

# Ultrasensitive isolation, identification and quantification of DNA–protein adducts by ELISA-based RADAR assay

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## ABSTRACT

Enzymes that form transient DNA–protein covalent complexes are targets for several potent classes of drugs used to treat infectious disease and cancer, making it important to establish robust and rapid procedures for analysis of these complexes. We report a method for isolation of DNA–protein adducts and their identification and quantification, using techniques compatible with high-throughput screening. This method is based on the RADAR assay for DNA adducts that we previously developed (Kiianitsa and Maizels (2013) *A rapid and sensitive assay for DNA–protein covalent complexes in living cells. Nucleic Acids Res.*, 41:e104), but incorporates three key new steps of broad applicability. (i) Silica-assisted ethanol/isopropanol precipitation ensures reproducible and efficient recovery of DNA and DNA–protein adducts at low centrifugal forces, enabling cell culture and DNA precipitation to be carried out in a single microtiter plate. (ii) Rigorous purification of DNA–protein adducts by a procedure that eliminates free proteins and free nucleic acids, generating samples suitable for detection of novel protein adducts (e.g. by mass spectroscopy). (iii) Identification and quantification of DNA–protein adducts by direct ELISA assay. The ELISA-based RADAR assay can detect Top1–DNA and Top2a–DNA adducts in human cells, and gyrase–DNA adducts in *Escherichia coli*. This approach will be useful for discovery and characterization of new drugs to treat infectious disease and cancer, and for development of companion diagnostics assays for individualized medicine.

## INTRODUCTION

We recently developed a rapid and sensitive assay for quantification of DNA–protein adducts in cells treated with drug, the RADAR assay (Rapid Assay of the DNA Adduct Response) (1). The RADAR assay detects DNA–protein covalent complexes (DPCCs) that are formed as transient intermediates, and that may be trapped by treatment with drug. Proteins that form transient covalent adducts to DNA are targets of life-saving antibiotics and robust cancer chemotherapeutics (2,3). These include prokaryotic DNA gyrase, the target of quinolone, fluoroquinolone and aminocoumarin antibiotics; human topoisomerase 1, the target of camptothecin derivatives such as topotecan and irinotecan; human topoisomerase 2, the target of etoposide (VP16) and doxorubicin; and DNA methyltransferases, targets of decitabine and azacitidine.

Despite the demonstrated effectiveness of drugs that trap DNA–protein adducts, there has been no systematic mechanism-based discovery of drug-target pairs in this class. Enzymes known to function by this mechanism that are potential new drug targets include human topoisomerase 3a (4); PARP-1 (5); Ku (6); DNA glycosylases including OGG1, TDG and NTH and NEIL family members (7,8); *O*<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) (9); Tyrosyl-DNA phosphodiesterase 1 (10); DNA Pol  $\beta$  (11) and Y family polymerases  $\iota$ ,  $\eta$  and  $\kappa$  (12) and bacterial topoisomerase 1 (13). Other enzymes involved in DNA metabolism may also employ this mechanism, but have not yet been shown to do so. The drugs in current use that function by trapping DNA–protein adducts are (or are derived from) natural products, suggesting that mechanism-based screening of large compound libraries could yield new drugs in this class.

The RADAR assay allows effective one-step precipitation of DPCCs and free nucleic acids and their immobilization on a membrane by slot blot, for detection by specific antibodies. Quantification of DNA–protein adducts had

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previously depended upon cumbersome protocols, typically involving ultracentrifugation to separate DPCCs from free protein and nucleic acid (14,15). The RADAR assay greatly increased throughput and sensitivity, and accelerated turnaround time, making it of clear use for mechanistic studies. However, it could not be applied directly to large numbers of samples in microtiter format, so it was not suitable for high-throughput applications.

Here we describe a new approach suitable for isolation of DPCCs and their detection by ELISA. The isolation protocol begins with cell lysis in chaotropic salts and detergent, as previously described (1), and employs three critical refinements that will be of use not only in this context but also more broadly. (i) Silica-assisted DNA precipitation: addition of silica slurry prior to isopropanol or ethanol precipitation allows a single microplate to be used for cell culture and DNA precipitation, with reproducible and efficient recovery of DNA and DNA–protein adducts at low centrifugal forces. (ii) Rigorous purification of DPCCs: a lysis buffer, composed of guanidinium thiocyanate, lithium chloride and detergents, is used to separate DNA and DPCC from free proteins, and nucleic acids are digested with Benzonase, generating sample sufficiently clean for identification of novel protein adducts, e.g. by mass spectroscopy. (iii) DPCC-ELISA: the purified DPCCs bind spontaneously and efficiently to plastic, enabling ultrasensitive detection by direct ELISA.

All steps in the ELISA-based RADAR assay are compatible with high-throughput screens. The assay can be reliably applied to small numbers of human cells grown in 96- or 384-well microtiter plates (40 ng DNA, or 10,000 cells), with a  $Z'$  factor  $> 0.5$  for detection of Top1–DNA complexes. We further show that the assay can be adapted to detect GyrA–DNA covalent complexes formed in *Escherichia coli* cells treated with ciprofloxacin. The RADAR assay thus offers potential for discovery and characterization of drugs for treatment of infectious disease and cancer.

## MATERIALS AND METHODS

### Cell culture, drug treatment and survival assays

Adherent cell lines HCT116, which derives from a colorectal tumor (ATCC CCL-247), and GM639, an SV40-transformed fibroblast line (gift of Dr Ray Monnat, University of Washington), were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Suspension cell lines CCRF-CEM (ATCC CCL-119) and MOLT-4 (ATCC CRL-1582), both derived from T cell acute lymphoblastic leukemias, were cultured in RPMI-1640 medium with 10% fetal calf serum. Cells were plated in flat bottom tissue culture plates 16–20 h prior to treatment with drugs and DPCC isolation. Cells were treated with 10  $\mu$ M camptothecin (CPT; Enzo Life Sciences) or topotecan (TPT; Enzo Life Sciences) for 30 min; or with 50  $\mu$ M VP16 (EMD Biosciences) for 15–30 min, unless otherwise indicated. Cell survival was quantified using the CellTiter-Glo<sup>®</sup> assay (Promega).

*Escherichia coli* K-12 strain MG1655 was a gift of Dr. Yuk-Ching Tse-Dinh, Florida International University. Log phase cells were treated with 20  $\mu$ g/ml ciprofloxacin

(Sigma) or with 100  $\mu$ g/ml nalidixic acid (TOKU-E) for 45 min.

### Cell lysis solutions

Key to the RADAR assay is cell lysis under conditions that preserve the DNA–protein covalent bond and that maintain protein epitopes for subsequent immunodetection. For isolation of topoisomerase 1 (Top1)–DNA adducts, cell lysis was carried out using a solution (LS1) composed of 1% Sarkosyl, 2% Nonidet P-40, 10 mg/ml DTT, 20 mM EDTA, 20 mM Tris-HCl (pH 8.0) and 0.1 M sodium acetate, to which guanidinium isothiocyanate (GTC), LiCl or urea were added at indicated concentrations. Final pH was adjusted to 6.5 using NaOH. We also tested two commercial cell lysis reagents supplemented with 1% Sarkosyl to facilitate separation of free proteins from DNA, DNAzol<sup>®</sup> genomic DNA isolation reagent (DZ; Invitrogen) and RNeasy<sup>®</sup> Plus lysis buffer (RLT; Qiagen).

For isolation of DNA topoisomerase 2a (Top2a)–DNA adducts, unless otherwise indicated, cell lysis was based on an alkaline lysis method previously used to isolate covalent Top1–DNA complexes for proteomic analyses (16). Cells were treated with an alkaline lysis solution, LS2, that contained 5 M GTC, 1% Sarkosyl, 1 M LiCl, 0.2 M NaOH and 1% beta-mercaptoethanol, and the solution immediately neutralized by addition of an equal volume of 3 M potassium acetate (pH 5.5). LS2 was also used for isolation of DNA gyrase (GyrA)–DNA adducts from *E. coli*.

### DNA isolation on custom silica filter plates

MultiScreen 96-well filter plates with 1.2  $\mu$ m Durapore membrane (Millipore) were pre-loaded with 4% slurry of silica gel fines (Sigma #288519) in the cell lysis solution (4–8 mg of dry silica per well). The plate was drained on the vacuum manifold (Bio-Rad), cell lysates corresponding to 2–3  $\times 10^5$  cells were applied and 0.5 volume of ethanol added. Plates were sealed and incubated on a plate shaker for 15 min. Three silica wash-drain cycles with 200  $\mu$ l 75% ethanol were performed, and silica fines were briefly air-dried and DNA was eluted in 100–200  $\mu$ l 8 mM NaOH.

### Silica-assisted DNA precipitation from whole cell lysates

The protocol is described for cells cultured in 100  $\mu$ l media per well in 96-well plates. Media was aspirated and 100  $\mu$ l lysis solution added to each well. Plates were sealed, shaken 5 min at room temperature on a plate shaker, heated at 55°C for 15 min and then shaken an additional 15 min. Silica gel fines were prepared as 4–16% slurry in lysis solution, depending on the desired final volume, and an amount of slurry corresponding to 4–8 mg of dry silica per well was added to the cell lysate. DNA was precipitated by addition of ethanol or isopropanol followed by centrifugation at RCF 2740 (3500 rpm) for 10 min. Supernatants were removed by careful aspiration, and pellets washed twice in 200–250  $\mu$ l 75% ethanol, centrifuging for 5 min at 3500 rpm between washes. Pellets, which contained DNA, RNA and DNA–protein adducts, were briefly air-dried and resuspended in 100–200  $\mu$ l of 8 mM NaOH. The typical yield was 300 ng DNA per well containing 4  $\times 10^4$  adherent cells.

### Quantification of DNA

DNA was quantified by measuring fluorescence of DNA-bound PicoGreen dye (Invitrogen) on a plate reader (FLU-Ostar Omega, BMG Labtech). DNA samples were diluted in 100  $\mu$ l TE to be within the dynamic range of this assay (5–500 ng/ml DNA) and briefly treated with RNase A (10  $\mu$ g/ml, 30 min at 37°C) prior to addition of PicoGreen. Measurements were standardized using a serial dilution of human genomic DNA for which concentration had been determined by measurement of  $A_{260}$ .

### Antibodies

Primary and secondary antibodies were diluted in 1  $\times$  ELISA Assay Diluent (Biolegend). Top1–DNA adducts were detected with polyclonal rabbit anti-human Top1 IgG (ab28432, Abcam) at 1:2000 dilution. Top2a–DNA adducts were detected with monoclonal mouse anti-human Top2a IgG (#611326, BD Transduction Laboratories) at 1:500. Gyrase–DNA adducts were detected with polyclonal rabbit anti-GyrA antibodies (PA001, Inspiralis) at 1:500 dilution. Secondary antibody was horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG at 1:5000 or 1:2500, respectively.

### Slot-blot immunoassay

Isolates, typically containing 200–500 ng DNA, were diluted in 25 mM sodium phosphate (pH 6.5) or TBS (Tris-Buffered Saline: 10 mM Tris, pH 7.5, 150 mM NaCl) and applied to nitrocellulose (Bio-Rad) or PDVF (Millipore) membranes using a vacuum slot-blot manifold (Bio-Rad). Membranes were blocked in 0.5% alkali-soluble casein (Novagen) dissolved in TBST (TBS containing 0.1% Tween-20) for at least 1 h; incubated with primary antibodies at least 3 h at room temperature or overnight at 4°C; and with secondary HRP-conjugated antibodies for 1 h at room temperature. After each incubation, membranes were washed three times for 5 min with TBST. Antibody signal was quantified by imaging the membrane on a Bio-Rad ChemiDoc XRS Plus Analyzer.

### DPCC adsorption to ELISA plates

Prior to treatment with Benzonase, samples were adjusted to 2 mM  $MgCl_2$  and then incubated with 12.5 U Benzonase nuclease (EMD Millipore) for 30 min at 37°C to digest DNA and RNA. Benzonase-treated samples were serially diluted in water, adjusted to 1  $\times$  ELISA Coating Buffer (Biolegend), loaded in 50–100  $\mu$ l volumes on an untreated 96-well ELISA plate (Nunc Maxi-Sorb) and incubated on a plate shaker for 1–2 h at room temperature or overnight at 4°C.

DNA-mediated adsorption of the samples was carried out using ELISA plates that had been pre-incubated for 1 h at room temperature with 50  $\mu$ l per well Reacti-Bind reagent (Pierce). The DPCC sample was added to the plate in 50  $\mu$ l, containing up to 20  $\mu$ g/ml DNA in TE, and the plates incubated at room temperature on a plate shaker for another 2 h.

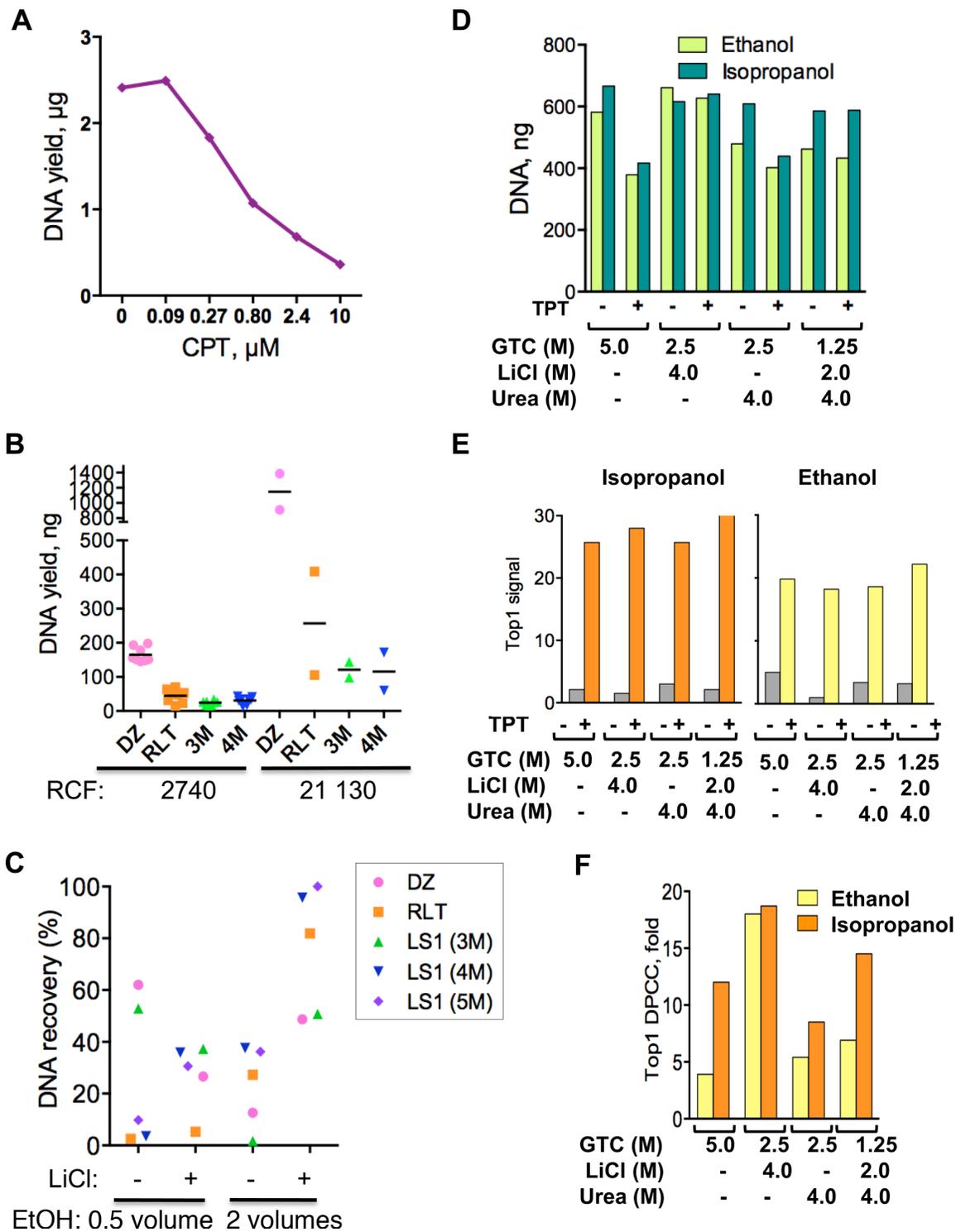
### DPCC-ELISA assays

Following DPCC adsorption, all washes and incubations were performed on a plate shaker at room temperature. Wells were washed three to four times with 150–200  $\mu$ l 1  $\times$  PBS and blocked for 1 h in 100  $\mu$ l of 1  $\times$  ELISA Assay Diluent. Samples were incubated with 60  $\mu$ l primary antibodies for 2–3 h; washed four times with 150  $\mu$ l 1  $\times$  ELISA Wash Buffer (Biolegend) or PBST (PBS containing 0.1% Tween-20); incubated with 60  $\mu$ l secondary HRP-conjugated antibodies for 30–45 min and washed four times with 150  $\mu$ l 1  $\times$  ELISA Wash Buffer or PBST. Following addition of 100  $\mu$ l of TMB High Sensitivity Substrate Solution (Biolegend), plates were incubated in the dark for 20 min and the reaction was stopped by addition of 50  $\mu$ l TMB Stop Solution (Biolegend). Absorbance was read at 450 nm, and the  $A_{450}$  reading was corrected for background by subtraction of  $A_{570}$ . Samples were run in duplicates or triplicates, and corrected for background by subtraction of signal from control wells that contained no DNA. The DPCC ELISA assay readily detects Top1-DPCC using samples prepared from human cells by lysis in RLT, DZ or LS1 containing 3.75 M GTC and 2 M LiCl, with no apparent difference in signal. DNA isolates contained little if any protease activity so that samples stored 6 months at 4°C did not exhibit substantial loss of immunoreactivity.

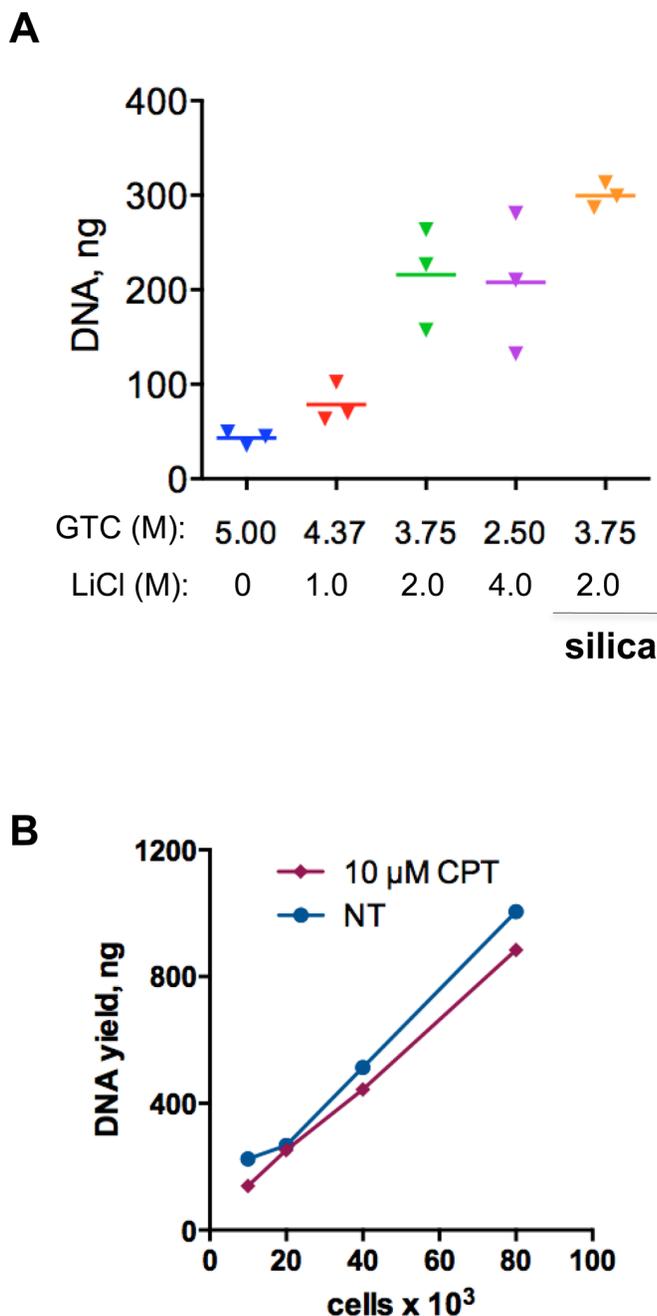
## RESULTS

### Miniscale DNA and DPCC recovery

Cells are very conveniently cultured on 96- or 384-well plates (0.2–2  $\times$  10<sup>5</sup> cells in 25–100  $\mu$ l). However, these plates do not tolerate high centrifugal forces, and the maximum centrifugal force for a rotor that handles microtiter plates is significantly below that of a benchtop microfuge (RCF 2740 and 21,130, respectively, for standard Eppendorf instruments). The DPCC isolation protocol we previously described (1) was designed for lysis in chaotropic salt solution followed by precipitation and recovery of nucleic acids in Eppendorf tubes using a benchtop microfuge. With the goal of avoiding centrifugation altogether, we first tested DNA recovery on silica filter plates, as in the presence of a chaotrope DNA has natural affinity for silica (17,18,19). The filter plates are designed for use with a vacuum manifold, which enables washing the sample to rid it of contaminating free protein and subsequent elution. We treated HCT116 cells with the Top1 poison, CPT, lysed them in DNAzol genomic DNA isolation reagent from Invitrogen supplemented with 1% Sarkosyl (DZ), and recovered DNA using 96-well silica filter plates from a customized source (20). Quite unexpectedly, we found that while yield of DNA from untreated cells was good, yield from samples from CPT-treated cells was reduced in a manner dependent upon the CPT dose (Figure 1A). Similar loss of DNA with increasing CPT dose was evident in analysis of cells lysed in LS1 containing 2.5–5.0 M GTC, using 96-well silica filter plates from a commercial (Pall Scientific) source (not shown). Thus reduced yield does not reflect the lysis buffer or source of silica filter plates. Reduced yield from CPT-treated cells could be due to impairment of DNA binding to silica plates by DPCCs, as this decrease was not evident



**Figure 1.** Low RCF precipitation enables quantitative recovery of DNA and DPCC from human cells treated with Top1 inhibitors. (A) DNA yield from HCT116 cells incubated for 30 min with CPT at indicated concentrations and lysed in DZ, followed by DNA recovery using custom-prepared 96-well silica filter plates. (B) DNA yield from HCT116 cells lysed in DZ, RLT or in LS1 containing either 3 or 4 M GTC, as indicated, followed by DNA precipitation with 0.5 volume ethanol, as recommended for DZ. RCF 2740, centrifugation directly in the culture plate (performed in two plates, in tetraplicates); RCF 21 130, centrifugation in Eppendorf tubes (performed in duplicates). (C) DNA recovery following precipitation at low RCF. Lysates of untreated HCT116 cells were prepared using a panel of five reagents, as indicated. DNA was precipitated in culture plates in the absence or presence of 1 M LiCl, using 0.5 or 2 volumes ethanol, as indicated. (D) Dependence of DNA recovery on lysis buffer and precipitation conditions. Effect of chaotrope and salt on silica-assisted recovery of DNA from HCT116 cells, either untreated or treated for 30 min with 10  $\mu\text{M}$  TPT, lysed in LS1 containing the indicated combination of chaotrope and salt and DNA and DPCC recovered in tubes by silica-assisted precipitation with 2 volumes ethanol or 1 volume isopropanol, as indicated. (E) Dependence of Top1 signal (normalized to DNA) on lysis buffer and precipitation conditions, as determined by slot blot. Data are for samples prepared in panel D. (F) Top1-DPCC induction in DNA isolated from treated versus untreated cells (panel D), calculated based on data from Panel E.



**Figure 2.** Validation of mini-scale DNA and DPCC isolation for high-throughput screening applications. **(A)** DNA yield from untreated CCRF-CEM cells lysed in LS1 containing 5 M GTC and adjusted to the indicated LiCl and GTC concentrations by addition of 8 M LiCl, and precipitated following addition of 1 volume isopropanol at low RCF; rightmost samples included 8 mg silica gel per well. **(B)** Efficiency of DNA recovery. DNA yield was determined from HCT116 cells which had been untreated (NT) or treated for 30 min with 10  $\mu$ M CPT, then lysed in LS1 containing 2.5 M GTC, 4 M LiCl and 8 mg silica gel per well, and DNA recovered by precipitation with 1 volume isopropanol. The x-axis indicates the number of cells seeded; assays were performed after overnight culture, so actual cell densities at treatment may be higher.

if the same samples were transferred to Eppendorf tubes for DNA precipitation (data not shown).

We then compared DNA recovery from cells lysed with four different lysis reagents: DZ, RNeasy Plus lysis buffer from Qiagen supplemented with 1% Sarkosyl (RLT) and our non-proprietary reagent LS1 (see Materials and Methods) containing 3 or 4 M GTC. We found that all were sub-optimal for DNA recovery at low RCF (2740). Among the four lysis reagents tested, DNAzol showed the best DNA yield at high RCF (21130), but yield was only 15% at low RCF (Figure 1B).

With the goal of optimizing conditions for DNA recovery at low RCF, we first varied three parameters: lysis reagent, organic solvent and salt. Testing DNA recovery along this three-parameter matrix showed that the best DNA recovery was achieved with lysis in LS1 containing 4 or 5 M GTC, followed by precipitation with 2 volumes of ethanol in the presence of high salt (Figure 1C).

We then modified buffer conditions to further improve recovery of DNA and Top1-DPCC signal. Cell lysis in high salt or urea has been shown to facilitate silica-based separation of DPCCs from non-covalently bound proteins for proteomic analyses (21). High salt and urea offered the further advantage that they might improve removal of non-covalently bound Top1 from DNA samples, thereby lowering background immunostaining and improving the sensitivity of DPCC detection. We therefore compared silica-assisted recovery of DNA and Top1-DPCC from untreated or TPT-treated HCT116 cells lysed in LS1 containing various combinations of GTC, LiCl and urea. With the further goal of limiting final volume, we concurrently compared recovery following precipitation with either 2 volumes of ethanol or 1 volume of isopropanol. We found that cell lysis in LS1 containing 2.5 M GTC and 4 M LiCl resulted in DNA recovery that was comparable to or better than other lysis solutions, and recovery was comparable of DNA from untreated cells and cells treated with 10  $\mu$ M TPT (Figure 1D).

Differences in composition of the lysis solution did not affect DPCC recovery, as assayed by measurement of the Top1 signal normalized to DNA in treated cells (Figure 1E). However, inclusion of LiCl appeared to reduce the background signal of DPCC in untreated cells, thereby increasing the apparent fold induction of Top1-DPCC as measured by the ratio of signals in treated versus untreated cells (Figure 1F). We conclude that LS1 supplemented with LiCl is a robust lysis reagent.

Precipitation with isopropanol resulted in better DNA recovery (Figure 1D) and Top1-DPCC signal (Figure 1E) than precipitation with ethanol, and the background signal from untreated cells was lower (Figure 1E, compare left and right). Isopropanol precipitation may improve signal either by improving DPCC recovery or by better preserving critical epitopes for immunodetection. Isopropanol has further advantage that it can be used at lower concentration than ethanol, minimizing total sample volume.

Further tests of lysis buffers showed some sample-to-sample variability that was minimized if silica slurry (8 mg) was included in each well (e.g. Figure 2A). To determine cell numbers necessary for efficient DNA recovery, we performed an experiment modeling a high-throughput chemi-

cal library screen. Varying numbers of cells cultured in a 96-well plate were untreated or briefly treated with 10  $\mu$ M CPT, lysed in LS1 containing 2.5 M GTC, 4 M LiCl and 8 mg silica gel per well, and DNA and DPCC recovered by precipitation with 1 volume of isopropanol and centrifugation of the microtiter plate at RCF 2740. Under these conditions, DNA was efficiently recovered from as few as  $1\text{--}2 \times 10^4$  cultured human cells, with a yield of about 140 ng per  $10^4$  cells, and the DNA yield was not substantially affected by treatment with CPT (Figure 2B). The ability to culture cells and recover DNA on the same microplate greatly streamlines workflow.

### Detection of DPCC by ELISA immunoassay

DPCC immunodetection by slot-blotting (1) is sensitive but requires loading of the sample onto a nitrocellulose or PVDF membrane with a vacuum slot-blot manifold. Formats of these devices are not compatible with high-throughput screens, and while microtiter plates with membrane-bottomed wells are commercially available, their cost is prohibitively high. We therefore modified the immunodetection step to use an ELISA rather than a slot blot assay.

In an ELISA, a primary antibody is used to detect its target immobilized in a well, and signal is amplified by detecting the primary antibody with a secondary antibody linked to an enzyme, such as horse radish peroxidase, activity of which can be measured by a simple colorimetric assay in solution. A variety of ELISA formats, including direct ELISA, competitive and 'sandwich' ELISA, have been developed to quantify specific proteins in complex mixtures (e.g. blood plasma or whole cell extracts) and for detection of low molecular weight adducts in genomic DNA (22–26). However, none of the current ELISAs for proteins or DNA adducts are suitable for DPCC detection. The main drawback is that protocols for enrichments of DPCC yield samples in which DNA constitutes over 98% of the mass, and protein constitutes only a small mass fraction, and DNA binds poorly to plastic yet limits protein access to the plastic surface. To surmount this difficulty, we asked if protein binding and DPCC signal could be improved by digestion of samples with Benzonase nuclease, which digests both DNA and RNA. We compared ELISAs performed on Benzonase-digested samples adsorbed to standard plates and on samples that had not been Benzonase-digested but had been adsorbed to plates coated with Reacti-Bind (Pierce), a reagent designed to facilitate capture of DNA and DNA adducts. We found that digestion with Benzonase followed by adsorption to standard plates resulted in an immunosignal ( $A_{450}$ ) 7-fold greater than that from undigested samples adsorbed to Reacti-Bind-coated plates DNA (Figure 3A). The signal was linear for samples from TPT-treated HCT116 cells containing the equivalent of 10–250 ng DNA per well (Figure 3B). Several biological replicates produced comparable results, including two different adherent cell lines, the colorectal cancer line HCT116 and the fibroblast line GM639; and assays of Top1–DNA adducts formed in response to two Top1 inhibitors, CPT and TPT (Figure 3C). To verify the specificity of Top1 adduct detection, we used the T cell line, CCRF-CEM, which is sensitive to both Top1

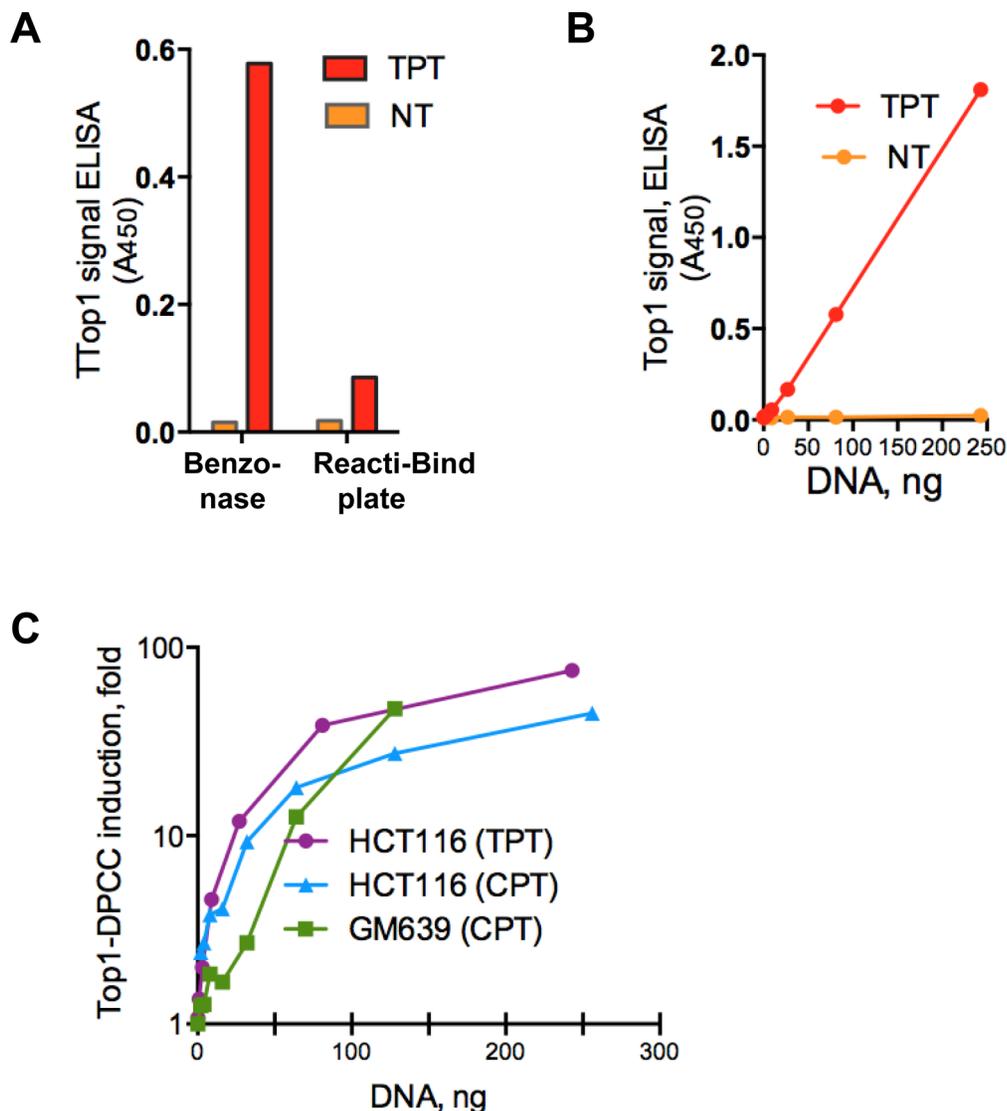
and Top2 inhibitors. Antibodies specific for Top1 produced a robust ELISA signal in CCRF-CEM DNA isolates that are treated with the Top1 poison TPT, but not in untreated cells or cells treated with the Top2a poison, VP16 (Figure 4A).

We carried out a side-by-side comparison of DPCC detection by DPCC-ELISA and slot-blot, assaying the same DPCC samples isolated from untreated or CPT-treated HCT116 cells. Comparison of the signal-to-noise ratio of the DPCC-ELISA immunoassay and the slot-blot showed that the DPCC-ELISA was as good or superior to this other assay (Figure 4B), but of course far more convenient.

The  $Z'$  factor is an important parameter of assay performance in a high-throughput screen. Assays with  $Z' > 0.5$  are considered excellent. We calculated the  $Z'$  factor of the DPCC-ELISA immunoassay for Top1-DPCC detection, and showed that the DPCC-ELISA reached the range of  $Z' > 0.5$  with amounts of DNA as low as 30 ng DNA per well (Figure 4C). The DPCC-ELISA immunoassay is therefore a robust and convenient method for quantification of DNA–protein adducts in samples derived from small numbers of cells.

### Kinetic analysis of Top1-DPCC repair by ELISA-based RADAR assay

The RADAR-based ELISA assay enables multiple samples to be assayed, and thus provides the opportunity for kinetic analyses that would be very tedious by other approaches. We took advantage of this to determine kinetics of Top1-DPCC repair in two different cell lines. The response of human cell lines to topoisomerase poisons is experimentally measured under a variety of conditions, some involving continuous culture with drug, and others involving brief exposure to drug followed by replacement of drug-containing media with media lacking drug ('wash-out'). Comparison of sensitivity of GM639 fibroblasts and HCT116 colorectal carcinoma cells in these two conditions showed that GM639 cells were much more resistant to brief exposure followed by wash-out, and HCT116 cells much more resistant to continuous exposure (Figure 5A). GM639 cells are normal human fibroblasts, with no known repair deficiencies, while HCT116 cells are mismatch repair deficient and express greatly reduced levels of the conserved repair factor, MRE11/RAD50. MRE11/RAD50 can cleave the 3'-phosphotyrosine bond formed between Top1 and its target *in vitro* (27), and it is critical to the CPT response in the yeast, *Saccharomyces cerevisiae* (28). This suggested that GM639 cells might repair Top1–DNA adducts more rapidly than HCT116 cells. We tested this by measuring kinetics of persistence of Top1–DNA adducts in each cell line after brief culture (30 min) with TPT followed by wash-out to remove drug. In GM639 cells, adduct levels were reduced to background levels within 15 min after drug removal; while in HCT116 cells, initially rapid repair occurred in the first 15 min after wash-out, but was followed by a period in which adducts persisted (Figure 5B). The biphasic kinetics in HCT116 cells could reflect importance of distinct pathways at different stages of the drug response, with MRE11/RAD50 important for later repair events.

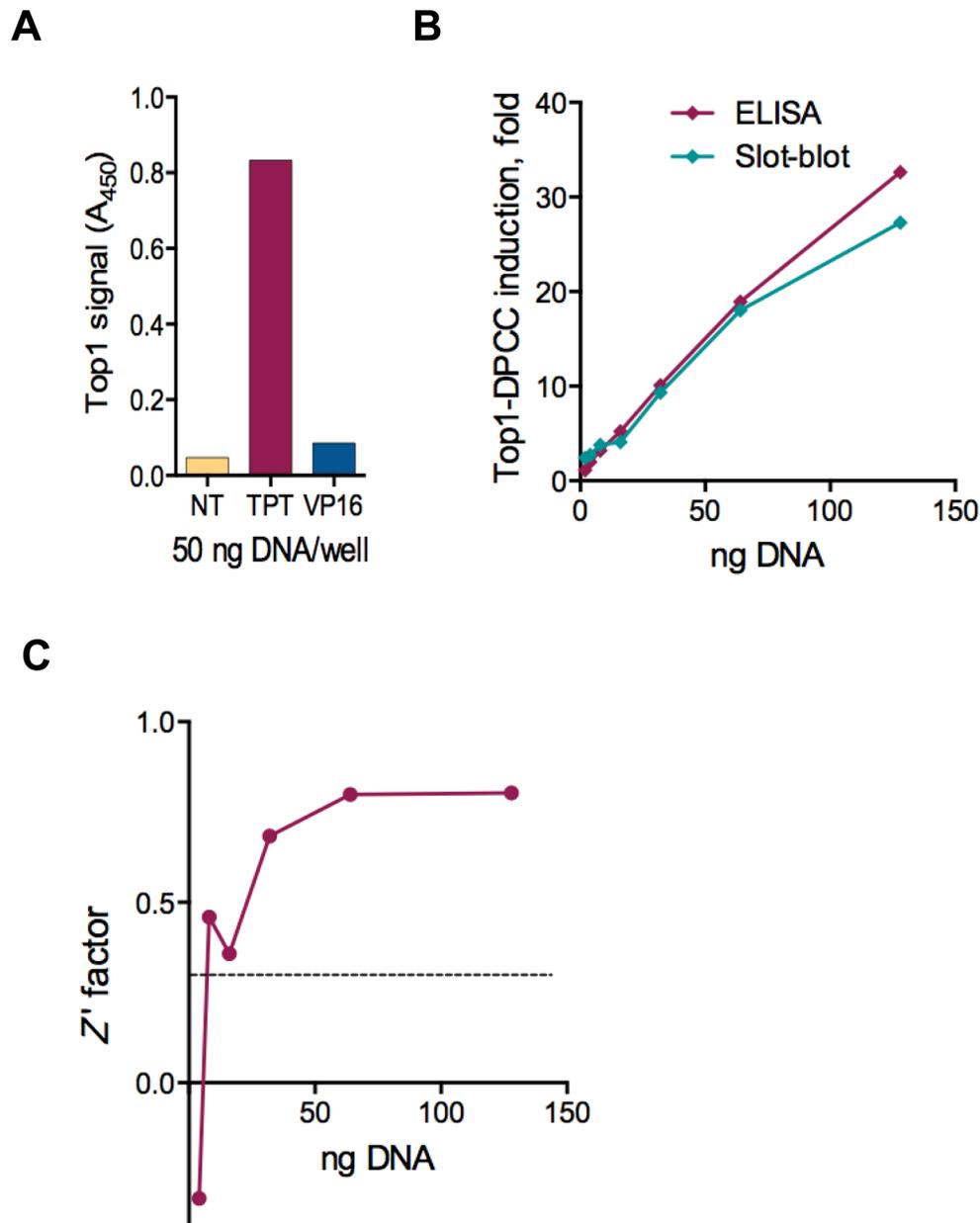


**Figure 3.** Detection of Top1-DPCC by a direct ELISA immunoassay. (A) Comparison of ELISA immunoassays for Top1-DPCC using DNA isolates (80 ng DNA per well) from untreated (NT) or TPT-treated HCT116 cells that were adsorbed to standard plates following digestion with Benzonase nuclease (left) or to Reacti-Bind-coated plates (right). (B) Linearity range and sensitivity of ELISA immunoassay for detection of Top1-DPCC in lysates from untreated (NT) or TPT-treated HCT116 cells, assayed in samples containing indicated initial amounts of DNA digested with Benzonase prior to adsorption to plates. (C) Fold induction of Top1-DPCC (ratio of treated to untreated samples) in HCT116 cells treated with CPT or TPT and GM639 cells treated with CPT, assayed in samples containing indicated initial amounts of DNA digested with Benzonase prior to adsorption to plates.

### Detection of human Top2a–DNA adducts by ELISA-based RADAR assay

Top2a is the target of VP16, doxorubicin and other drugs used to treat human leukemias. In vertebrate cells, the amount of Top2a protein is tightly controlled during the cell cycle (29). Top2a is less abundant than Top1, and detection of Top2a–DNA complexes has been reported to require significantly more sample than necessary for detection of Top1–DNA adducts. For example, Top2a–DNA adducts are barely detectable by the ICE immunoassay if  $<1 \mu\text{g}$  DNA is loaded per slot (14), and robust detection may require as much as  $30 \mu\text{g}$  DNA per slot (30), equivalent to  $4 \times 10^6$  human cells. To ask if the ELISA-based RADAR assay can detect Top2a–DNA adducts, we first validated commercial antibodies specific for Top2a, show-

ing that they produced a robust ELISA signal in assays with CCRF-CEM cells treated with the Top2 poison VP16, but not in untreated cells or cells treated with the Top1 poison, TPT (Figure 6A). We then assayed kinetics of induction of Top2a–DNA adducts in CCRF-CEM and MOLT4, another T-ALL derived cell line, following brief treatment with  $50 \mu\text{M}$  VP16. DNA and DPCC were isolated following cell lysis with LS2, nucleic acids digested with Benzonase and the equivalent of 500 ng DNA adsorbed per well of an ELISA plate. An increase in Top2a–DNA complexes was evident within 15 min of VP16 treatment, with a more rapid response and greatest induction evident in CCRF-CEM cells (Figure 6B). These results show that the ELISA-based RADAR assay readily detects Top2a–DPCC adducts in samples containing  $<1 \mu\text{g}$  DNA per well, from  $10^5$  hu-



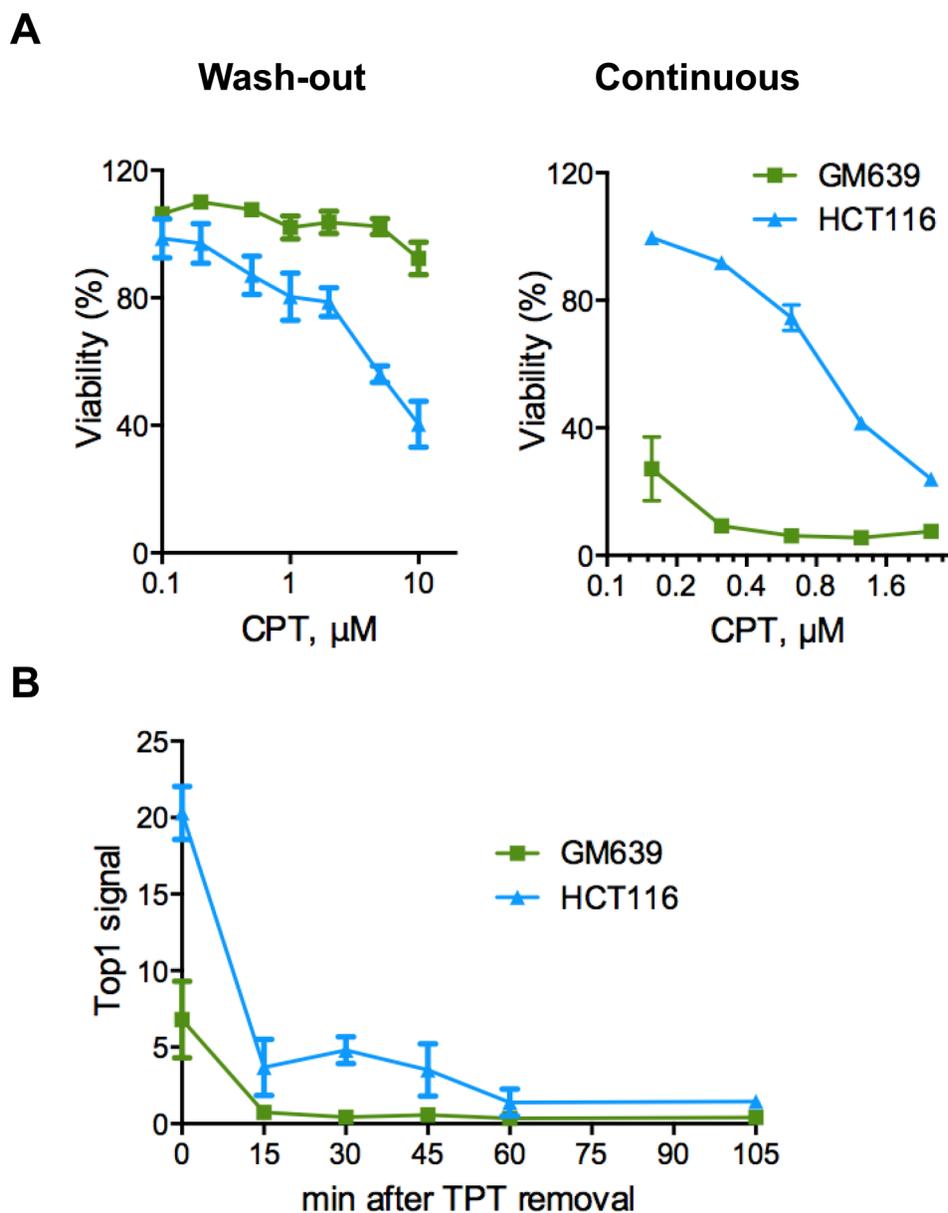
**Figure 4.** Reproducibility of detection of Top1-DPCC by ELISA-based RADAR assay. (A) Specificity of detection by ELISA assay using an anti-Top1 antibody. ELISA assays were performed on DNA isolates of CCRF-CEM treated with cognate (TPT) versus non-cognate (VP16) topoisomerase poison, and compared to an untreated control (NT). (B) Comparison of ELISA and slot-blot immunoassays of fold induction of Top1-DPCC, assayed in samples from HCT116 cells treated with CPT, containing indicated initial amounts of DNA. (C) Z' factor of Top1 DPCC detection by ELISA-based RADAR assay. Z' factor was calculated for each DNA dilution indicated using replicates of HCT116 cells treated with CPT and untreated controls.

man cells. Several relatively simple approaches can be used to increase assay sensitivity and thereby reduce required cell numbers (see 'Discussion' section).

#### Detection of *E. coli* gyrase-DNA adducts by ELISA-based RADAR assay

Some of the most widely used drugs for treatment of infectious disease are fluoroquinolones like ciprofloxacin, which poison bacterial DNA gyrase. However, there has been no mechanism-based discovery of gyrase poisons, probably because of the cumbersome nature of earlier assays

(31). To show that the ELISA-based RADAR assay can be applied to bacterial gyrase, we took advantage of the fact that tyrosyl-DNA bonds of topoisomerase covalent complexes are resistant to alkali (32). We showed that alkaline lysis in LS2, which contains GTC and detergents, produces DNA isolates suitable for immunodetection with anti-gyrase (GyrA) antibodies. Under these conditions, we recovered approximately 1  $\mu\text{g}$  of chromosomal DNA per  $10^9$  untreated cells. We compared levels of GyrA-DNA adducts in samples from *E. coli* MG1655 that had been untreated or treated for 45 min with either of two gyrase in-

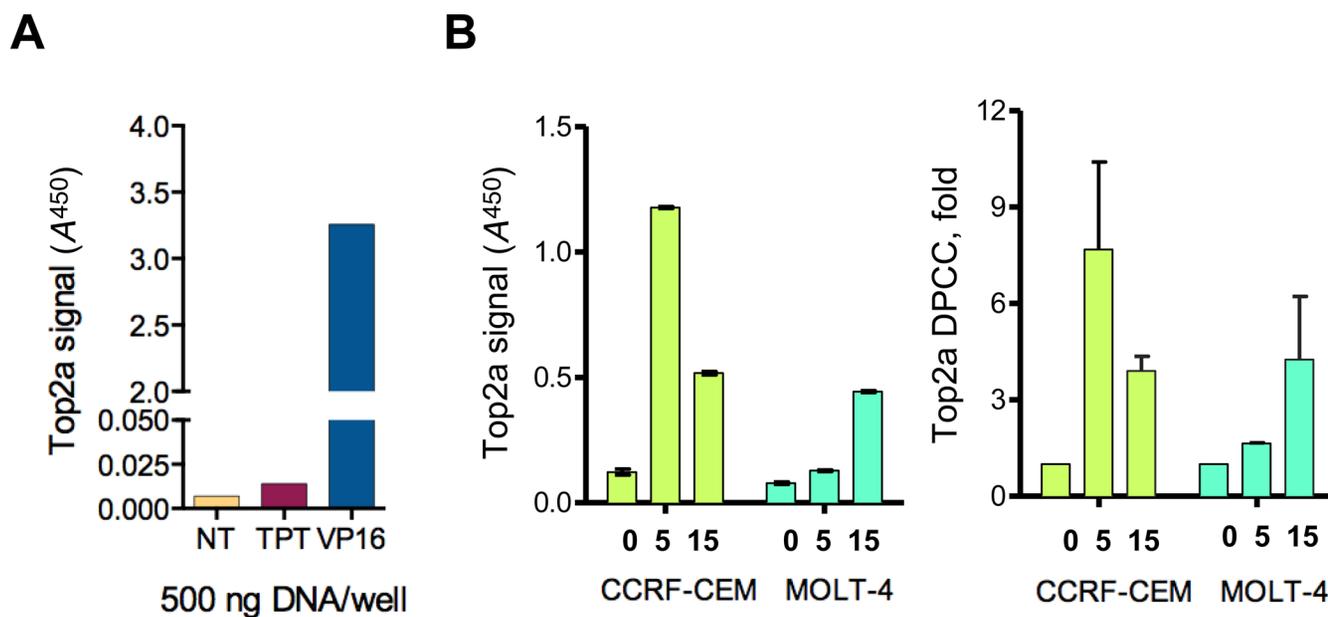


**Figure 5.** Kinetic analysis of Top1 DPCC repair. (A) Comparison of survival of GM639 and HCT116 cells treated with indicated concentrations of CPT for 2 h, washed with fresh media and incubated for 96 h ('Wash-out', left); or treated continuously with indicated concentrations of CPT for 32 h ('Continuous', right). Surviving fraction was calculated relative to the untreated control. Survival assays were performed in triplicate on 5000 cells/well; error bars represent standard deviation. (B) Top1 DPCC levels were assayed in cells incubated with 10  $\mu$ M TPT for 30 min, followed by wash-out and incubation for the indicated amount of time. Assays were performed in triplicate on 20 000 cells/well; error bars represent standard deviation.

inhibitors, nalidixic acid or ciprofloxacin. Both drugs effectively arrested cell growth, as evidenced by cell density (not shown) and decreased DNA yield (Figure 7A). We also observed a substantial loss of DNA viscosity, particularly in the ciprofloxacin-treated sample. GyrA-DNA adducts were quantified by ELISA assay of samples containing 0.2  $\mu$ g DNA, equivalent to  $2 \times 10^8$  cells. The GyrA signal was increased 4-fold in the nalidixic acid-treated samples and 9-fold in the ciprofloxacin-treated samples, relative to samples containing DNA from untreated cells (Figure 7B). The ability of the ELISA-based RADAR assay to detect gyrase-DNA adducts extends its potential use to applications in infectious disease.

## DISCUSSION

The ELISA-based RADAR assay that we describe here provides an approach for isolation, identification and quantification of DNA-protein adducts that is sensitive and compatible with high throughput. This approach should be readily applicable to discovery of better drugs or drug combinations that trap proteins known to form covalent complexes with DNA. We have demonstrated applicability to three different bacterial and human topoisomerases, Top1, Top2 and bacterial DNA gyrase. It should be possible to extend the ELISA-based RADAR assay to other proteins that form covalent DNA adducts.



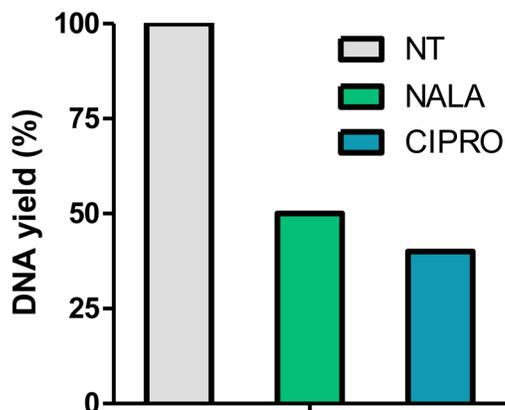
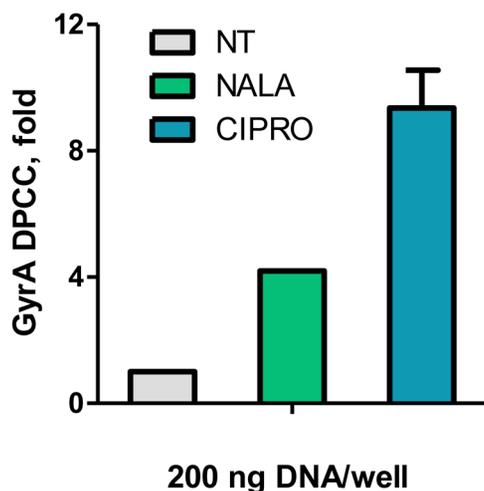
**Figure 6.** Detection of Top2a–DNA adducts by ELISA-based RADAR assay. (A) Specificity of detection by ELISA assay using an anti-Top2a antibody. ELISA assays were performed with DNA isolates of CCRF-CEM cells treated with cognate (VP16) or non-cognate (TPT) topoisomerase poison, and compared to an untreated control (NT). (B) Kinetics of Top2a–DPCC formation. Top2a-DPCC were detected by ELISA-based RADAR of DNA isolates (0.5  $\mu$ g DNA per well) from CCRF-CEM and MOLT4 cells treated with 50 mM VP16 for indicated times and lysed in LS2. Left panel shows averages of replicates of  $A_{450}$  reading from a representative ELISA experiment with anti-Top2a antibodies. Right panel shows averages of fold Top2a-DPCC induction calculated from two ELISA experiments performed on different days. Error bars represent standard deviation.

Three key components of the approach we describe may also have independent uses in other contexts. Silica-assisted DNA precipitation enables DNA to be recovered on the same microplate that was used for cell culture. This can streamline workflow for assays in which reproducible recovery is essential but only a small amount of DNA is needed for analyses. Silica-assisted precipitation has the potential to eliminate some column-based DNA isolation procedures, as well as procedures that use silica filter microplate, where it provides the important advantage of eliminating the filtration step that depends upon a vacuum manifold, and substituting centrifugation. This greatly facilitates adaptation of a protocol to a high-throughput robotic workstation, as vacuum filtration involves costly installation of a robotic gripper, a non-standard part of this equipment.

We have shown that the ELISA-based RADAR assay can quantify adducts in human cells treated with either Top1 or Top2a inhibitors. Sensitivity of detection probably varies among cell types and adducts, but the sensitivity we have demonstrated for two prominent drug targets, human Top1 and Top2a, compares quite favorably with other assays. The DPCC-ELISA detected Top1–DNA adducts in samples containing <50 ng DNA, reproducibly obtainable from cells cultured in 96-well plates, and it detected Top2a–DNA adducts in samples containing <1  $\mu$ g DNA. The DPCC-ELISA step should be applicable to assays of nucleic acid adducts independent of the method used to isolate these complexes, provided that Benzonase digestion can be used to eliminate most nucleic acids to facilitate adsorption to the plastic ELISA plate.

We have not optimized sensitivity of the ELISA step for protein detection, but it should be possible to do so by several simple modifications. For example, a 384-well microplate will produce a signal comparable to that of a 96-well microplate using one-fourth as much adsorbed antigen, so use of a 384-well plate would permit a 4-fold signal increase (or corresponding reduction in antigen). Another simple strategy for signal increase would be to use biotinylated antibodies for either primary or secondary detection, then decorate these with multivalent streptavidin-HRP conjugates prior to addition of HRP substrate. It should also be possible to increase signal by expression of recombinant tagged proteins, which will increase the level of adducts and provide a well-defined epitope for recognition by high affinity commercial antibodies.

The chaotropic lysis protocol upon which the RADAR assay (1) was based was first developed for proteomic analyses (21). The further refinements reported here, notably lysis in a solution containing high salt, chaotrope and detergents followed by silica-assisted isopropanol precipitation and Benzonase digestion, will increase the stringency of purification, and yield samples even more readily analyzed by mass spectroscopy. Thus this approach will be suitable not only for detection of proteins known to be targets of specific drugs, but also for identification of new proteins that form covalent DNA adducts. This will be useful for defining enzymatic mechanisms. It may also enable discovery of new classes of drugs that trap these intermediates and that will be useful in treatment of cancer or infectious disease.

**A****B**

**Figure 7.** Detection of GyrA–DNA adducts by ELISA-based RADAR assay. DNA and DNA–protein adducts were recovered and GyrA quantified in samples containing 0.2  $\mu$ g DNA from untreated *E. coli* (gray) or *E. coli* treated with nalidixic acid (100  $\mu$ g/ml) or ciprofloxacin (20  $\mu$ g/ml) for 45 min. (A) Relative DNA recovery expressed as percentage of untreated sample. (B) Averages of fold GyrA–DNA DPCC induction calculated from triplicates of a representative ELISA experiment with anti-GyrA antibodies. Error bars represent standard deviation.

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## REFERENCES

- Kiiianitsa, K. and Maizels, N. (2013) A rapid and sensitive assay for DNA–protein covalent complexes in living cells. *Nucleic Acids Res.*, **41**, e104.
- Li, T.K. and Liu, L.F. (2001) Tumor cell death induced by topoisomerase-targeting drugs. *Annu. Rev. Pharmacol. Toxicol.*, **41**, 53–77.
- Pommier, Y., Leo, E., Zhang, H. and Marchand, C. (2010) DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem. Biol.*, **17**, 421–433.
- Champoux, J.J. (2001) DNA topoisomerases: structure, function, and mechanism. *Annu. Rev. Biochem.*, **70**, 369–413.
- Khodyreva, S.N., Prasad, R., Ilina, E.S., Sukhanova, M.V., Kutuzov, M.M., Liu, Y., Hou, E.W., Wilson, S.H. and Lavrik, O.I. (2010) Apurinic/aprimidinic (AP) site recognition by the 5'-dRP/AP lyase in poly(ADP-ribose) polymerase-1 (PARP-1). *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 22090–22095.
- Roberts, S.A., Strande, N., Burkhalter, M.D., Strom, C., Havener, J.M., Hasty, P. and Ramsden, D.A. (2010) Ku is a 5'-dRP/AP lyase that excises nucleotide damage near broken ends. *Nature*, **464**, 1214–1217.
- Barker, S., Weinfeld, M., Zheng, J., Li, L. and Murray, D. (2005) Identification of mammalian proteins cross-linked to DNA by ionizing radiation. *J. Biol. Chem.*, **280**, 33826–33838.
- Hazra, T.K., Das, A., Das, S., Choudhury, S., Kow, Y.W. and Roy, R. (2007) Oxidative DNA damage repair in mammalian cells: a new perspective. *DNA Repair (Amst.)*, **6**, 470–480.
- Tubbs, J.L., Pegg, A.E. and Tainer, J.A. (2007) DNA binding, nucleotide flipping, and the helix–turn–helix motif in base repair by O6-alkylguanine–DNA alkyltransferase and its implications for cancer chemotherapy. *DNA Repair (Amst.)*, **6**, 1100–1115.
- Interthal, H., Chen, H.J. and Champoux, J.J. (2005) Human Tdp1 cleaves a broad spectrum of substrates, including phosphoamide linkages. *J. Biol. Chem.*, **280**, 36518–36528.
- Gao, Z., Maloney, D.J., Dedkova, L.M. and Hecht, S.M. (2008) Inhibitors of DNA polymerase beta: activity and mechanism. *Bioorg. Med. Chem.*, **16**, 4331–4340.
- Haracska, L., Prakash, L. and Prakash, S. (2003) A mechanism for the exclusion of low-fidelity human Y-family DNA polymerases from base excision repair. *Genes Dev.*, **17**, 2777–2785.
- Tse-Dinh, Y.C. (2009) Bacterial topoisomerase I as a target for discovery of antibacterial compounds. *Nucleic Acids Res.*, **37**, 731–737.
- Nitiss, J.L., Soans, E., Rogojina, A., Seth, A. and Mishina, M. (2012) Topoisomerase assays. *Curr. Protoc. Pharmacol.*, **3**, 3.3.1–3.3.27.
- Shoukamy, M.I., Nakano, T., Ohshima, M., Hirayama, R., Uzawa, A., Furusawa, Y. and Ide, H. (2012) Detection of DNA–protein crosslinks (DPCs) by novel direct fluorescence labeling methods: distinct stabilities of aldehyde and radiation-induced DPCs. *Nucleic Acids Res.*, **40**, e143.
- Kanagasabai, R., Liu, S., Salama, S., Yamasaki, E.F., Zhang, L., Greenchurch, K.B. and Snapka, R.M. (2009) Ubiquitin-family modifications of topoisomerase I in camptothecin-treated human breast cancer cells. *Biochemistry*, **48**, 3176–3185.
- Vogelstein, B. and Gillespie, D. (1979) Preparative and analytical purification of DNA from agarose. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 615–619.
- Marko, M.A., Chipperfield, R. and Birnboim, H.C. (1982) A procedure for the large-scale isolation of highly purified plasmid DNA using alkaline extraction and binding to glass powder. *Anal. Biochem.*, **121**, 382–387.
- Boom, R., Sol, C.J., Salimans, M.M., Jansen, C.L., Wertheim-van Dillen, P.M. and van der Noordaa, J. (1990) Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.*, **28**, 495–503.
- Elphinstone, M.S., Hinten, G.N., Anderson, M.J. and Nock, C.J. (2003) An inexpensive and high-throughput procedure to extract and purify total genomic DNA for population studies. *Mol. Ecol. Notes*, **3**, 317–320.
- Barker, S., Murray, D., Zheng, J., Li, L. and Weinfeld, M. (2005) A method for the isolation of covalent DNA–protein crosslinks suitable for proteomics analysis. *Anal. Biochem.*, **344**, 204–215.
- Pfister, T.D., Reinhold, W.C., Agama, K., Gupta, S., Khin, S.A., Kinders, R.J., Parchment, R.E., Tomaszewski, J.E., Doroshow, J.H. and

- Pommier, Y. (2009) Topoisomerase I levels in the NCI-60 cancer cell line panel determined by validated ELISA and microarray analysis and correlation with indenoisoquinoline sensitivity. *Mol. Cancer Ther.*, **8**, 1878–1884.
23. Divi, R.L., Beland, F.A., Fu, P.P., Von Tungeln, L.S., Schoket, B., Camara, J.E., Ghei, M., Rothman, N., Sinha, R. and Poirier, M.C. (2002) Highly sensitive chemiluminescence immunoassay for benzo[a]pyrene-DNA adducts: validation by comparison with other methods, and use in human biomonitoring. *Carcinogenesis*, **23**, 2043–2049.
24. Schild, L.J., Divi, R.L., Beland, F.A., Churchwell, M.I., Doerge, D.R., Gamboa da Costa, G., Marques, M.M. and Poirier, M.C. (2003) Formation of tamoxifen-DNA adducts in multiple organs of adult female cynomolgus monkeys dosed with tamoxifen for 30 days. *Cancer Res.*, **63**, 5999–6003.
25. Georgiadis, P., Kovacs, K., Kaila, S., Makedonopoulou, P., Anna, L., Poirier, M.C., Knudsen, L.E., Schoket, B. and Kyrtopoulos, S.A. (2012) Development and validation of a direct sandwich chemiluminescence immunoassay for measuring DNA adducts of benzo[a]pyrene and other polycyclic aromatic hydrocarbons. *Mutagenesis*, **27**, 589–597.
26. Kremer, D., Metzger, S. and Kolb-Bachofen, V. (2012) Quantitative measurement of genome-wide DNA methylation by a reliable and cost-efficient enzyme-linked immunosorbent assay technique. *Anal. Biochem.*, **422**, 74–78.
27. Sacho, E.J. and Maizels, N. (2011) DNA repair factor MRE11/RAD50 cleaves 3'-phosphotyrosyl bonds and reseals DNA to repair damage caused by topoisomerase I poisons. *J. Biol. Chem.*, **286**, 44945–44951.
28. Hamilton, N.K. and Maizels, N. (2010) MRE11 function in response to topoisomerase poisons is independent of its function in double-strand break repair in *Saccharomyces cerevisiae*. *PLoS One*, **5**, e15387.
29. Heck, M.M., Hittelman, W.N. and Earnshaw, W.C. (1988) Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 1086–1090.
30. Gao, H., Huang, K.C., Yamasaki, E.F., Chan, K.K., Chohan, L. and Snapka, R.M. (1999) XK469, a selective topoisomerase IIbeta poison. *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 12168–12173.
31. Aedo, S. and Tse-Dinh, Y.C. (2012) Isolation and quantitation of topoisomerase complexes accumulated on *Escherichia coli* chromosomal DNA. *Antimicrob. Agents Chemother.*, **56**, 5458–5464.
32. Champoux, J.J. (1981) DNA is linked to the rat liver DNA nicking-closing enzyme by a phosphodiester bond to tyrosine. *J. Biol. Chem.*, **256**, 4805–4809.