **2008**, *8*, 816–821.
23. Lohmann, J. S.; Stougaard, M.; Koch, J. a New Enzymatic Route for Production of Long 5′-Phosphorylated Oligonucleotides Using Suicide Cassettes and Rolling Circle DNA Synthesis. *BMC Biotechnol.* **2007**, *7*, 49.
24. Stougaard, M.; Lohmann, J. S.; Zajac, M.; Hamilton-Dutoit, S.; Koch, J. *In Situ* Detection of Non-polyadenylated RNA Molecules Using Turtle Probes and Target Primed Rolling Circle PRINS. *BMC Biotechnol.* **2007**, *7*, 69.
25. Champoux, J. J. Mechanism of Catalysis by Eukaryotic DNA Topoisomerase I. *Adv. Pharmacol.* **1994**, *29A*, 71–82.
26. Pommier, Y. Topoisomerase I Inhibitors: Camptothecins and beyond. *Nat. Rev. Cancer* **2006**, *6*, 789–802.
27. Liao, Z.; Robey, R. W.; Guirouilh-Barbat, J.; To, K. K.; Polgar, O.; Bates, S. E.; Pommier, Y. Reduced Expression of DNA Topoisomerase I in SF295 Human Glioblastoma Cells Selected for Resistance to Homocamptothecin and Diflomotecan. *Mol. Pharmacol.* **2008**, *73*, 490–497.
28. Soret, J.; Gabut, M.; Dupon, C.; Kohlhagen, G.; Stévenin, J.; Pommier, Y.; Tazi, J. Altered Serine/Arginine-Rich Protein Phosphorylation and Exonic Enhancer-Dependent Splicing in Mammalian Cells Lacking Topoisomerase I. *Cancer Res.* **2003**, *63*, 8203–8211.
29. Taniguchi, K.; Kohno, K.; Kawanami, K.; Wada, M.; Kanematsu, T.; Kuwano, M. Drug-Induced down-Regulation of Topoisomerase I in Human Epidermoid Cancer Cells Resistant to Saintopin and Camptothecins. *Cancer Res.* **1996**, *56*, 2348–2354.
30. Desai, S. D.; Li, T. K.; Rodriguez-Bauman, A.; Rubin, E. H.; Liu, L. F. Ubiquitin/26S Proteasome-Mediated Degradation of Topoisomerase I as a Resistance Mechanism to Camptothecin in Tumor Cells. *Cancer Res.* **2001**, *61*, 5926–5932.
31. Xu, Y.; Villalona-Calero, M. A. Irinotecan: Mechanisms of Tumor Resistance and Novel Strategies for Modulating Its Activity. *Ann Oncol.* **2002**, *13*, 1841–51.
32. Taneja, B.; Schnurr, B.; Slesarev, A.; Marko, J. F.; Mondragón, A. Topoisomerase V Relaxes Supercoiled DNA by a Constrained Swiveling Mechanism. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 4670–4675.
33. Koster, D. A.; Palle, K.; Bot, E. S.; Bjornsti, M. A.; Dekker, N. H. Antitumour Drugs Impede DNA Uncoiling by Topoisomerase I. *Nature* **2007**, *448*, 213–217.
34. Bonven, B. J.; Gocke, E.; Westergaard, O. A High Affinity Topoisomerase I Binding Sequence is Clustered at DNAase I Hypersensitive Sites in Tetrahymena R-Chromatin. *Cell* **1985**, *41*, 541–551.
35. Lisby, M.; Krogh, B. O.; Boege, F.; Westergaard, O.; Knudsen, B. R. Camptothecins Inhibit the Utilization of Hydrogen Peroxide in the Ligation Step of Topoisomerase I Catalysis. *Biochemistry* **1998**, *37*, 10815–10827.
36. Andersen, A. H.; Christiansen, K.; Westergaard, O. Uncoupling of Topoisomerase-Mediated DNA Cleavage and Religation. *Methods Mol. Biol.* **2001**, *95*, 101–117.
37. Frøhlich, R. F.; Andersen, F. F.; Westergaard, O.; Andersen, A. H; Knudsen, B. R. Regions within the N-Terminal Domain of Human Topoisomerase I Exert Important Functions during Strand Rotation and DNA Binding. *J. Mol. Biol.* **2004**, *336*, 93–103.
38. Knudsen, B. R.; Straub, T.; Boege, F. Separation and Functional Analysis of Eukaryotic DNA Topoisomerases by Chromatography and Electrophoresis. *J. Chromatogr. B* **1996**, *684*, 307–321.
39. Christiansen, K; Knudsen, B R; Westergaard, O. The Covalent Eukaryotic Topoisomerase I—DNA Intermediate Catalyzes pH-Dependent Hydrolysis and Alcoholysis. *J. Biol. Chem.* **1994**, *269*, 11367–11373.
40. Lehman, I. R. DNA Ligase: Structure, Mechanism, And Function. *Science* **1974**, *186*, 790–797.
41. Ceska, T. A.; Sayers, J. R. Structure-Specific DNA Cleavage by 5′ Nucleases. *Trends Biochem. Sci.* **1998**, *23*, 331–336.
42. Champoux, J. J. Proteins That Affect DNA Conformation. *Annu. Rev. Biochem.* **1978**, *47*, 449–479.
43. Hede, M. S.; Petersen, R. L.; Frøhlich, R. F.; Krüger, D.; Andersen, F. F.; Andersen, A. H.; Knudsen, B. R. Resolution of Holliday Junction Substrates by Human Topoisomerase I. *J. Mol. Biol.* **2007**, *365*, 1076–1092.
44. Andersen, F. F.; Andersen, K. E.; Kusk, M.; Frøhlich, R. F.; Westergaard, O.; Andersen, A. H.; Knudsen, B. R. Recombinogenic Flap Ligation Mediated by Human Topoisomerase I. *J. Mol. Biol.* **2003**, *330*, 235–246.