

DCTD Standard Operating Procedure (SOP)

Title:	PBMC Protein Extraction for TOP1 Immunoassay			Page 1 of 12
Doc. #:	SOP340703	Revision:	B	Effective Date: 2/20/2013

National Clinical Target Validation Laboratory (NCTVL)

Applied Developmental Directorate

SAIC-Frederick, Inc.

Frederick National Laboratory for Cancer Research

Technical Reviewer:	<u>Yiping Zhang</u>	Date:	<u>2-21-13</u>
NCTVL Approval:	<u>Jiuping Ji</u>	Date:	<u>2-21-13</u>
IQC Approval:	<u>Katherine E. Ferry-Galow</u>	Date:	<u>2-25-13</u>
LHTP Approval:	<u>Ralph E. Parchment</u>	Date:	<u>Feb 25, 2013</u>
DCTD OD Approval:	<u>Joseph E. Tomaszewski</u>	Date:	_____

Change History

Revision	Approval Date	Description	Originator	Approval
--	5/27/2011	New Document. Separate protein extraction steps from SOP340701. Create Batch Record. Assay transfer complete.	YAE	JJ, RJK
A	9/24/2012	Move PhosSTOP to critical reagents. Minimum PBMC number required to run the TOP1 Immunoassay defined. Requirements for digital sample tables added to SOP Step 5.3.	YAE, YZ	JJ
B	2/20/2013	New extraction buffer to be consistent with new biopsy extraction process. Change improves assay dilution linearity and increases maximum protein loaded per well in immunoassay	KFG, JJ	JJ

Please check for revision status of the SOP at

<http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm>

and be sure to use the current version.



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OVERVIEW OF TOP1 IMMUNOASSAY SAMPLE PROCESSING

PBMC Processing

SOP340503: PBMC Collection, Preparation, and Freezing for Protein Extraction	<ul style="list-style-type: none"> Collect PD blood sample from clinical site Purify PBMCs and determine total viable PBMCs/mL
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Ship to Certified Assay Site



SOP340703: PBMC Protein Extraction for TOP1 Immunoassay	<ul style="list-style-type: none"> Extract protein from PBMC cell pellet to a final relative concentration of 1×10^7 cells/mL
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SOP340701: Topoisomerase 1 Immunoassay	<ul style="list-style-type: none"> Perform ELISA with clinical samples, rTOP1 standards, and controls Using Tecan Microplate reader, determine relative signal of all samples
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SOP340704: TOP1 Immunoassay Quality Control, Data Analyses, and Reporting	<ul style="list-style-type: none"> Determine the Top1 concentration in all samples and apply quality control standards to verify utility of assay Prepare a Clinical Sample Data Report for each set of unknown samples and send to the clinical protocol Principal Investigator
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Tumor Biopsy Processing

SOP340507: Tumor Frozen Needle Biopsy Collection and Handling	<ul style="list-style-type: none"> Collect fresh needle biopsy from clinical site
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Ship to Certified Assay Site



SOP340702: Biopsy Protein Extraction for TOP1 Immunoassay	<ul style="list-style-type: none"> Extract protein from tumor biopsy Determine total protein concentration for all samples
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SOP340701: Topoisomerase 1 Immunoassay	<ul style="list-style-type: none"> Perform ELISA with clinical samples, rTOP1 standards, and controls Using Tecan Microplate reader, determine relative signal of all samples
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SOP340704: TOP1 Immunoassay Quality Control, Data Analyses, and Reporting	<ul style="list-style-type: none"> Determine the Top1 concentration in all samples and apply quality control standards to verify utility of assay Prepare a Clinical Sample Data Report for each set of unknown samples and send to the clinical protocol Principal Investigator
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1.0 PURPOSE

To standardize the method for preparing lysates of peripheral blood mononuclear cells (PBMC) to enable quantification of topoisomerase 1 (TOP1) levels with an enzyme-linked immunosorbent assay (ELISA) in pharmacodynamic (PD) studies of TOP1 inhibitors.

To date, NCI and SAIC-F have not been able to measure changes in Top1 levels in PBMCs that would provide a surrogate response of drug effect compared to Top1 levels measured in tumor biopsy samples.

2.0 SCOPE

This procedure applies to all personnel involved in the use of the TOP1 as a PD marker during clinical trials and in the preparation of samples for the analysis of TOP1 levels by the TOP1 Immunoassay (SOP340701). The goal of the SOP and associated training is to ensure consistency in TOP1 measurement across samples and clinical sites.

3.0 ABBREVIATIONS

CEB	=	Cell Extraction Buffer
DCTD	=	Division of Cancer Treatment and Diagnosis
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
HRP	=	Horse Radish Peroxidase
IA	=	Immunoassay
IQC	=	Internal Quality Control
LHTP	=	Laboratory of Human Toxicology and Pharmacology
NCTVL	=	National Clinical Target Validation Laboratory
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
PBMC	=	Peripheral Blood Mononuclear Cell
PD	=	Pharmacodynamic
PI	=	Protease Inhibitor
PMSF	=	Phenylmethanesulfonyl Fluoride
RT	=	Room Temperature
SDS	=	Sodium Dodecyl Sulfate
SOP	=	Standard Operating Procedure
TOP1	=	Topoisomerase 1

4.0 INTRODUCTION

The TOP1 Immunoassay (SOP340701) has been developed to measure the effect of TOP1 inhibitors on TOP1 levels in a variety of biospecimen types, including PBMCs and tissue/tumor biopsies. An ELISA is used to first capture TOP1 protein from total protein extracts on plates coated with a TOP1 capture monoclonal antibody. The captured protein is then detected using a TOP1 polyclonal antibody detection antibody followed by an HRP-conjugate to allow chemiluminescent readout and quantitation of TOP1 levels.

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5.0 ROLES AND RESPONSIBILITIES

Laboratory Director/Supervisor The Laboratory Director/Supervisor, directs laboratory operations, supervises technical personnel and reporting of findings, and is responsible for the proper performance of all laboratory procedures. The Laboratory Director/Supervisor oversees the personnel who follow the SOPs within the laboratory and is responsible for ensuring the personnel are certified and have sufficient experience to handle clinical samples.

Certified Assay Operator A Certified Assay Operator may be a Laboratory Technician/Technologist, Research Associate, or Laboratory Scientist who has been certified through DCTD training on this SOP. The Certified Assay Operator works under the guidance of the Laboratory Director/Supervisor. This person performs laboratory procedures and examinations in accordance with the current SOP(s), as well as any other procedures conducted by a laboratory, including maintaining equipment and records and performing quality assurance activities related to performance.

- 5.1** It is the responsibility of the Laboratory Director/Supervisor to ensure that all personnel have documented training and qualification on this SOP prior to the actual handling and processing of samples from clinical trial patients. The Laboratory Director/Supervisor is responsible for ensuring the Certified Assay Operator running the SOP has sufficient experience to handle and analyze clinical samples.
- 5.2** The Certified Assay Operator responsible for conducting the assay is to follow this SOP and complete the required tasks and associated documentation. The Batch Record ([Appendix 1](#)) must be completed in *real-time* for each experimental run, with each page *dated and initialed*, and placed with the clinical sample information.
- 5.3** Digital versions of the sample table in the Batch Record (Appendix 1, Sections 2) can be created for logging sample information as long as all column information exactly matches the table in the Batch Record. A copy of the completed, digital sample table must be printed and attached to the Batch Record in order to maintain a complete audit trail.
- 5.4** All responsible personnel are to check the DCTD Biomarkers Web site (<http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm>) to verify that the most recent version of the SOP for the assay is being used.

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6.0 MATERIALS AND EQUIPMENT REQUIRED

- 6.1** Pipettors (200-1000 μ L, 50-200 μ L, 2-20 μ L) and tips
- 6.2** Electronic pipette
- 6.3** 10- and 25-mL pipettes, sterile, individually wrapped (Fisher Scientific, Cat#:13-675-20 and 13-668-2)
- 6.4** 1.5-mL Sarstedt o-ring screw cap, conical tubes (Fisher Scientific, Cat#: 72.692.005)
- 6.5** 50-mL polypropylene tubes (Becton Dickinson, Cat#: 352098)
- 6.6** Printable microcentrifuge tube labels
- 6.7** 81-place freezer storage boxes (Fisher Scientific, Cat#: 12-565-182)
- 6.8** Ice bucket
- 6.9** UltraPure DNase/RNase-free distilled water (e.g., Invitrogen, Cat#: 10977-015) or Milli-Q water
- 6.10** Protease Inhibitor Cocktail (Sigma-Aldrich, Cat#: P-2714 or Roche, Cat#: 11697498001)
- 6.11** Phenylmethanesulfonyl fluoride solution, 0.1 M (PMSF; Sigma-Aldrich, Cat#: 93482-50ML-F)
- 6.12** Tris, ultra pure (e.g., MP Biomedicals, Cat#: 04819620 or 04819623)
- 6.13** Sodium chloride, ReagentPlus grade (e.g., Sigma-Aldrich, Cat#: S9625)
- 6.14** Glycerol, 100% w/v (e.g., Sigma-Aldrich, Cat#: G5516)
- 6.15** EDTA, 0.5 M, pH 8.0 (e.g., Boston BioProducts, Cat#: BM-150)
- 6.16** Magnesium chloride, anhydrous (e.g., Sigma-Aldrich, Cat#: M8266)
- 6.17** β -Glycerol phosphate disodium salt, pentahydrate (e.g., Sigma-Aldrich, Cat#: 50020)
- 6.18** Sodium fluoride, ACS grade (e.g., Sigma-Aldrich, Cat#: 201154)
- 6.19** Triton X-100, non-ionic, aqueous solution, 10% w/v, stored according to manufacturer's direction (e.g., Roche Applied Science, Cat#: 11332481001)
- 6.20** Liquid nitrogen or dry ice/ethanol bath
- 6.21** Sorvall Fresco centrifuge, refrigerated (Fisher Scientific)
- 6.22** Vortex Genie 2 (Daigger, Cat#: EF3030A)
- 6.23** Ultrasonic processor, 130 watt with 3 mm probe (Cole-Parmer Instruments, Cat#: EW-04714-52)
- 6.24** -20°C and -80°C freezer
- 6.25** PBMC frozen cell pellets prepared following SOP340503 (PBMC Sample Collection, Preparation, and Freezing for Protein Extraction)

* If instruments and/or reagents differ from those specified above, the Certified Assay Laboratory performing the assay must prove their comparability or equivalence to those recommended using the manufacturer's specifications and experimental validation data.

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7.0 OPERATING PROCEDURES

7.1 All reagents for an individual assay are to be prepared for use in one experimental run, and only in the amounts required for the specific assay. All excess reagents are to be discarded following appropriate safety procedures. Process a single patient's **batched** samples to ensure consistent sample handling.

7.2 Record the name and certification number of the Certified Assay Operator and the facility running the SOP in the Batch Record (Appendix 1). Include reference clinical protocol number(s), if applicable.

7.3 Record equipment serial numbers that will be used in the assay in the Batch Record (Appendix 1, Section 1A) and prepare the reagents outlined in the Batch Record (Appendix 1, Section 1B).

Note: Do not prepare the CEB with PI cocktail and PMSF (**with** PIs) until noted in the SOP.

7.4 Cell Lysis

7.4.1 Fill in the Sample Information Table in the Batch Record (Appendix 1, Section 2A) with the Sample ID and starting PBMC cell number for each PBMC vial. Samples will have either 3.0×10^6 or 1.5×10^6 PBMCs/pellet based on sample preparation in SOP340503.

7.4.1.1 The Sample ID should include the CTEP protocol number followed by a unique patient identifier and a sequential specimen ID (NCI blood collections for PD sampling are series 300).

7.4.2 Record the total volume of CEB (**with** PIs) needed for each sample in the Sample Information Table in the Batch Record (Appendix 1, Section 2A); 100 μ L of CEB (**with** PIs) is added per 1×10^6 PBMCs.

7.4.2.1 **Do not use** samples with $< 0.3 \times 10^6$ PBMCs for the TOP1 Immunoassay. A sample containing 0.3×10^6 PBMCs would have been prepared as a deviation in SOP340503 and could be resuspended in 30 μ L to create a 1×10^7 PBMCs/mL solution; this sample would only be sufficient to run the TOP1 Immunoassay once.

7.4.2.2 **Do not** prepare samples at $< 1 \times 10^7$ PBMCs/mL for the TOP1 Immunoassay.

7.4.3 Using the calculations in Appendix 1, Section 3B and the volumes recorded in the Sample Information Table prepare sufficient buffer for the assay; keep CEB (**with** PIs) on ice.

7.4.4 Place the frozen PBMC pellets on ice and add 100 μ L of the CEB (**with** PIs) per 1×10^6 PBMCs to fresh or frozen cell pellets and record the volume CEB (**with** PIs) used for each sample in the Sample Information Table (Appendix 1, Section 2A). This should yield a relative cell concentration of 1×10^7 PBMCs/mL.

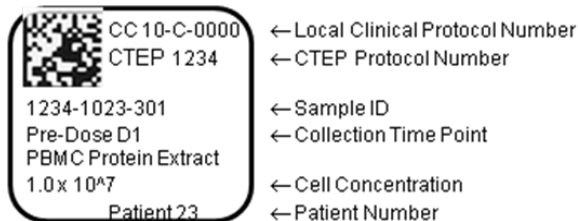
7.4.5 Let the tube stand on ice for 5 min and record the start and stop times for the incubation in the Batch Record (Appendix 1, Section 3). Vortex samples 3 to 5 sec at medium speed (setting 5-6 on Vortex Genie 2); ensure the cell pellet is dislodged and mixing gently.

7.4.6 Sonicate the cells at an output of 02-03 watts for 5 to 10 sec and keep on ice for 15 sec. Repeat up to 3 times as required letting sit on ice between each sonication. Keep the specimen tube on ice while sonicating so samples do not heat up. Record the actual output setting for the sonicator in the Batch Record (Appendix 1, Section 3).

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- 7.4.7** Following sonication, let the tubes stand on ice for 10 to 15 min and record the start and stop times of the incubation in the Batch Record (Appendix 1, Section 3). Vortex for 3 to 5 sec at medium speed (setting 5-6 on Vortex Genie 2).
- 7.4.8** Clarify the lysate by centrifugation at 12,000 x g for 5 min at 2°C to 8°C. Transfer the cleared lysate into a 1.5-mL Sarstedt tube, label as the **stock lysate** tube, and include the relative cells/mL (transfer step may not be necessary as lysate is often clear). Keep **stock lysate** on ice. Discard the original tube with any precipitated material in the appropriate waste container. Example pre-printed label:



- 7.5** If the **stock lysates** will be used within 8 h, store on ice or at 2°C to 8°C.
- 7.6** **Stock lysates** not used immediately for the TOP1 immunoassay can be snap-frozen in liquid nitrogen or a dry ice/ethanol bath and then stored in an 81-place freezer box, batched by patient, at -80°C until analysis. Record the date and time lysates are frozen in the Batch Record (Appendix 1, Section 4).
- 7.7** Review and finalize the Batch Record (Appendix 1) and obtain required signatures. Document ANY and ALL deviations from this SOP in the Batch Record (Appendix 1, Section 5).
- 7.8** The Laboratory Director/Supervisor should review the Batch Record and print and sign their name affirming the data contained within are correct (Appendix 1, Section 6).

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APPENDIX 1: BATCH RECORD

NOTE: Record times using **military time** (24-h designation); for example, specify 16:15 to indicate 4:15 PM.

Certified Assay Operator: _____

Certification Number: _____

Facility/Laboratory Running SOP: _____

Clinical Protocol Number: _____

Equipment and Preparation of Reagents

A. Equipment

Ultrasonic Processor: Make/Model : _____

Serial #: _____

B. Reagents

Buffers should be prepared based on volumes needed to complete all the steps preparing at least 10% excess volume of buffer to ensure adequate volume to complete the study.

- a. Cell Extraction Buffer (CEB) (**without** PIs): Prepare 500 mL of buffer at a time by adding the following reagents to 350 mL ultrapure DNase/RNase-free water. Once all reagents have been added, adjust volume to 500 mL with additional ultrapure DNase/RNase-free water. Sterile filter and store at 2°C to 8°C for no longer than 3 mo.

Reagent	Molecular Weight/ Concentration	Amount Needed	Final Concentration
Tris	121.14	3028.5 mg	50 mM Tris
NaCl	58.44	8766 mg	300 mM NaCl
Glycerol	100%	50 mL	10% Glycerol
EDTA	0.5 M	3 mL	3 mM EDTA
MgCl ₂	95.22	47.5 mg	1 mM MgCl ₂
β-Glycerol	306.11	3061.2 mg	20 mM β-Glycerol
NaF	41.99	524.75 mg	25 mM NaF
Triton X-100	10%	50 mL	1% Triton

- b. Protease Inhibitor Cocktail Tablets: Dissolve one PI cocktail tablet in 2 mL ddH₂O (25X stock). The 25X stock solution is stable for 1 wk at 2°C to 8°C or 12 wk at -15°C to -25°C. If stored frozen, the material must be prepared as single-use aliquots to prevent repeat freeze-thaw.

Lot#: _____ Expiration Date: _____

- c. PMSE: Manufacturer's stock solution supplied at 100 mM. Label vial with date of receipt from manufacturer; the expiration date should be considered 6 mo after receipt.

Lot#: _____ Expiration Date: _____

BATCH RECORD: INITIALS _____

DATE: _____

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2. **Protein Extraction for PBMC Cell Pellets**

A. Sample Information Table

Sample No.	Sample ID	Lyse Pellet at 1×10^7 PBMCs/mL		Notes
		PBMCs/Pellet	Vol. CEB (with PI) (μ L)	
Ex:	1234-1025-300	3×10^6	300	
Ex:	1234-1025-301	1.5×10^6	150	
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				

B. Calculations for preparation of CEB (with PIs)

Vol. to prepare: (____ # of 3×10^6 vials + 2) * 300 μ L _____ μ L

Vol. to prepare: (____ # of 1.5×10^6 vials + 2) * 150 μ L + _____ μ L

TOTAL VOL. CEB (with PIs) to prepare = _____ μ L

Vol. 25X PI stock: (*TOTAL VOL. CEB* /25; 1X final) _____ μ L PI

Vol. 100 mM PMSF: (*TOTAL VOL. CEB* /100; 1 mM final) + _____ μ L PMSF

Vol. CEB (**without PI**): (*Total Vol. CEB* – Vol. 25X PI stock
 - Vol. 100 mM PMSF) + _____ μ L CEB
(without PI)

***Once PMSF and PI added to CEB (without PI), keep on ice.**

BATCH RECORD: INITIALS _____ DATE: _____

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3. PBMC Cell Lysis

Incubate PBMCs at on ice for 5 min: Start Time: _____ : _____ Stop Time: _____ :

Sonicate tissue at a setting of _____ watts for 10-30 sec; repeat 3 times on ice.

Incubate lysate on ice for 10-15 min: Start Time: _____ : _____ Stop Time: _____ :

4. PBMC Stock Lysate Storage

Cell extract frozen in liquid nitrogen or dry ice/ethanol bath: Date _____ Time _____ :

Sarstedt tubes placed into -80°C storage Date _____ Time _____ :

5. Notes, Including any Deviations From the SOP:

6. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

BATCH RECORD: INITIALS _____ DATE: _____

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Date: ___ / ___ / ___

BATCH RECORD:

INITIALS _____

DATE: _____