Measurement of Drug-Stabilized Topoisomerase II Cleavage Complexes by Flow Cytometry

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The poisoning of Topoisomerase II (Top2) has been found to be useful as a therapeutic strategy for the treatment of several tumors. The mechanism of Top2 poisons involves a drug-mediated stabilization of a Top2-DNA complex, termed Top2 cleavage complex (Top2cc), which maintains a 5′ end of DNA covalently bound to a tyrosine from Top2 through a phosphodiester group. Drug-stabilized Top2cc leads to Top2-linked-DNA breaks, which are believed to mediate their cytotoxicity. Several time-consuming or cell type-limiting assays have been used in the past to study drug-stabilized Top2cc. Here, we describe a flow cytometry-based method that allows a rapid assessment of drug-induced Top2cc, which is suitable for high throughput analysis in almost any kind of human cell. The analyses of the drug-induced Top2cc in the cell cycle context and the possibility to track its removal are additional benefits from this methodology. © 2017 by John Wiley & Sons, Inc.

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INTRODUCTION

Accumulating evidence through the last two decades points to drug-mediated stabilization of Topoisomerase II cleavage complexes (Top2cc) as the main cause of persistent DNA damage and cytotoxicity induced by Top2 poisons (Bandele & Osheroff, 2008; Nitiss & Beck, 1996). This fact has encouraged the development of new Top2 targeting agents and has aroused the interest for studying the cellular mechanisms for the removal of these complexes. As Top2 enzyme has structural roles in addition to their catalytic activity in the cellular nucleus, several biochemical and immunological methods have been designed to detect the catalytically active fraction of the enzyme (Agostinho et al., 2004; Cowell, Tilby, & Austin, 2011; Subramanian, Furbee, & Muller, 2001).

In this unit, we describe an easy and quick protocol to assess drug-stabilized Top2cc in different human cell types. By using a versatile heparin- and detergent-based extraction protocol, the removal of unbound- or weakly-bound Top2 from DNA is achieved. The immunolabeling of a specific isoform of drug-stabilized Top2cc is then performed and the cell cycle context co-analyzed by the DNA counterstaining.

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BASIC PROTOCOL

DETECTION OF STABILIZED TOP2 CLEAVAGE COMPLEXES IN HUMAN CELLS FOLLOWING EXPOSURE TO TOP2 POISONS

Spontaneous Top2 cleavage complexes are short-lived intermediate structures formed through the catalytic cycle of the enzyme during their regulatory role of the topological state of DNA. Spontaneous or drug-induced failures interrupt its catalysis, increasing the half-life of these complexes and the likelihood of resulting in a persistent DNA double-stranded break. As both α and β isoforms of human Top2 may be targeted by Top2 poisons during different metabolic processes with dissimilar consequences, interest on the removal of these covalently bound structures has emerged.

Despite the availability of numerous useful methods for studying Top2 cleavage complex formation and removal, there is an urgent need for a high-throughput compatible assay to simultaneously test several experimental conditions with uniform performance across samples of different origin. The method described here is intended to satisfy these goals and was successfully used and validated by the authors with several human cell lines and blood cell samples.

Materials

HL-60 cell line (ATCC, #CCL-640)
Etoposide (Sigma, cat. no. 33419–42-0)
Ice
1× PHEM buffer (see recipe)
Phenylmethanesulfonyl fluoride (PMSF; Sigma)
2× Extraction buffer (see recipe)
4% paraformaldehyde solution (in PBS)
1× Phosphate-buffered saline (PBS; Gibco, cat. no. 10010031)
Blocking buffer (see recipe)
Rabbit anti-Top2α (H-231, Santa Cruz Biotechnology) or mouse anti-Top2β (H-8, Santa Cruz Biotechnology) primary antibodies
Alexa Fluor 488-conjugated goat anti-rabbit (Life Technologies) or DyLight 488-conjugated goat anti-mouse (Thermo Scientific) secondary antibodies
RNase A solution
Propidium iodide solution
RPMI 1640 (with phenol red, sodium bicarbonate, and L-glutamine)
Fetal bovine serum

Micropipettes
37°C, 5% CO_2 incubator
1.5-ml tubes
Microcentrifuge
Rotating microtube mixer
Flow cytometer with at least a blue laser (488 nm)
Cell Quest software or any other analysis software

Prepare the cells

1. Using a micropipet, seed the cells at a density of 0.5–1 × 10^6 cells/treatment 24 hr before the experiments in RPMI 1640 medium containing 10% fetal bovine serum. Incubate the cells in a 37°C, 5% CO_2 incubator.
2. Treat the cells with vehicle or Etoposide (ETO) for 1 hr.

   It is strongly recommended to add an additional sample as a no extracted control to evaluate the total Top2α or Top2β content.

   The authors used 10 μg/ml of ETO. The ETO-stabilized Top2cc are detected as soon as 15 min post-treatment, although lower incubation times were not assayed. If other drugs are going to be assayed, a range of doses should be analyzed.

3. Harvest the cells by transferring them into a 1.5-ml tube and keep on ice.

Perform protein extraction

4. Centrifuge the cells for by 5 min at 224 × *g*, 4°C.
5. Discard the supernatant and resuspend the cells in 500 µl of cold PHEM buffer containing 2 mM PMSF.
6. Add an equal volume (500 µl) of 2× extraction buffer and mix the tubes gently under a rotating microtube mixer for 5 min at 4°C.

   In the authors´ experience, the extraction buffer can be stored at 4°C for more than a week. However, it is strongly recommended to add the unfractionated heparin to the extraction buffer on the same day of the experiment.

   It is strongly recommended to perform the extraction by 5 min as a shorter extraction time, in the authors' experience, did not result in a complete removal of non-covalently bound Top2.

7. Add 325 µl of 4% paraformaldehyde solution per tube and incubate them for 30 min at room temperature.

   The authors suggest preparing fresh paraformaldehyde as the reagent is unstable in solution.

8. Centrifuge the cells for 5 min at 500 × *g*, room temperature, and discard the supernatant.
9. Resuspend the cells in 1 ml PBS and centrifuge the cells for 5 min at 500 × *g*, room temperature. Discard the supernatant.
10. Repeat step 9.

Top2 labeling

11. Add 50 µl blocking buffer per tube and resuspend the cells. Incubate the cells in blocking buffer by 1 hr at room temperature.
12. Centrifuge the cells for 5 min at 500 × *g*, room temperature, and discard the supernatant.
13. Dilute the anti-Top2α or the anti-Top2β primary antibody 1:200 in blocking buffer.

    The dilutions for anti-Top2α or anti-Top2β antibodies were determined for clones from Santa Cruz Biotechnology H-231 and H-8, respectively. If other antibodies are going to be assayed, the dilutions should be determined empirically.

14. Add 50 µl of the diluted primary antibody solution per sample and incubate 2 hr at room temperature.
15. Centrifuge the cells for 5 min at 500 × *g*, room temperature, and discard the supernatant. Resuspend the cells in 1 ml PBS and centrifuge again for 5 min at 500 × *g*, room temperature. Discard the supernatant.
16. Dilute the appropriate secondary antibody 1:100 in blocking buffer.

    The dilutions for the Alexa Fluor488-conjugated anti-rabbit or the DyLight488-conjugated anti-mouse antibodies were determined for clones from Life Technologies and Thermo Scientifics, respectively. If a different secondary antibody is going to be used, the dilution should be determined empirically.

17. Add 50 µl of the diluted secondary antibody solution to each sample and incubate 1 hr at room temperature in the dark.

18. Add 950 µl PBS per sample and centrifuge for 5 min at 500 × *g*, room temperature. Discard the supernatant.

DNA labeling

19. Resuspend the cells in 300 µl PBS.

20. Add 5 µl of a 12 mg/ml RNase A solution and incubate for 30 min at room temperature.

21. Add 20 µl/ml propidium iodide solution and incubate 15 min at room temperature.

Flow cytometry

22. Turn on the flow cytometer and verify operation of lasers and fluidics.

23. Run a control sample and set the forward versus side scatter gains.

24. Construct two-parameter dot plots for the combination of fluorochromes. Add an FL3-A versus FL3-W dot plot to discriminate doublets. Set voltages for each detector to ensure that fluorescent signals are on scale.

25. Analyze the samples.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes.

Blocking buffer, 1 ml

Mix 300 µl of a 10% bovine serum albumin solution, 100 µl of a 5% Triton-X100 solution in phosphate-buffered saline (PBS) and 600 µl of 1× PBS. This solution should be prepared fresh and stored at 4°C. Use the day of the experiment.

Extraction buffer, 2×

The 2× extraction buffer is a PHEM buffer containing 1% Triton-X100 and 400 U/ml of unfractionated heparin. The heparin should be added in the day of the experiment to the 2× extraction buffer. This solution should be prepared fresh and stored at 4°C for no more than a week.

PHEM buffer, 100 ml

Mix 3 ml of 1 M HEPES, 10 ml of 650 mM PIPES, 2 ml of 500 mM EDTA, and 200 µl 1 M $MgCl_2$. Add 84.8 ml of distillated water and adjust the pH to 6.9 using a 5 M NaOH solution. PHEM buffer final concentration: 65 mM PIPES, 30 mM HEPES, 10 mM EDTA, 2 mM $MgCl_2$, pH 6.9. Add a 1:100 dilution of a 200 mM PMSF solution to the volume of PHEM buffer to be used in the day. Store up to 2 weeks at 4°C.

COMMENTARY

Background Information

Several different methods have been used for the detection of drug-stabilized Top2cc. Biochemical methods for separating Top2 adducts are very used but are hard and time-consuming; thus, inappropriate for high-throughput analysis. The salt- and detergent-based methods for the extraction of proteins non-covalently bound to DNA have also been widely used during the last decades for fluorescence microscopy (Mirzoeva & Petrini, 2001; Mladenov, Anachkova, & Tsaneva, 2006; Zellweger et al., 2015). Several proteins have been analyzed by this methodology, including Top2 (Agostinho et al., 2004; Cowell et al., 2011). However, the release of histone-free DNA loops by the use of high-salt conditions has been reported in non-adherent growing

cells (Iarovaia, Akopov, Nikolaev, Sverdlov, & Razin, 2005). This fact restricts the use of this method to certain cell types, preventing the standardization of this technique, but also limiting the development of high-throughput analysis approaches. The assay described in this unit is intended to overcome these limitations to analyze drug-stabilized Top2cc in almost any kind of human cells. The procedure uses unfractionated heparin, in place of high-salt containing buffers, for removing non-covalently bound proteins from DNA by competition. Heparin is a natural anionic polymer with an extremely high negative charge density, and it has also been used in the past for removing proteins from DNA in biochemical experiments (Adolphs, Cheng, Paulson, & Laemmli, 1977). The assay described here utilize a PIPES containing buffer, which is considered a good buffer, and has been used previously to reveal sub-nuclear details of the association of Top2 to chromatin and chromosome regions (Agostinho et al., 2004).

The assay described in this unit takes the advantage of using flow cytometry to evaluate several thousands of events quickly but also allowing the visualization of the cell cycle context by using a fluorescent DNA intercalating agent. This is particularly important for Top2α, as its expression and activity are differently regulated throughout the cell cycle.

The assay has the potential of multiplexing with the labeling of a subset of cells to be studied from a mixed population or the labeling of a DNA damage marker, such as γH2AX, to follow up the removal of the drug-induced Top2cc and the appearance of DNA damage signals.

Critical Parameters

Controls

As with any flow cytometry experiment, proper controls are essential. Good negative and positive controls are important to verify the performance of the assay, particularly during early development. A negative or untreated control should be included in all experiments; keeping in mind that a small number of spontaneous Top2cc in control samples are expected, but should be minimal when comparing with a population exposed to a proper concentration of a Top2 poison. Thus, a good positive control is also essential. An internal control for Top2 total content is also recommended. In this control, the proteins should not be extracted but fixed and then permeabilized. This internal control should always show higher levels of Top2 signals, and will allow detecting any problem during protein extraction with the other extracted samples.

Cell preparation and protein extraction

The results are always better when cells are healthy and in good physiological conditions. Actively growing cells are ideal for assessing drug-stabilized Top2cc involving α isoform. In this sense, non-cycling cells can show low levels of Top2cc formation. Care should be taken to maintain the harvested cells in cold solutions while handling before the fixation step. This is to avoid the cellular removal of Top2cc.

The presence of PMSF during the protein extraction step is recommended to prevent proteases-mediated Top2 degradation. The use of a microtube rotating mixer is also important to obtain a similar and efficient extraction of proteins between samples. The temperature at 4°C during this process diminishes the unintended proteolysis of Top2cc.

In the authors' experience, the incubation time with unfractionated heparin is critical. A reduced incubation time results in a partial removal of Top2, but an extensive one may result in the release of DNA loops and the aggregation of the cells.

Top2 immunolabeling

The authors' protocol utilizes commercially available antibodies for the detection of drug-stabilized Top2α or Top2β cleavage complexes. These antibodies have proven to work properly for detecting Top2 in fluorescence microscopy and flow cytometry experiments. If different antibodies are going to be assayed, it should be checked they are suitable for this approach; and both primary and secondary antibodies should be titrated. Optimal incubation times and blocking buffers should also be determined empirically.

In the authors' experience, good results are obtained when counterstaining DNA with propidium iodide. However, many other fluorescent dyes can be used according to the spectral compatibilities of the fluorescent dyes and the number of lasers of the flow cytometer to be used.

Anticipated Results

Representative dot plots from positive and negative controls of HL-60 cells that were heparin- and detergent-extracted and then immunolabeled for Top2αcc and

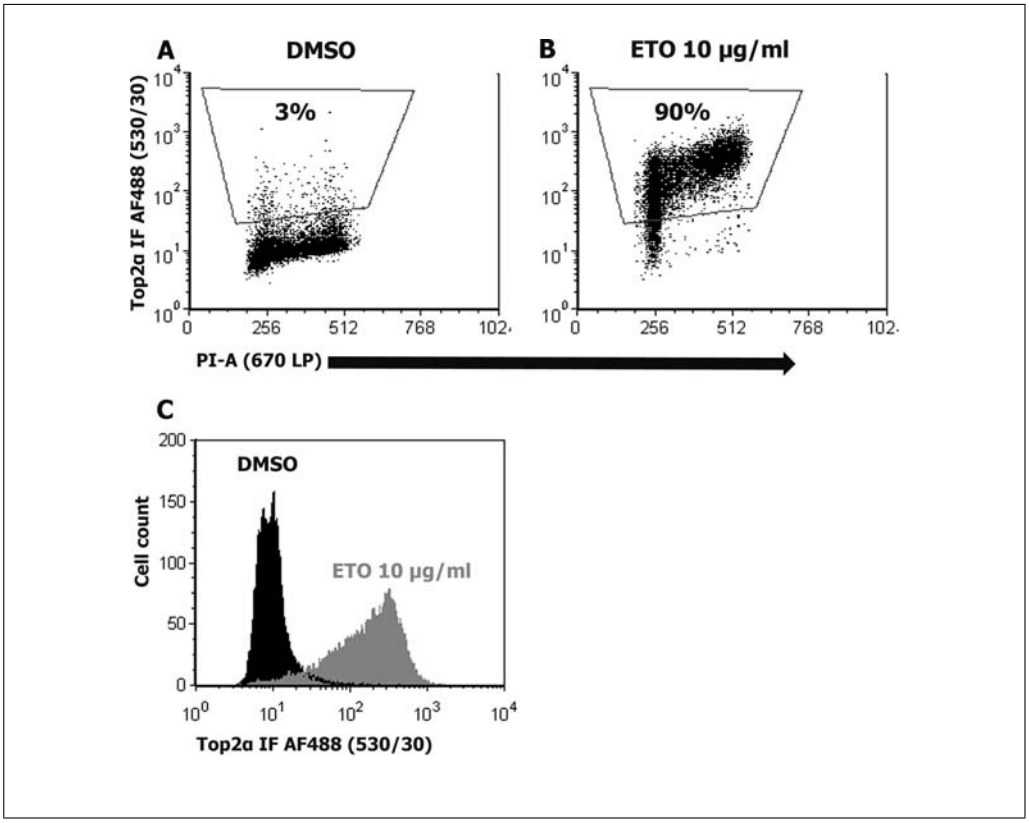

Figure 7.48.1 Etoposide-stabilized Top2α cleavage complexes measurement by flow cytometry. HL-60 cells have been treated by 1 hr with vehicle (DMSO 0.5%) or etoposide (ETO; 10 μg/ml) and then heparin- and detergent-extracted and immunolabeled for Top2α. Following the identification of the cell population and the exclusion of doublets in a FL3-A versus FL3-W dot plot, daughter dot plots were generated to detect stabilized Top2αcc (*y*-axis) versus DNA content (*x*-axis). (**A**) Represents a negative control sample where spontaneously generated Top2αcc are shown. (**B**) Represents a positive control sample where ETO-stabilized Top2αcc are shown. (**C**) represents an overlapped histogram from the negative and positive controls shown in A and B, respectively. Abbreviations: IF = Intensity of fluorescence. PI = Propidium iodide.

counterstained with propidium iodide are depicted in Figure 7.48.1. Depending on the treatment and the proliferation status, a positive population separated from the negative one, should be easily recognized. The negative control is useful to identify the appropriate boundaries necessary to define the positive population. In addition, an overlapped histogram can also be used to analyze the median intensity of fluorescence of the positive and negative controls or to define a marker for the positive population.

In Figure 7.48.2, representative dot plots are shown for the immunolabeling of Top2βcc. A similar strategy as described for Top2αcc can be used to define the appropriate boundaries of the positive population. An overlapped histogram to analyze the median intensities of fluorescence can be performed but it may also serve to identify the positive populations. In both cases, Top2α or Top2β immunolabeling, the positive control is expected to show a higher percentage of positive cells and an increased median intensity of fluorescence.

Time Considerations

This assay is a relatively easy procedure; and thus, compatible with a high-throughput analysis. Depending on the number of samples to be handled, it can be performed in a single working day. In the authors´ experience, when too many samples are going to be used, an additional day may be required. The procedure of doing treatments with Top2 poisons, obtaining the extracted samples and then fixing them requires about 2 hr. An additional 4 to 5 hr should be used for Top2 immunolabeling and DNA counterstaining. The next 1 to 2 hr should be spent to acquire the samples in the flow cytometer and to analyze the results.

The main variation in times should be related to the use of different antibodies or the processing of a lot of samples.

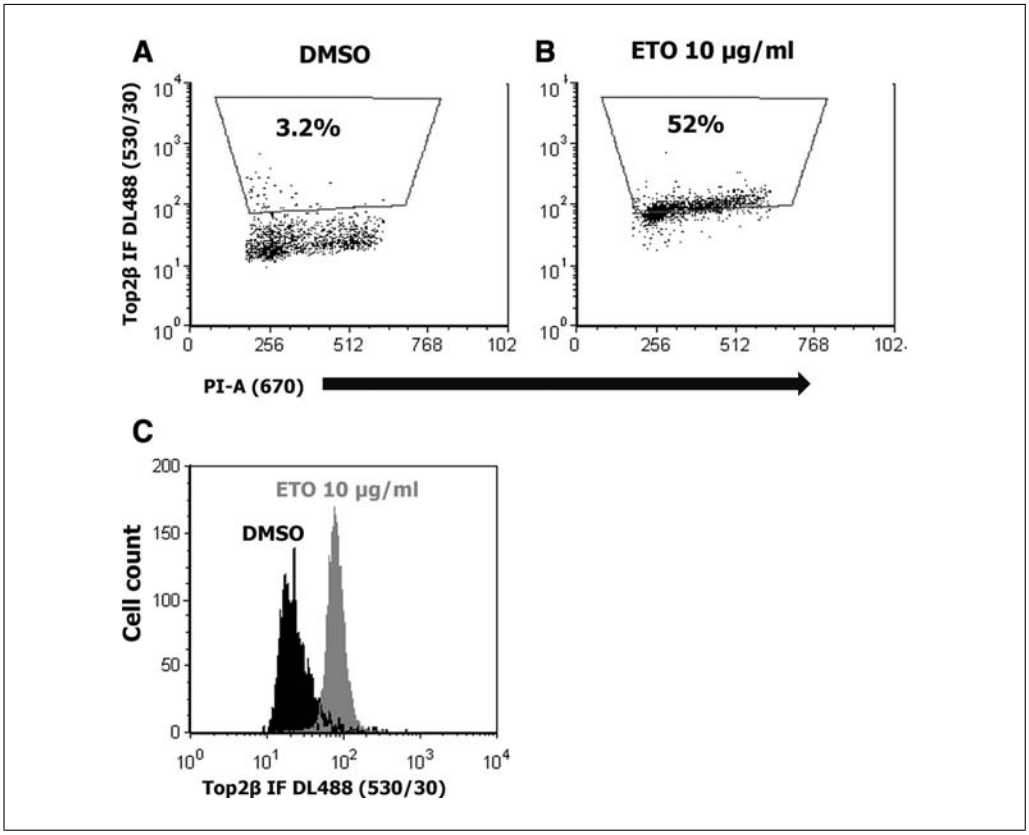

Figure 7.48.2 Etoposide-stabilized Top2β cleavage complexes measurement by flow cytometry. HL-60 cells have been treated by 1 hr with vehicle (DMSO 0.5%) or etoposide (ETO; 10 μg/ml) and then heparin- and detergent-extracted and immunolabeled for Top2β. Following the identification of the cell population and the exclusion of doublets in a FL3-A versus FL3-W dot plot, daughter dot plots were generated to detect stabilized Top2βcc (*y*-axis) versus DNA content (*x*-axis). (**A**) Represents a negative control sample where spontaneously generated Top2βcc are shown. (**B**) Represents a positive control sample where ETO-stabilized Top2βcc are shown. (**C**) Represents an overlapped histogram from the negative and positive controls shown in A and B, respectively. Abbreviations: IF = Intensity of fluorescence. PI = Propidium iodide.

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