

SOP340701: Topoisomerase I Immunoassay

Effective Date: 02/28/2024

Please check for revision status of the SOP at
<http://dctd.cancer.gov/drug-discovery-development/assays/validated-biomarker-assays>
and be sure to use the current version.

TABLE OF CONTENTS

OVERVIEW OF TOP1 IMMUNOASSAY SAMPLE PROCESSING	3
1.0 PURPOSE	4
2.0 SCOPE.....	4
3.0 ABBREVIATIONS	4
4.0 INTRODUCTION.....	5
5.0 ROLES AND RESPONSIBILITIES.....	5
6.0 MATERIALS, CRITICAL REAGENTS, AND EQUIPMENT REQUIRED	6
7.0 OPERATING PROCEDURES	8
APPENDIX 1: PLATE MAP DESIGN.....	18
APPENDIX 2: BATCH RECORD	20

Version History

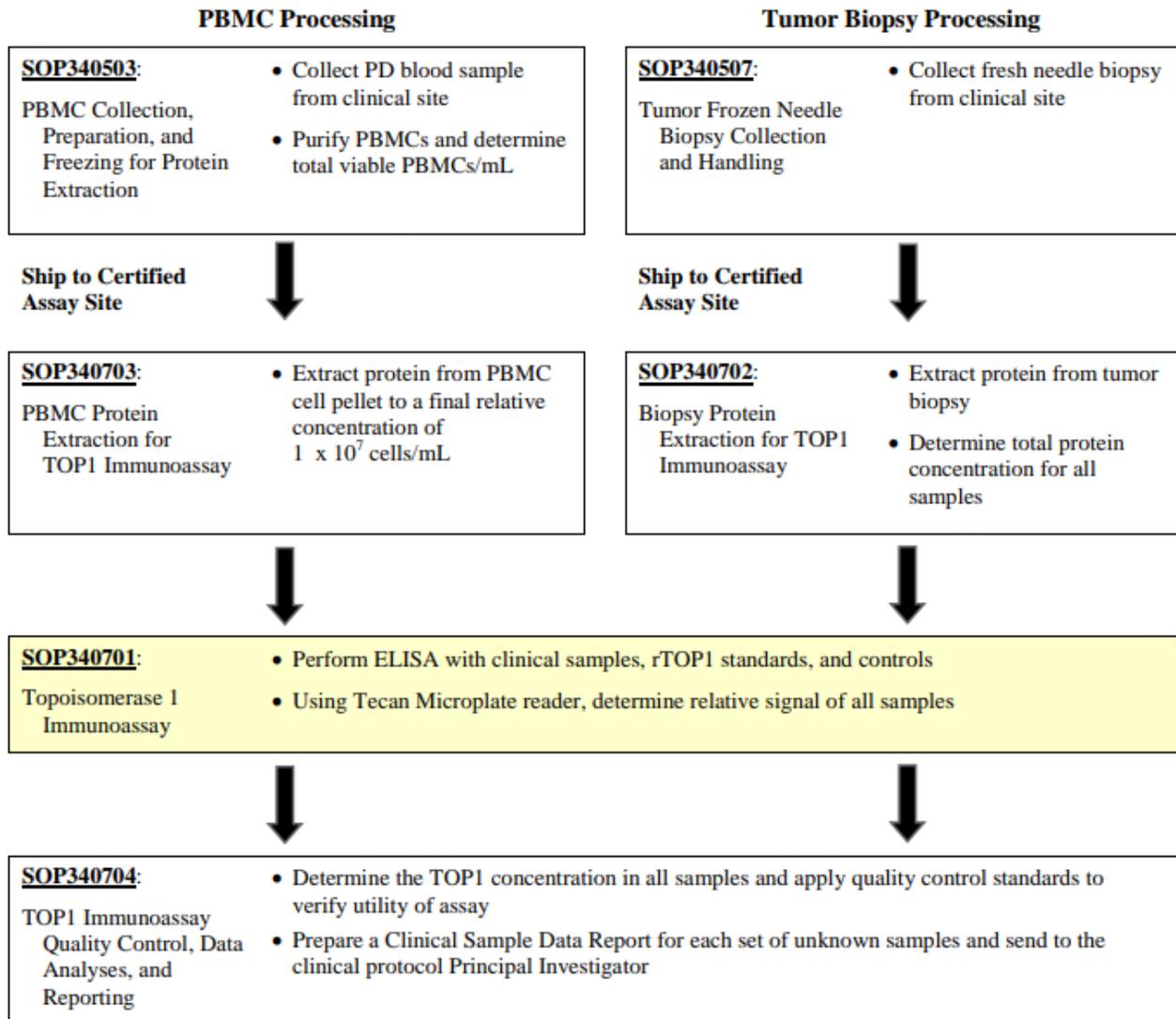
1. Approvals

Technical Reviewer:	<u>Yiping Zhang</u>	Date:	<u>2-28-14</u>
NCTVL Approval:	<u>Jiuping Ji</u>	Date:	<u>3-4-14</u>
IQC Approval:	<u>Katherine E. Ferry-Galow</u>	Date:	<u>3-4-14</u>
LHTP Approval:	<u>Ralph E. Parchment</u>	Date:	<u>6/11/2014</u>
DCTD OD Approval:	<u>Joseph E. Tomaszewski</u>	Date:	<u>06/11/2014</u>

2. Change History

Revision	Approval Date	Description	Originator	Approval
D	2/28/2014	Thermoconductive plate holder used for all 37°C and 25°C incubation steps; all incubations in fixed-temperature incubators. Additional 5-sec soak time added to plate washing method to decrease assay variability. Define room temperature. Specify that matched biopsy samples should be loaded at the same concentration. PBS Casein added as a critical reagent.	YAE, KFG	KFG
C	2/20/2013	New coating buffer and plate washer settings used to minimize background variation and plate drift. Protein load per well increased and volume preparations for lysates modified to reflect updates in biopsy processing SOP340702 and PBMC processing SOP340703.	KFG	JJ
B	9/24/2012	Removed Appendix 3 with assigned ranges of quality control samples; will be provided by lot number. Tween 20 and PhosSTOP moved to critical reagents. Total protein load requirements for different dilution loads defined in Section 7.6.1. PBMC load increased following first-in-human results. Quality control, data analysis, and clinical reporting moved to new SOP340704. Requirements for digital sample tables added.	YZ, YAE	JJ
A	5/27/2011	Reformat SOP, define critical reagents, and expand Batch Record. Assay transfer complete.	YAE	JJ,RJK
--	7/14/2009	New document	YZ	JJ

OVERVIEW OF TOP1 IMMUNOASSAY SAMPLE PROCESSING



1.0 PURPOSE

To standardize an enzyme-linked immunosorbent assay (ELISA) method for quantifying topoisomerase 1 (TOP1) levels as a pharmacodynamic (PD) measure of TOP1 inhibitors.

2.0 SCOPE

This procedure applies to all personnel involved in the use of the TOP1 Immunoassay during clinical trials. The goal of the SOP and associated training is to ensure consistency in TOP1 measurement across samples and clinical sites.

3.0 ABBREVIATIONS

C	=	Control
CV	=	Coefficient of Variation
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
mAb	=	Monoclonal Antibody
NCTVL	=	National Clinical Target Validation Laboratory
pAb	=	Polyclonal Antibody
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
PBMC	=	Peripheral Blood Mononuclear Cell
PBS	=	Phosphate Buffered Saline
PBS-Casein	=	Phosphate Buffered Saline/Casein Block and Diluent
PD	=	Pharmacodynamic
PI	=	Protease Inhibitors
PMSF	=	Phenylmethanesulfonyl fluoride
RLU	=	Relative Light Units
SD	=	Standard Deviation
SOP	=	Standard Operating Procedure
TOP1	=	Topoisomerase 1

4.0 INTRODUCTION

The TOP1 Immunoassay has been developed to measure the effect of TOP1 inhibitors on TOP1 levels in a variety of biospecimen types, including peripheral blood mononuclear cells (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 detection antibody followed by an HRP-conjugate to allow chemiluminescent readout and quantitation of TOP1 levels.

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. Oversees the personnel running 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 and reports to 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 Plate Map Design ([Appendix 1](#)) and Batch Record ([Appendix 2](#)) 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 tables in the Batch Record (Appendix 2, Sections 5 and 6) can be created for logging sample information as long as all column information exactly matches the tables in the Batch Record. A copy of the completed, digital sample tables 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.

6.0 MATERIALS, CRITICAL REAGENTS, AND EQUIPMENT REQUIRED

6.1 Critical Reagents

- 6.1.1 TOP1 mouse monoclonal antibody affinity purified, Clone C-21.2, 0.5 mg/mL (TOP1 mAb; BD Biosciences, Cat#: 556597)
 - 6.1.2 TOP1 rabbit polyclonal antibody affinity purified, 1 mg/mL (TOP1 pAb; Abcam Cat#: ab28432)
 - 6.1.3 Recombinant TOP1 (rTOP1) standard (EMD Biosciences, Cat#: 614850).
 - 6.1.4 Goat anti-rabbit HRP-conjugated pAb, 1 mg/mL (KPL, Cat#: 074-15-061)
 - 6.1.5 Tumor cell lysate control (custom preparation prepared to a target TOP1 range)
 - 6.1.6 SuperSignal ELISA Pico Chemiluminescent Substrate (Thermo Scientific Pierce, Cat#: 37070)
 - 6.1.7 PDA II Antibody Coating Buffer (custom order; Trevigen, Cat#: 4520-960-13)
 - 6.1.8 Phosphate Buffered Saline/Casein Block and Diluent, 5X concentrate (5X PBS-Casein; BioFfx, Cat#: PBSC-0100-01)
- 6.2 Pipettors (100-1000 μ L, 50-200 μ L, 2-20 μ L) and tips
 - 6.3 Multichannel pipettors (50-300 μ L, 5-50 μ L) and tips
 - 6.4 Reagent reservoirs (e.g., Fisher Scientific, Cat#: 21-381-27C)
 - 6.5 1.5-mL Sarstedt o-ring screw cap tubes (e.g., Sarstedt, Cat#: 72.692.005)
 - 6.6 15-mL polypropylene tubes (e.g., Becton Dickinson, Cat#: 352097)
 - 6.7 50-mL polypropylene tubes (e.g., Becton Dickinson, Cat#: 352098)
 - 6.8 Reacti-Bind white opaque 96-well plate (Thermo Scientific Pierce, Cat#: 15042)
 - 6.9 Acetate microtiter plate sealers (Thermo Scientific Pierce, Cat #: 3501)
 - 6.10 Aluminum foil
 - 6.11 Ice bucket
 - 6.12 UltraPure DNase/RNase-free distilled water (e.g., Invitrogen, Cat#: 10977-015) or Milli-Q water
 - 6.13 Protease Inhibitor Cocktail (Sigma-Aldrich, Cat#: P-2714 or Roche, Cat#: 11697498001)
 - 6.14 Phenylmethanesulfonyl fluoride solution, 0.1 M (PMSF; Sigma-Aldrich, Cat#: 93482-50ML-F)
 - 6.15 Tris, ultra pure (e.g., MP Biomedicals, Cat#: 04819620 or 04819623)
 - 6.16 Sodium chloride, ReagentPlus grade (e.g., Sigma-Aldrich, Cat#: S9625)
 - 6.17 Glycerol, 100% w/v (e.g., Sigma-Aldrich, Cat#: G5516)
 - 6.18 EDTA, 0.5 M, pH 8.0 (e.g., Boston BioProducts, Cat#: BM-150)
 - 6.19 Magnesium chloride, anhydrous (e.g., Sigma-Aldrich, Cat#: M8266)
 - 6.20 β -Glycerol phosphate disodium salt, pentahydrate (e.g., Sigma-Aldrich, Cat#: 50020)
 - 6.21 Sodium fluoride, ACS grade (e.g., Sigma-Aldrich, Cat#: 201154)
 - 6.22 Tween 20 non-ionic, aqueous solution, 10% w/v (Roche Applied Science, Cat#: 11332465001)
 - 6.23 Triton X-100, non-ionic, aqueous solution, 10% w/v, stored according to manufacturer's direction (e.g., Roche Applied Science, Cat#: 11332481001)
 - 6.24 10X Phosphate Buffered Saline pH 7.2 (PBS; e.g., Invitrogen, Cat#: 70013-073)
 - 6.25 Mouse serum (Sigma-Aldrich, Cat#: M5905)
 - 6.26 Vortex Genie 2 (Daigger, Cat#: 3030A)
 - 6.27 Sorvall Fresco centrifuge, refrigerated (Fisher Scientific)
 - 6.28 BioTek ELx405 Select Microplate Washer (BioTek Instruments)
 - 6.29 Infinite® M200 Microplate Reader (Tecan US; M200 PRO and M1000 also acceptable)
 - 6.30 BioCision CoolSink XT 96F thermoconductive plate for flat bottom plates (VWR, Cat#: 89239-504); minimum of two are needed per assay (one at 25°C and the other at 37°C)

- 6.31** Non-humidified, fixed temperature incubator able to maintain 25°C (\pm 3°C)
- 6.32** 37°C non-humidified incubator
- 6.33** -80°C freezer
- 6.34** 2°C to 8°C refrigerator
- 6.35** PBMC samples processed following SOP340703 (PBMC Protein Extraction for TOP1 Immunoassay) or biopsy samples processed following SOP340702 (Biopsy Protein Extraction for TOP1 Immunoassay)

7.0 OPERATING PROCEDURES

7.1 Prior to beginning the assay, refer to the Plate Map Design and Batch Record to review all actions required for successful assay setup ([Appendices 1 and 2](#)).

7.2 Record the name and certification number of the Certified Assay Operator, facility running the SOP, and associated clinical protocol number in the Batch Record ([Appendix 2](#)).

7.3 Critical Reagents

7.3.1 All Critical Reagents are to be labeled with date of receipt and stored under the specified conditions for no longer than the recommended duration.

- Storage conditions and expiration dates for all Critical Reagents are provided on the package insert.
- Do not exchange reagents from one set of qualified Critical Reagents with a set of reagents qualified separately.

7.3.2 Record the date of receipt, lot number, stock/supplied reagent concentration, recommended working dilution/concentration, and expiration date for the Critical Reagents in the Batch Record (Appendix 2, Section 1).

7.3.2.1 **Recombinant TOP1 Standard:** 50 ng/mL stock solution in 1X PBS-Casein.

7.3.2.2 **Tumor Lysate Control:** Lysates prepared from a well characterized human breast tumor cell line (MCF7) provided at 1×10^7 cells/mL in lysis buffer. The control lysate is prepared such that TOP1 levels fall within a pre-determined analyte range.

7.3.2.3 **PDA II Antibody Coating Buffer:** Stock solution qualified from the manufacturer.

7.3.2.4 **PBS-Casein Block and Diluent (PBS-Casein):** 5X stock solution qualified from the manufacturer. Individual lots are qualified and released with a TOP1 reagent set. Dilute to 1X per SOP instructions.

7.3.2.5 **TOP1 Capture mAb:** Stock solution qualified from the manufacturer. Lots are qualified as a matched set with the TOP1 Detection pAb. The recommended dilution for the SOP is provided with reagent.

7.3.2.6 **TOP1 Detection pAb:** Stock solution qualified from the manufacturer. Lots are qualified as a matched set with the TOP1 Capture mAb. The recommended dilution for the SOP is provided with reagent.

7.3.2.7 **HRP-Conjugated pAb:** Supplied as a 1 mg/mL stock solution in HRP Stabilizer (KPL, Cat#: 54-15-01).

7.3.2.8 **Chemiluminescent Substrate Solutions:** Stock solutions (Peroxide and Pico Luminol/Enhancer Solutions) qualified from the manufacturer. Protect from light.

7.4 Plate Map and Buffer Preparation

7.4.1 Based on the number of patient samples to be analyzed, generate a Plate Map (Appendix 1) to define the location and replicates of unknown samples, tumor lysate controls, and rTOP1 standards. A single patient's **batched** samples should be contained on one 96-well plate, not split over two, to ensure consistent sample handling.

Important: The data analysis template (SOP340704) is based on the 96-well sample designations in the Plate Map (Appendix 1). To prevent user errors, always load the plate according to the plate map well designations.

7.4.2 Once the number of wells is known, determine the amount of reagents required for the assay using the Batch Record in Appendix 2. Once these calculations are complete, check that sufficient reagents and supplies are on hand to complete the assay.

7.4.3 Record serial numbers of equipment in the Batch Record (Appendix 2, Section 2A). Prepare the reagents as outlined in the Batch Record (Appendix 2, Section 2B).

7.4.4 Place a sufficient volume of PDA II Antibody Coating Buffer to be used for the coating step and 1X PBS-Casein to be used for the blocking and sample loading steps on the bench top to warm 2 h prior to the initiation of the assay.

7.4.5 Both 37°C and 25°C incubation steps for the TOP1 IA will be carried out in fixed-temperature incubators. Each 96-well plate will be placed on a CoolSink thermoconductive plate during these incubation steps.

7.4.5.1 Place a sufficient number of CoolSink thermoconductive plates inside each incubator **at least 1 h prior** to the initiation of each incubation step. For each assay plate, one thermoconductive plate will be placed in a 25°C incubator and one in a 37°C incubator.

7.4.5.2 These plates should be placed horizontally inside the incubator in direct contact with the incubator bottom or shelf and should not be stacked. The assay plate will be placed and carefully centered onto a prewarmed thermoconductive plate inside the incubator for each incubation step.

IMPORTANT:

- For all of the following wash and aspiration steps, do not let the plate dry out.
- Casein solutions are very sticky, follow the directions for rinsing the BioTek Microplate Washer after every plate wash.

7.5 Plate Preparation

7.5.1 Use the calculations in the Batch Record (Appendix 2, Section 3A) to prepare 11 mL TOP1 mAb Coating Solution for the assay. This is sufficient for one 96-well plate (preparing enough for 110 wells). Dilute TOP1 mAb with ambient temperature Coating Buffer; do not allow to sit for an extended period.

7.5.1.1 If more than one 96-well plate is to be coated, pool the aliquots and then dilute appropriately. This will ensure that all plates are exposed to identical coating antibody. Discard excess diluted antibody.

- 7.5.2 Add 100 μL of the TOP1 mAb Coating Solution per well using a multichannel pipettor, cover the plate with an acetate sheet, and incubate at 37°C for 2 h on a prewarmed CoolSink thermoconductive plate. Record the coating antibody incubation conditions in the Batch Record (Appendix 2, Section 3B).
- 7.5.3 Following incubation with the coating antibody, aspirate the plate using a plate washer (for the BioTek Plate Washer, use the *Aspirate* program). After aspiration, tap the plate on a paper towel to remove any residual liquid.
- 7.5.4 Add 250 μL of 1X PBS-Casein to each well. Cover the plate with an acetate sheet and incubate in a fixed-temperature 25°C incubator for 2 h on a prewarmed CoolSink thermoconductive plate. Record the incubation conditions in the Batch Record (Appendix 2, Section 4).

7.6 Prepare Working Dilutions of Unknown Biopsy Lysates

- 7.6.1 Biopsy samples with total protein concentration of < **0.25 $\mu\text{g}/\mu\text{L}$** should not be used in the TOP1 Immunoassay and will be reported as Not Reportable, Insufficient Protein (NR, IP) in the Clinical Sample Data report (SOP340704).
- 7.6.2 Place all stock biopsy lysates to be assayed on ice. Based on the protein measurements for the **stock tumor lysate** ($\mu\text{g}/\mu\text{L}$), prepare one of the following **Working Lysates** in CEB (**with** PIs). Do not pipette less than 2 μL . If the calculations below yield volumes of stock lysate of less than 2 μL , prepare sufficient volume of a 1:5 pre-dilution of the lysate before proceeding.

Important: Pre and post-treatment biopsies from a single patient should be prepared with matched protein concentrations (matched to sample with lowest concentration).

7.6.2.1 For unknown stock lysates with stock protein concentrations \geq **0.5 $\mu\text{g}/\mu\text{L}$** :

- Prepare 85 μL of a **0.5 $\mu\text{g}/\mu\text{L}$ Working Lysate** as follows:

$\frac{0.5 \mu\text{g}/\mu\text{L}}{\text{Working Lysate}} * 85 \mu\text{L} = \frac{\text{XXX } \mu\text{g}/\mu\text{L}}{\text{Conc. Stock Lysate}} \text{ Vol. Stock Lysate to use}$

- In labeled 1.5-mL tube, add sufficient CEB (**with** PIs) to the calculated volume of stock lysate needed to bring the total volume to 85 μL .
- Record the volumes stock lysate and CEB (**with** PIs) and final concentration of **Working Lysate** in the Batch Record (Appendix 2, Section 5).

7.6.2.2 For unknown stock lysates with stock protein concentrations **between 0.25 and 0.5 µg/µL**:

- Prepare 115 µL of a **0.25 µg/µL Working Lysate** as follows:

$\frac{0.25 \mu\text{g}/\mu\text{L}}{\text{Working Lysate}} * 115 \mu\text{L} = \frac{\text{XXX } \mu\text{g}/\mu\text{L}}{\text{Conc. Stock Lysate}} = \text{XX } \mu\text{L Vol. Stock Lysate to use}$
--

- In a labeled 1.5-mL tube, add sufficient CEB (**with PIs**) to the calculated volume of stock lysate needed to bring the total volume to 115 µL.
- Record the volumes stock lysate and CEB (**with PIs**) and final concentration of **working lysate** in the Batch Record (Appendix 2, Section 5).

7.6.2.3 Keep working lysates on ice. Flash freeze remaining stock lysates and return them to -80°C storage.

7.7 Preparation of Unknown Tumor Biopsy or PBMC Lysate Samples

7.7.1 Important: If both tumor biopsy and PBMC samples are being run on the same plate, due to the format of the Data Analysis calculations in SOP340704, load tumor biopsies first and then load PBMC samples. For example, load pre-dose biopsy dilutions in S1 - S3, post-dose in S4 - S6, and PBMC samples in S7 - S20. Stock lysates for tumor lysates are prepared according to SOP340702 and PBMCs (1 x 10⁷ cells/mL) are prepared according to SOP340703.

7.7.1.1 PBMC samples with ≤ **1 x 10⁶ PBMCs/mL** (originally <0.3 x 10⁶ PBMCs total) should not be used in the TOP1 Immunoassay and will be reported as Not Reportable, Insufficient Protein (NR, IP) in the Clinical Sample Data report (SOP340704).

7.7.2 Place all unknown sample **Working Lysates** to be assayed on ice. Record the sample/patient IDs for all lysates in the Batch Record (Appendix 2, Section 6A). Each unknown biopsy lysate will take up 3 sample wells (e.g., S1, S2, and S3). For tumor biopsy lysates, record the **Working Lysate** concentration; for PBMC lysates, record the total cells/mL in the lysate (Appendix 2, Section 6A; center and right portions of table, respectively).

7.7.3 Tumor biopsy lysate samples

7.7.3.1 Biopsy samples are prepared according to the total protein concentration of the **Working Lysate** prepared (0.25 or 0.5 µg/µL) as described below; however, pre- and post-dose samples from a single patient should be prepared with matched protein concentrations (matched to sample with lowest concentration).

7.7.3.2 For unknown **Working Lysates** with protein concentrations of **0.5 µg/µL**,

- Perform the following calculation to prepare 3 different lysate dilutions (6, 3, or 1.5 µg/well) in 175 µL total volume with 1X PBS-Casein. This is

sufficient volume to run each dilution in triplicate (plus 0.5 well extra). Clearly label all tubes with the sample number (e.g., S1, S2).

- Record volume **Working Lysate** and 1X PBS-Casein used to prepare each **Diluted Lysate** in the Batch Record (Appendix 2, Section 6A).

7.7.3.3 For unknown **Working Lysates** with protein concentrations of **0.25 µg/µL**,

- Perform the following calculation to prepare 3 different lysate dilutions (4, 2, and 1 µg/well) in 175 µL total volume 1X PBS-Casein. This is sufficient volume to run each dilution in triplicate (plus 0.5 well extra). Clearly label all tubes with the sample number (e.g., S1, S2).

$\frac{(6, 3, \text{ or } 1.5) \mu\text{g/well}}{0.5 \mu\text{g}/\mu\text{L}} \times 3.5 \text{ wells} = (42, 21 \text{ or } 10.5) \mu\text{L}$
$\frac{\text{Diluted Lysate}}{\text{Working Lysate}} \times 3.5 \text{ wells} = \text{Working Lysate}$

- Record volume **Working Lysate** and 1X PBS-Casein used to prepare each **Diluted Lysate** in the Batch Record (Appendix 2, Section 6A).

$\frac{(4, 2, \text{ and } 1) \mu\text{g/well}}{0.25 \mu\text{g}/\mu\text{L}} \times 3.5 \text{ wells} = (56, 28 \text{ or } 14) \mu\text{L}$
$\frac{\text{Diluted Lysate}}{\text{Working Lysate}} \times 3.5 \text{ wells} = \text{Working Lysate}$

7.7.3.4 Discard remaining **working lysate**.

7.7.4 PBMC lysate samples

7.7.4.1 Stock lysates for PBMCs (1×10^7 cells/mL) are prepared according to SOP340703.

7.7.4.2 Prepare PBMC **working lysates** by adding 100 µL stock lysate (into 100 µL 1X PBS-Casein (final 5×10^6 cells/mL in 200 µL). Record the dilution information in the Sample Calculation Table (Appendix 2, Section 6A). Clearly label all tubes with the sample number (e.g., S7, S8).

7.7.4.3 Lysates will be diluted an additional 2-fold with 1X PBS-Casein once loaded into the 96-well plate yielding a relative load of 2.5×10^5 cells/well.

7.7.5 Keep all unknown samples on ice until use. Only aliquot enough of each unknown sample for the assay.

7.8 Preparation of rTOP1 Standards and Tumor Lysate Controls

7.8.1 Preparation of rTOP1 standards; run in duplicate

- 7.8.1.1 For one 96-well plate, retrieve one rTOP1 standard stock tube (50 ng/mL) from the -80°C freezer and thaw on ice. Vortex and mix by inverting 5-8 times before use. Label eight 1.5-mL Sarstedt tubes, numbered 1 through 8, for the rTOP1 standards.
- 7.8.1.2 Prepare the rTOP1 standards by serial dilution as outlined in the Batch Record (Appendix 2, Section 6B) with final concentrations ranging from 25,000 to 195.3 pg/mL in 1X PBS-Casein.
- 7.8.1.3 Keep samples on ice until use. Only make enough standards for the experiment and discard any excess. Standards will be diluted an additional 2-fold when added to the 96-well plate to generate a reference curve ranging from 12,500 to 97.7 pg/mL rTOP1 standard.

7.8.2 Preparation of tumor lysate controls; run in triplicate

- 7.8.2.1 For one 96-well plate, retrieve one tumor lysate control vial (1×10^7 cells/mL) from the -80°C freezer and thaw on ice. Vortex and mix by inverting 5-8 times before use.
- 7.8.2.2 Use the dilution scheme in the Batch Record (Appendix 2, Section 6C) to prepare 1:25, 1:125, and 1:625 dilutions of the stock tumor lysate control to represent the High-Control (-C), Mid-C, and Low-C tumor controls, respectively. Label the tubes accordingly.
- 7.8.2.3 Controls will be diluted an additional 2-fold with 1X PBS-Casein once loaded into the 96-well plate for final dilutions of 1:50, 1:250, and 1:1250.
- 7.8.2.4 Keep samples on ice until use. Only make enough controls for the experiment and discard any excess.

7.9 TOP1 Protein Capture

7.9.1 Following incubation the blocking step with 1X PBS-Casein (SOP Step 7.5.4), the plates are aspirated and washed once with 350 μ L of 1X PBS-0.1% Tween using a plate washer.

For the BioTek Microplate Washer, the settings are:

*Recommended initial setting, optimize Aspirate Height and Horizontal ASPR POS to optimize complete aspiration for an individual unit following manufacturer’s recommendations.

METHOD	
Number of Cycles:	1
Soak/Shake:	Yes
Soak Time	5 sec
Shake before soak:	No
Prime after soak:	No
DISPENSE	
Dispense Volume:	350 μL/well
Dispense Flow Rate:	06
Dispense Height:	120 (15.240 mm)
Horizontal DISP POS:	00 (0.000 mm)
Bottom Wash First:	No
Prime Before Start:	No
ASPIRATE	
Aspirate Height:	031 (3.937 mm)
Horizontal ASPR POS:	-20 (-0.914 mm)
Aspiration Rate:	05 (6.4 mm/sec)
Aspirate Delay:	1000 MSec
Crosswise ASPIR:	No
Final Aspiration:	Yes
Final Aspirate Delay:	1000 MSec

7.9.2 After the wash, tap the plate on a paper towel to remove residual buffer. Proceed immediately to the next step; do not allow the plate to dry out.

7.9.3 Immediately, add 50 μ L of 1X PBS-Casein (equilibrated to ambient temperature) to each well using a multichannel pipettor. Each well will hold a final volume of 100 μ L after sample addition.

- 7.9.4** Use the Plate Map Design (Appendix 1) and the Unknown Sample Calculation Table (Appendix 2, Section 6A) as a guide to set up the 96-well plate for incubation with unknown samples (SOP Step 7.7), rTOP1 standards (SOP Step 7.8.1), and tumor lysate controls (SOP Step 7.8.2). Pipette reagents in the following order; **do not deviate** from order of addition:

Order	SAMPLE/REAGENT
1	50 μ L of 1X PBS-Casein into each of the Background wells
2	50 μ L of specified concentrations of rTOP1 standards into designated duplicate wells
3	50 μ L each of tumor lysate controls (Low-C, Mid-C, and High-C) into designated triplicate wells
4	50 μ L of each unknown sample, biopsy or PBMC, into designated triplicate wells

- 7.9.5** Cover the plate with an acetate sheet and incubate at 2°C to 8°C for 18 \pm 2 h. Record the date, start time, and incubation temperature in the Batch Record (Appendix 2, Section 7).

- 7.9.5.1 Once the plate is incubating, run the DAY_RINSE maintenance program on the BioTek Microplate Washer using deionized water and then run the OVERNIGHT_LOOP program. Re-prime the lines with 1X PBS-0.1% Tween before the next aspiration/wash step.

7.10 TOP1 Detection (next day)

- 7.10.1** Prepare a sufficient amount of the TOP1 detection pAb 1 h before washing the plate (next step) that has been incubating with samples.

- 7.10.1.1 Using the calculations in Appendix 2, Sections 8A, prepare the TOP1 pAb working solution in cold 1X PBS-Casein; be sure to record the lot number of mouse serum used.

- 7.10.1.2 Allow the prepared TOP1 pAb to incubate for in a fixed-temperature incubator for 1 h at 25°C and record the incubation conditions in the Batch Record (Appendix 2, Section 8A).

- 7.10.2** After the 18-h incubation is complete, aspirate and wash the wells 4 times with 350 μ L of 1X PBS-0.1% Tween (same wash program as SOP Step 7.9.1, except run for 4 cycles). Record the date and stop time of sample incubation in the Batch Record (Appendix 2, Section 7).

- 7.10.3** After the wash, tap plate on a paper towel to remove residual liquid. Proceed immediately to the next step; do not allow the plate to dry out.

- 7.10.4** Add 100 μ L TOP1 pAb working solution per well using a multichannel pipettor, cover the plate with an acetate sheet, and incubate for 2 h on a prewarmed CoolSink thermoconductive plate in a fixed-temperature 25°C incubator. Discard residual working solution and record incubation conditions in the Batch Record (Appendix 2, Section 8B).

7.10.4.1 Once the plate is incubating, run the DAY_RINSE maintenance program on the BioTek Microplate Washer using deionized water and then run the OVERNIGHT_LOOP program. Re-prime the lines with 1X PBS-0.1% Tween before the next aspiration/wash step.

7.10.5 One hour before the incubation with TOP1 detection pAb is complete, prepare a sufficient amount of HRP conjugate for the assay.

7.10.5.1 Using the calculations in Appendix 2, Section 9A, prepare the HRP conjugate working solution in 1X PBS-Casein; record the lot number of mouse serum used. Wrap the tube in aluminum foil to keep solution in the dark.

7.10.5.2 Allow the prepared HRP conjugate to incubate in a fixed-temperature 25°C incubator for 1 h and record the incubation conditions (Appendix 2, Section 9A).

7.10.6 After the 2 h incubation with the TOP1 pAb is complete, aspirate and wash the wells 4 times with 350 µL of 1X PBS-0.1% Tween (same wash program as SOP Step 7.9.1, except run for 4 cycles). Tap plate on a paper towel to remove residual liquid and proceed immediately to the next step.

7.10.7 Add 100 µL of the HRP conjugate working solution per well using a multichannel pipettor. Cover the plate with an acetate sheet and then in aluminum foil and incubate for 1 h on a prewarmed CoolSink thermoconductive plate in a fixed-temperature 25°C incubator. Record the incubation conditions in the Batch Record (Appendix 2, Section 9B). Discard residual working solution and record the incubation conditions in the Batch Record (Appendix 2, Section 9B).

7.10.7.1 Once the plate is incubating, run the DAY_RINSE maintenance program on the BioTek Microplate Washer using deionized water and then run the OVERNIGHT_LOOP program. Re-prime the lines with 1X PBS-0.1% Tween before the next aspiration/wash step.

7.11 Signal Detection

7.11.1 Turn on the Tecan Infinite Plate Reader at least 30 min before use.

7.11.1.1 Under “Instrument,” select “Heating” and set a Target Temperature of 25°C.

7.11.1.2 For chemiluminescent readings, the plate reader should be set to the following reading parameters:

Shaking duration:	5 sec
Mode:	Linear
Amplitude:	1 mm
Attenuation:	OD1
Integration Time:	100 ms

7.11.2 Immediately prior to the final wash step (next step), prepare the Chemiluminescent Substrate Solution as outlined in Appendix 2, Section 10A, being sure to note the time of preparation. Cover with aluminum foil.

- 7.11.3** After the 1 h HRP conjugate incubation is complete, aspirate and wash the wells 4 times with 350 μ L of 1X PBS-0.1% Tween (same wash program as SOP Step 7.9.1, except run for 4 cycles). Tap plate on a paper towel to remove excess buffer and proceed immediately to the next step.
- 7.11.4** Add 100 μ L of the freshly made Chemiluminescent Substrate solution to each well using a multichannel pipettor, noting the time of addition to wells (Appendix 2, Section 10B).
- 7.11.5** Immediately place the plate into the Tecan plate reader.
- 7.11.5.1 Perform the first chemiluminescent reading at approximately 1 min after substrate addition. Record the time of the initial relative light unit (RLU) reading in the Batch Record (Appendix 2, Section 10B).
- 7.11.5.2 Take a second reading at approximately 5 min after substrate addition (4 min after first reading) using the same instrument settings.
- 7.11.5.3 Use readings from the readout with the highest RLU values for analysis.
- 7.11.5.4 In some cases the signal may be too high (no read-out, invalid read-out) from the initial reading, wait an additional 5-10 min and read the plate again at the same instrument setting. Maximum Chemiluminescent Substrate incubation on the plate is 30 min.
- 7.11.5.5 Record time final RLU reading is taken in Appendix 2, Section 10B.
- 7.11.6** Save the resulting readings in an Excel file to a secure computer; recommended to label with a unique file name (e.g., SOP number + “Tecan” + run date + unique plate identifier). Print a paper copy of the raw Tecan data for inclusion with the Batch Record.
- 7.12** Proceed to SOP340704 for quality control, data analyses, and preparation of the Clinical Sample Data Report to send to the clinical protocol Principal Investigator.
- 7.13** Review and finalize the Batch Record (Appendix 2) and obtain required signatures. Document ANY and ALL deviations from this SOP in the Batch Record (Appendix 2, Section 11).
- 7.14** The Laboratory Director/Supervisor should review the Batch Record and sample reports and sign the Batch Record affirming the data contained within the reports are correct (Appendix 2, Section 12).

APPENDIX 1: PLATE MAP DESIGN

	1	2	3	4	5	6	7	8	9	10	11	12
A	97.7	1X PBS-Casein (no sample) – Background										
B	195.3	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	
C	390.6											
D	781.3											
E	1562.5	S2	S4	S6	S8	S10	S12	S14	S16	S18	S20	
F	3125											
G	6250											
H	12500	Low-C			Mid-C			High-C			Blank	

rTOP1
Standards,
Duplicate

Controls and Unknown Samples, Triplicate

- S1 through S20 are unknown sample (S) wells in triplicate. If fewer samples are run, fill the empty sample wells with 1X PBS-Casein and ignore for data analysis.
 - **Important:** If both tumor biopsy and PBMC samples are being run on the same plate, due to the format of the Data Analysis calculations in SOP340704, load tumor biopsy samples first and then load PBMC samples. For example, load pre-dose biopsy dilutions in S1 - S3; post-dose in S4 - S6; and PBMC samples in S7 - S20.
- Background wells are loaded with 1X PBS-Casein only (no sample).
- Document the sample/patient IDs and other pertinent information in the Batch Record (Appendix 2, Section 5 and 6A).

Important: This Plate Map design and well designation is assumed for the format of the Tecan output file that will be used in SOP340704: TOP1 Immunoassay Quality Control, Data Analysis, and Reporting. Manual adjustment of the output well data is outlined in the SOP if a different Plate Map is used.

THIS PAGE LEFT BLANK ON PURPOSE

APPENDIX 2: 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: _____

Plate ID (optional): _____

1. Critical Reagents

Be sure the lot numbers and recommended working solution concentrations on each of the reagents match those determined for the Critical Reagent. Reagents from one set of qualified Critical Reagents **should not** be exchanged with a set of reagents qualified separately

Reagent Name	Date Received	Lot Number	Provided Reagent/ Concentration	Recommended Dilution/Conc. for Working Solution	Expiration Date
rTOP1 Standard	/ /		50 ng/mL	N/A	/ /
Tumor Lysate Control	/ /		cells/mL	N/A	/ /
PDA II Antibody Coating Buffer	/ /		N/A	N/A	/ /
PBS-Casein	/ /		5X	1X	
TOP1 Capture mAb	/ /		mg/mL	1 :	/ /
TOP1 Detection pAb	/ /		mg/mL	1 :	/ /
Goat Anti-Rabbit HRP-Conjugate	/ /		1 mg/mL	1:	/ /
SuperSignal Chemiluminescent Substrate	/ /		N/A	N/A	/ /

2. Equipment and Preparation of Reagents

A. Equipment

BioTek Plate Washer: Make/Model: _____

Serial #: _____

Microplate Reader Make/Model: _____

Serial #: _____

B. Buffers

Buffers should be prepared based on volumes needed to complete all the steps with the number of 96-well plates in the experimental run. Always prepare at least 10% excess volume of buffer to ensure adequate volume to complete the study (scale-up appropriately for additional plates).

- a. 1X PBS-Casein (Blocking Buffer): For one 96-well plate (preparing enough for 110 wells), prepare 80 mL of 1X PBS-Casein in two 40-mL aliquots as follows. Pipette 8 mL 5X PBS-Casein and 32 mL of distilled water into a 50-mL tube yielding 40 mL 1X PBS-Casein Blocking Buffer. Keep one 40-mL aliquot at 2°C to 8°C and the other at ambient temperature. Discard unused buffer at end of the experimental run.

5X PBS-Casein Lot#: _____

- b. 1X PBS- 0.1% Tween: To prepare 1 L of buffer pipette 100 mL 10X PBS and 10 mL 10% Tween 20 into 890 mL deionized water. Keep at ambient temperature, use within 1 week of preparation.

- c. 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: _____

- d. 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: _____

- e. Cell Extraction Buffer (CEB): Manufacturer's 1X solution; store in aliquots at -20°C.

Lot#: _____ Expiration Date: _____

- f. 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

- g.** CEB (with PIs): To prepare 2 mL of CEB (**with** PIs), pipette 20 μ L 100 mM PMSF and 80 μ L 25X PI cocktail and in 1.9 mL CEB (**without** PIs). Keep on ice or at 2°C to 8°C and discard excess buffer at the end of the experimental run.

3. Capture Antibody: TOP1 mAb

A. Preparation of TOP1 mAb Coating Solution

Remove antibody from -20°C freezer and thaw on ice.

For one 96-well plate, prepare 110 wells: $(100 \mu\text{L}/\text{well} * 110) / (1000 \mu\text{L}/\text{mL}) = 11 \text{ mL}$. Prepare **TOP1 mAb Coating Solution** using the following calculations:

a. Recommended dilution of TOP1 mAb **STOCK** = 1: _____

e.g., TOP1 mAb **STOCK** recommended dilution for Lot# 73181, 44003, and 82288 of 1:500.

$\frac{11 \text{ mL}}{\text{Recommended dilution of TOP1 mAb STOCK}} * 1000 \mu\text{L}/\text{mL} = \underline{\text{XX}} \mu\text{L TOP1 mAb STOCK}$

$$\frac{11 \text{ mL}}{\text{(dilution factor)}} * 1000 \mu\text{L}/\text{mL} = \underline{\quad} \mu\text{L TOP1 mAb STOCK}$$

b. Place the following in a 15-mL polypropylene tube and mix by inversion 5 to 8 times.

11 mL	PDA II Antibody Coating Buffer
____ μL	TOP1 mAb STOCK

B. Incubation Conditions for Coating Plate

Add 100 μL **TOP1 mAb Coating Solution** to each well, and incubate at 37°C for 2 h on a prewarmed CoolSink thermoconductive plate.

Date: _____ Start Time: _____ : _____ Stop Time: _____ : _____ Incubation Temp: _____ °C

4. Block Step

Following the washing step after the capture antibody incubation, add 250 μL 1X PBS-Casein in each well and incubate in a fixed-temperature 25°C incubator for 2 h on a prewarmed CoolSink thermoconductive plate.

Incubation conditions for blocking plate:

Date: _____ Start Time: _____ : _____ Stop Time: _____ : _____ Incubation Temp: _____ °C

5. Preparation of Working Dilutions of Unknown Biopsy Lysates

Normalize unknown biopsy lysates to either a 0.25 or 0.5 $\mu\text{g}/\mu\text{L}$ working dilution prior to preparation of samples for the immunoassay. Pre- and post-dose samples from a single patient should be prepared with matched protein concentrations.

<u>Sample No.</u>	<u>Sample/Patient ID</u>	<u>Stock Lysate Conc.</u> xx $\mu\text{g}/\mu\text{L}$	<u>Working Lysate Conc.</u> 0.25 or 0.5 $\mu\text{g}/\mu\text{L}$	<u>Vol. Stock Lysate</u> (μL)	<u>Vol. CEB (with PIs)</u> (85 (or 115) μL - Vol. Stock Lysate used)
S1					
S2					
S3					
S4					
S5					
S6					
S7					
S8					
S9					
S10					
S11					
S12					
S13					
S14					
S15					
S16					
S17					
S18					
S19					
S20					

6. Preparation of Unknown Samples (A), rTOP1 Standards (B), and Tumor Lysate Controls (C)

A. Unknown Sample Calculation Table: Unknown samples are run in triplicate, 50 μL sample/well (preparing 0.5 - 1 well extra). Sample numbers correspond to those on the Plate Map Design in Appendix 1.

Important: If both tumor biopsy and PBMC samples are being run on the same plate, due to the format of the Data Analysis calculations in SOP340704, load tumor biopsy samples first and then load PBMC samples. For example, load pre-dose biopsy dilutions in S1 - S3, post-dose in S4 - S6, and PBMC samples in S7 - S20.

All Samples		Tumor Biopsy Samples				PBMC Samples		
Sample No.	Sample/Patient ID	Working Lysate Conc. 0.25 or 0.5 $\mu\text{g}/\mu\text{L}$	Diluted Lysate, (6, 3, and 1.5 $\mu\text{g}/\text{well}$) OR (4, 2, and 1 $\mu\text{g}/\text{well}$)			Stock Cell Number 1×10^7 cells/mL	Stock Lysate Vol. (μL) 100 μL	Vol. 1X PBS-Casein (μL) 100 μL
			Vol. Working Lysate (μL)	Vol. 1X PBS-Casein (175 μL - Vol. Lysate)	Final conc. ($\mu\text{g}/\text{well}$)			
S1								
S2								
S3								
S4								
S5								
S6								
S7								
S8								
S9								

Continued on next page.

Continued from previous page

All Samples		Tumor Biopsy Samples				PBMC Samples		
Sample No.	Sample/Patient ID	Working Lysate Conc. 0.25 or 0.5 µg/µL	Diluted Lysate, (6, 3, and 1.5 µg/well) OR (4, 2, and 1 µg/well)			Stock Cell Number 1 x 10 ⁷ cells/mL	Stock Lysate Vol. (µL) 100 µL	Vol. 1X PBS-Casein (µL) 100 µL
			Vol. Working Lysate (µL)	Vol. 1X PBS-Casein (175 µL - Vol. Lysate)	Final conc. (µg/well)			
S10								
S11								
S12								
S13								
S14								
S15								
S16								
S17								
S18								
S19								
S20								

B. Recombinant TOP1 Standards

Serial dilutions of the rTOP1 stock are used to generate a reference curve ranging from 12500 to 97.7 pg/mL upon pipetting 50 µL of each diluted standard into wells containing 50 µL 1X PBS-Casein (1:2 dilution). Label tubes with final concentration of standard.

Tube # (Plate Row)	Vol. and Source of Concentrated Standard	Volume 1X PBS-Casein	Resulting Diluted Conc. of Standard	Conc. of Standard in Plate (1:2 Dilution)
Stock	rTOP1 Stock Solution	N/A	50 ng/mL (50000 pg/mL)	N/A
1 (H)	140 µL of Stock tube	140 µL	25000 pg/mL	12500 pg/mL
2 (G)	140 µL of tube #1	140 µL	12500 pg/mL	6250 pg/mL
3 (F)	140 µL of tube #2	140 µL	6250 pg/mL	3125 pg/mL
4 (E)	140 µL of tube #3	140 µL	3125 pg/mL	1562.5 pg/mL
5 (D)	140 µL of tube #4	140 µL	1562.5 pg/mL	781.25 pg/mL
6 (C)	140 µL of tube #5	140 µL	781.25 pg/mL	390.6 pg/mL
7 (B)	140 µL of tube #6	140 µL	390.6 pg/mL	195.3 pg/mL
8 (A)	140 µL of tube #7	140 µL	195.3 pg/mL	97.7 pg/mL
9 (Background Control)	0 µL	600 µL	0 pg/mL	0 pg/mL

C. Tumor Lysate Controls

The High-, Mid- and Low-C tumor lysate controls are dilutions prepared from a 1×10^7 cells/mL stock lysate. Controls will be diluted an additional 2-fold when added to the 96-well plate to generate final dilutions at 1:50, 1:250, and 1:1250.

Control Tube Dilution	Number of Tumor Cells/Well	Vol. and Source of Control Lysate	Volume 1X PBS-Casein	Conc. of Control in Plate (1:2 Dilution)
High-C (1:25)	20000 cells/well	<u>12</u> µL of Tumor Cell Stock	288 µL	High-C (1:50)
Mid-C (1:125)	4000 cells/well	<u>60</u> µL of High-C	240 µL	Mid-C (1:250)
Low-C (1:625)	800 cells/well	<u>60</u> µL of Mid-C	240 µL	Low-C (1:1250)

7. Plate Incubation

Add 50 µL unknown samples, tumor controls, and rTOP1 standards to the 96-well plate (wells contain 50 µL 1X PBS-Casein [equilibrated to ambient temperature]), cover plate, and incubate at 2°C to 8°C for 18 ± 2 h.

Date: _____ Start Time: _____ : _____ Incubation Temp: _____ °C

Date: _____ Stop Time: _____ : _____

8. Detection Antibody: TOP1 pAb

A. Preparation of TOP1 pAb Working Solution (100 µL/well)

For one 96-well plate, prepare 110 wells: $(100 \mu\text{L/well} * 110) / (1000 \mu\text{L/mL}) = 11 \text{ mL}$. Prepare **TOP1 pAb Working Solution** using the following calculations:

a. Recommended dilution of TOP1 pAb STOCK = 1: _____

e.g., TOP1 pAb STOCK recommended dilution for Lot# 856307 and Lot# 950661 is 1:250.

$\frac{11 \text{ mL}}{\text{Recommended dilution of TOP1 pAb STOCK}} * 1000 \mu\text{L/mL} = \underline{\text{XX}} \mu\text{L TOP1 pAb STOCK}$
--

$$\frac{11 \text{ mL}}{\text{(dilution factor)}} * 1000 \mu\text{L/mL} = \underline{\quad} \mu\text{L TOP1 pAb STOCK}$$

b. Place the following in a 15-mL polypropylene tube:

11 mL	Cold 1X PBS-Casein	
11 µL	Mouse serum (1:1000)	Lot#: _____
___ µL	TOP1 pAb STOCK	

c. Mix by inversion 5 to 8 times, and incubate in a fixed-temperature 25°C incubator in the dark for 1 h before use.

Start Time: _____ : _____ Stop Time: _____ : _____ Incubation Temp: _____ °C

B. Addition of Prepared TOP1 pAb Working Solution

Add 100 µL of the **TOP1 pAb Working Solution** to each well and incubate for 2 h in a fixed-temperature 25°C incubator on a prewarmed CoolSink thermoconductive plate.

Start Time: _____ : _____ Stop Time: _____ : _____ Incubation Temp: _____ °C

9. Reporter: HRP Conjugate

A. Preparation of HRP Conjugate Working Solution (100 µL/well)

For one 96-well plate, prepare 110 wells: $(100 \mu\text{L/well} \times 110) / (1000 \mu\text{L/mL}) = 11 \text{ mL}$. Prepare **HRP Conjugate Working Solution** using the following calculations:

- a. Recommended dilution of Goat Anti-Rabbit HRP Conjugate **STOCK** = 1: _____
 e.g., HRP Conjugate **STOCK** recommended dilution for Lot# 110373 is 1:1000.

$\frac{11 \text{ mL}}{\text{Recommended dilution of HRP Conjugate STOCK}} * 1000 \mu\text{L/mL} = \underline{\text{XX}} \mu\text{L HRP Conjugate STOCK}$
--

$$\frac{11 \text{ mL}}{\text{(dilution factor)}} * 1000 \mu\text{L/mL} = \underline{\quad} \mu\text{L HRP Conjugate STOCK}$$

- b. Place the following in a 15-mL polypropylene tube:

11 mL	Cold 1X PBS-Casein	
11 µL	Mouse serum (1:1000)	Lot#: _____
___ µL	HRP Conjugate STOCK	

- c. Mix by inversion 5 to 8 times, and incubate in the dark in a fixed-temperature 25°C incubator for 1 h before use.

Start Time: _____ : _____ Stop Time: _____ : _____ Incubation Temp: _____ °C

B. Addition of HRP Conjugate Working Solution

Add 100 µL of the **HRP Conjugate Working Solution** to each of the washed wells and incubate in the dark in a fixed-temperature 25°C incubator for 1 h on a prewarmed CoolSink thermoconductive plate.

Start Time: _____ : _____ Stop Time: _____ : _____ Incubation Temp: _____ °C

10. Chemiluminescent Substrate

A. Preparation of Substrate Solution (100 µL/well)

Calculate volume of substrate required for the experimental run. For one 96-well plate, prepare 110 wells: $(100 \mu\text{L/well} * 110) / (1000 \mu\text{L/mL}) = 11 \text{ mL}$.

Prepare the following in a 15-mL polypropylene tube wrapped with aluminum foil. Mix by inversion 5 to 8 times and keep in the dark until use.

5.5 mL	Pico Stable Peroxide	$(50 \mu\text{L/well} * 110) / (1000 \mu\text{L/mL})$
5.5 mL	Pico Luminol/Enhancer	$(50 \mu\text{L/well} * 110) / (1000 \mu\text{L/mL})$

Time of Substrate Preparation: _____ :

B. Substrate Solution Addition and RLU Reading Times

Time of Substrate Addition to Wells: _____ :

Time Initial RLU Reading is Captured: _____ :

Time Final RLU Reading is Captured (opt): _____ :

11. Notes, Including any Deviations From the SOP:

12. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

Date: ____ / ____ / ____