Boston Medical Center
Boston MA 02118
Department of Pathology and Laboratory Medicine

BARC PRO 015 BARC PRO 015 Prothrombin F1+2 SOP

Copy of version 2.1 (approved and current)

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Description
Not final format

Comments for version 2.0 (last major revision)
Manufacture instruction and actual practice for standard reconstitution. This is an error correction to remove DI water as diluent. Also, minor number changes and location of some procedural notes were made for clarity.

Comments for version 2.1 (this revision)
Typos and clarification of process

Approval and Periodic Review Signatures

Type	Description	Date	Version	Performed By	Notes
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Approval	Quality Approval	10/5/2017	2.0	ERDuffy Elizabeth Duffy	
Approval	Primary Investigator	8/23/2017	1.4	Chris Andry, PhD Chris Andry	
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Version History

Version	Status	Type	Date Added	Date Effective	Date Retired
2.1	Approved and Current	Minor revision	8/20/2018	8/20/2018	Indefinite
2.0	Retired	Major revision	10/4/2017	10/5/2017	8/20/2018
1.4	Retired	Minor revision	8/23/2017	8/28/2017	10/5/2017
1.3	Retired	Minor revision	8/23/2017	8/23/2017	8/28/2017
1.2	Retired	Minor revision	8/23/2017	8/23/2017	8/23/2017
1.1	Retired	Minor revision	8/23/2017	8/23/2017	8/23/2017
1.0	Retired	First version in Document Control	3/23/2017	3/23/2017	8/23/2017

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1.0 **PURPOSE AND SCOPE**

1.1. The purpose of this SOP is to provide standardized instructions and guidance for measurement of human Prothrombin Fragment 1+2 in human plasma in the Pathology and Laboratory Medicine Department of Boston Medical Center.

1.2. This procedure applies to all personnel involved in the use of this assay during the study. The goal of the SOP and associated training is to ensure consistency in measurement across samples.

2.0 **OVERVIEW**

2.1. PRINCIPLE OF THE ASSAY: This assay employs the quantitative sandwich immunoassay technique. A monoclonal antibody specific for human Prothrombin Fragment 1+2 (PF1+2) has been pre-coated onto a microplate. Standards, samples and Control are pipetted into the wells followed by a biotin-conjugated secondary antibody. An avidin-horseradish peroxidase conjugate and substrate are the detection reagents and color is developed which is proportional to analyte concentration. The color development is stopped and the intensity of the color is measured. Assay quality control criteria are applied to the background, calibrator and control samples to validate the assay run. Quality control criteria are then applied to the unknown samples and data reporting guidelines are defined.

2.2. CLINICAL SIGNIFICANCE: Prothrombin fragment 1+2 is released when coagulation Factor Xa cleaves precursor prothrombin to thrombin, signaling *in vivo* thrombin generation. Thus, PF1+2 levels are a measure of coagulation activation. The levels of this biomarker are upregulated in patients with breast, prostate, gynecologic and lung cancer with clinical thrombosis.

2.3. SPECIMEN REQUIREMENT: Human platelet-poor plasma (citrate, heparin, or EDTA anticoagulant). Samples require a 10-fold dilution in the assay. A minimum of fifty microliters (50 µl) plasma is needed for each sample.

3.0 **RESPONSIBILITY**

3.1. Principal Investigator. It is the responsibility of the Principal Investigator (PI) at BMC to ensure that project personnel have been trained in accordance with this SOP, that the training is documented, and that this procedure is followed.

3.2. Project Personnel. It is the responsibility of the project lab personnel to ensure he/she has read, understands, and follows the SOP when working with blood samples and the data.

3.3. It is the responsibility of the project staff designated by the PI or Biospecimen Source Site (BSS) to ensure that all the required case report forms (CRFs) in the Comprehensive Data Resource (CDR) are completed.

3.4. Any planned deviation or change from this SOP, known prior to a collection, should be approved by the Biospecimen Research Group-Quality Management (BRG-QM) and Leidos Technical Project Manager (TPM) and ***well documented by the site***.

3.5. *Any unplanned deviation that is unexpected or identified during or after a collection should be well documented by the site*. Such deviations should be submitted to the TPM and BRG-QM along with a corrective action description for documentation.

4.0 **DEFINITIONS and ACRONYMS**

4.1. Acronyms: See Table I

Table I. Acronyms

Acronym	**Name**
PF1+2, PF1.2	Prothrombin Fragment 1+2
CV	coefficient of variation
HBSS	Hank's balanced salt solution
ID	Identification/ Identifier
LLQ	lower limit of quantification
PBS	phosphate buffered saline
SD	standard deviation
SOP	standard operating procedure
UA	unanalyzable
ULQ	upper limit of quantification

4.2 Assay Procedure Summary

Prepare all reagents, samples and standards.

Add 100 µl of diluted Sample, Standard, Control or Blank to well; incubate 90 minutes at 37 °C.
Aspirate liquid from each well without washing.

Add 100 µl of 1X Biotinylated Detection Antibody
Incubate for 1 hour at 37 °C.

Aspirate and wash 3 times.

Add 100 µl of 1X HRP Conjugate
and Incubate for 30 minutes at 37 °C.

Aspirate and wash 5 times.

Add 90 µl of TMB Substrate solution
and Incubate for 15 minutes at 37 °C.

Add 50 µl of Stop Solution

Read immediately at 450 nM

5.0 **ENVIRONMENTAL HEALTH & SAFETY**

5.1. Universal Safety Precaution will be followed.

6.0 **CRITICAL REAGENTS, MATERIALS, AND EQUIPMENT REQUIRED**

6.1. Human platelet-poor plasma sample(s) handled as per SOP BARC PRO 023 (Blood processing, storage, and shipping). Samples can be anticoagulated with citrate, heparin or EDTA from blood obtained in standard vacutainer collection tubes.

6.2. Critical reagents

6.2.1. Human Prothrombin Fragment 1+2 ELISA Kit (Catalog number: LS-F23736, LifeSpan BioSciences, Inc. Seattle, WA 98121, USA). Store all kit components at 2-8 ^{o}C). The substrate should never be frozen. Once individual reagents are opened it is recommended that the kit be used within 1 month. Unused Strip Plate wells should be

stored at 2-8°C in a sealed bag containing desiccant in order to minimize exposure to moisture. Do not use the kit beyond its expiration date. See Table II for kit contents.

6.2.2. Other critical reagents: see Table II

Table II: Critical Reagents

Reagent	Vendor	Catalog #	Storage	Notes
Hank's balanced salt solution (HBSS)	ThermoFisher Scientific	14025-092	keep stock solution bottles at room temp (~25°C)	Store in sterile 10mL aliquots at 4°C. Use once, then discard.
Normal human pooled plasma in 4% trisodium citrate	Sigma-Aldrich	P9523-5ML	2-8°C, sterile	Prepare BMC Control
PF1.2 ELISA standard from 2 separate kits	LifeSpan BioSciences, Inc. Seattle, WA	LS-F21825	2-8°C, sterile	Prepare BMC Controls
Sample Diluent from 2 separate kits	LifeSpan BioSciences, Inc. Seattle, WA	LS-F21825	2-8°C, sterile, Supplied in ELISA kit	Prepare BMC Controls
Coated 96-well Strip Plate	LifeSpan BioSciences, Inc. Seattle, WA	LS-F23736,	2-8°C, Supplied in ELISA kit	1 plate
Standard (Lyophilized)			2-8°C, Supplied in ELISA kit	2 vials
Sample Diluent			2-8°C, Supplied in ELISA kit	1 vial x 20 ml
Biotinylated Detection Antibody (100x)			2-8°C, Supplied in ELISA kit	1 vial x 120 µl

Biotinylated Detection Antibody Diluent			2-8°C, Supplied in ELISA kit	1 vial x 10 ml
HRP Conjugate (100x)			2-8°C, Supplied in ELISA kit	1 vial x 120 µl
HRP Conjugate Diluent			2-8°C, Supplied in ELISA kit	1 vial x 10 ml
Wash Buffer (25x)			2-8°C, Supplied in ELISA kit	1 vial x 30 ml
TMB Substrate			2-8°C, Supplied in ELISA kit	1 vial x 10 ml
Stop Solution			2-8°C, Supplied in ELISA kit	1 vial x 10 ml

6.3. Reagent Comments:

6.3.1. PF1.2 recognized in this immunoassay is not available commercially, so standards from two separate kits are used to prepare the BMC controls.

6.4. Consumables: see Table III

Table III. Consumables

Item	Range / Capacity	Quantity	Suggested Vendor / Catalog #
Pipet tips	100-1000 µL	1 box	
Pipet tips	20-200 µL	1 box	
Pipet tips	0.5-10 µL	1 box	
Volumetric pipette with dispenser or bulb	5ml	at least 2	
Polystyrene round bottom test tubes	12x75mm	about 20	
500uL tubes, O-ring screw cap, conical bottom, sterile	1.5 mL		Sarstedt 72.692.005
Polypropylene conical tubes, sterile	15 mL		VWR 21008-918
Polypropylene conical tubes, sterile	50 mL		VWR 21008-951

Sealing tape for 96 well plates			Thermo Fisher 15036
Disposable reagent reservoirs			ThermoFisher 95128095

6.5. Equipment: see Table IV

Table IV: Equipment

Equipment	Range/Capacity	Manufacturer	Model	Serial No
Pipettor	100-1000 μL			
Pipettor	20-200 μL			
Pipettor	0.5-20 μL			
Multichannel Pipettor	30-300 μL			
Microplate Washer		BioTek	ELx50	259186
Microplate Reader		Molecular Device	VersaMax	BNR06440
Refrigerator	2°C to 8°C			
Incubator, dry	37°C (36-38°C)			

6.6. Reagent storage and stability

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

6.6.2. Unopened reagents are stable until the expiration date shown on the vial when stored at 2-8°C.

6.6.3. All critical reagents are to be labelled with date of receipt and stored under the specified conditions for no longer than the recommended duration.

6.6.3.1. Check dates on all vials and replace any that are ready to expire and/or expired.

6.6.3.2. Storage conditions and expiration dates for all Critical Reagents are provided on the package insert.

6.6.3.3. Do not exchange reagents from one set of qualified Critical Reagents with a set of reagents qualified separately.

6.6.3.4. Do not use any materials past expiration date.

7.0 . OPERATING PROCEDURE

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 and the facility running the SOP in the Batch Record (Appendix 2: Batch Record). Include reference to 96-well plate ID, if applicable.

7.3. Plate Map

7.3.1. Based on the number of patient samples to be analyzed, generate a Plate Map (Appendix 1) to define the location and replicates of clinical samples, control samples, and PF1+2 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 analyses template 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.

7.3.2. Once the number of wells is known, determine the amount of reagents required for the assay. Once these calculations are complete, check that sufficient reagents and supplies are on hand to complete the assay.

7.3.3. Record serial numbers of equipment in the Batch Record (Appendix 2, Section 5)

7.4. Pre-Assay Reagent Preparation

7.4.1. Prepare BMC Control for aliquot storage. PF1.2, compatible with LSBiosciences PF1.2 ELISA Kit, is not available commercially. Two kits are purchased and its stock standards are used for preparation of BMC Control aliquots. Sample diluent from the kit is used to reconstitute the antigen; all other components from these kits are discarded.

7.4.1.1. To each PF1.2 ELISA kit standard stock vial, add 1.0 mL of sample diluent to reconstitute (stock 3000 pg/mL). Allow to sit for 10 minutes at room temperature with occasional gentle mixing to ensure complete resuspension. Pool these four reconstituted standards into a 50mL conical tube. Label as "PF1.2 Control."

7.4.1.2. Reconstitute lyophilized human plasma in 5.0 mL of deionized water. Allow to sit for 15 minutes at room temperature. Gently swirl to mix. Add 2.0 mL of plasma to the PF1.2 Control conical tube.

7.4.1.3. Add 14.0 mL of HBSS to PF1.2 Control conical tube for a final volume of 20 mL (net 10% plasma). Gently swirl to mix.

7.4.1.4. Divide into 350 µl aliquots using 0.5 mL cryovials. This should make about 57 vials. The control concentration of PF1.2 will be approximately 600 pg/mL higher than normal 1:10 diluted plasma.

7.4.1.5. For remainder of normal human pooled plasma, make 100 µL aliquots in screw cap tubes with O-ring. Label and put in -80°C to freeze rapidly.

7.4.1.6. Store frozen at -80°C. Document lot number of kit and standard used for preparation of the Control. Controls are used once and excess is discarded.

7.5. **Reagent Preparation on Assay Day: Reagents should be brought to room temperature without the use of additional heat**

7.5.1. Preparation of Detection Antibody: Calculate the required amount needed before beginning the experiment. Briefly centrifuge the stock tube before use. Dilute the concentrated Biotinylated Detection Antibody to the working concentration using Biotinylated Detection Antibody Diluent (1:100, v/v). For one 96-well plate, mix 100 µL of Antibody Concentrate with 9.9 mL Diluent.

7.5.2. Preparation of Wash buffer: If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved.

7.5.2.1. Add 30 mL of 25X Wash Buffer Concentrate to 720 ml of deionized water to prepare 750 mL of Wash Buffer. After use, wash buffer can be stored at 4°C once prepared.

7.5.3. Preparation of HRP Conjugates: Calculate the required amount needed before beginning the experiment. Briefly centrifuge the stock tube before use. Dilute the concentrated HRP Conjugate to the working concentration using HRP Conjugate Diluent (1:100, v/v). For one 96-well plate and use of a multi-channel pipettor, mix 100 µL of Antibody Concentrate with 9.9 mL Diluent.

7.6. Preparation of Standards (for triplicate on each plate)

7.6.1. Retrieve one vial of PF1+2 Standard from the kit stored at 4°C. Reconstitute Standard just before use with 1.0 ml of sample diluent. Allow vial to sit at room

temperature for at least 10 minutes and mix by gentle inversion and swirling until all contents are completely dissolved. Vigorous agitation and foaming should be avoided.

7.6.2. Label eight 12x75 mm test tubes, numbered 1 through 8, for the PF1+2 standards. Prepare the PF1+2 standards #1-7 by serial dilution with final concentrations of 3000 to 46.88 pg/mL in sample diluent (Table V: Preparation of Standards). Tube 8 will contain only sample diluent.

Table V. Preparation of Standards

Standard #	Concentration (pg/mL)	Volume Diluent (µL)	Volume PF1+2 (µL)	Final concentration in assay (pg/mL)
1	3000	0	800	3000
2	1500	400	400 of tube #1	1500
3	750	400	400 of tube #2	750
4	375	400	400 of tube #3	375
5	187.5	400	400 of tube #4	187.5
6	93.75	400	400 of tube #5	93.75
7	46.88	400	400 of tube #6	46.88
8	0	400	0	0

7.6.3. Serially diluted Standards will be added directly to the 96-well plate with no further dilution. Diluted standards should be used within 1 hour.

7.7. Preparation of Unknowns (plasma samples)

7.7.1. Thaw plasma samples rapidly at 37°C, then keep at room temperature. Do not expose samples to elevated temperature for longer than the time needed to thaw the plasma.

7.7.2. All plasma samples must be diluted 10-fold into Sample Diluent with suggested ratio of 40 µL of plasma + 360 µL of Sample Diluent to ensure enough sample for triplicates.

7.7.3. In some cases, sample may need further dilution in order to lower the concentration of PF1+2.

7.8. Assay procedure

7.8.1. Add 100 µL of each sample, control, and standard to wells as shown in the Plate Map (Appendix 1).

7.8.2. Cover the plate with an adhesive plate sealer.

7.8.3. Incubate plate at 37°C for 90 minutes. Record the date, start time, and incubation temperature in the Batch Record (Appendix 2, Section 3A).

7.8.4. Aspirate the liquid from each well by flipping the plate, followed by tapping the plate on 5 layers of paper towels to remove residual buffer. **Do not wash** and do not allow the plate to dry out.

7.8.5. Add Biotinylated Detection Antibody

7.8.5.1. Add 100 µL of diluted Biotinylated Detection Antibody to wells using a multichannel pipettor. Cover plate with adhesive tape to seal.

7.8.5.2. Incubate at 37°C for 1 hour. Record the date, start time, and incubation temperature in the Batch Record (Appendix 2, Section 3B).

7.8.6. Wash

7.8.6.1. Aspirate the plate using a plate washer (for the BioTek Plate Washer, use the *ELISA* program). Immediately wash the plate 3 times with 300 µL Wash Buffer, aspirating the plate between each wash and being sure no residual liquid remains.

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

7.8.6.3. For the BioTek Microplate Washer, the settings are:

METHOD	ELx405 Select	ELx405
Number of Cycles:	3 (First Wash) 5 (Second Wash)	3 (First Wash) 5 (Second Wash)

Soak/Shake:	Yes	Yes
Soak Time:	5 sec	5 sec
Dispense Volume:	350 µL/well	350 µL/well

7.8.7. Adding HRP Conjugates

7.8.7.1. Add 100 µL of 1x HRP Conjugate to wells using a multichannel pipettor. Cover plate with adhesive tape to seal.

7.8.7.2. Incubate for 30 minutes at 37°C. Record the date, start time, and incubation temperature in the Batch Record (Appendix 2, Section 3C).

7.8.8. Wash

7.8.8.1. Aspirate the plate using a plate washer (for the BioTek Plate Washer, use the *ELISA* program). Immediately wash the plate 5 times with 300 µL Wash Buffer, aspirating the plate between each wash and being sure no residual liquid remains.

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

7.8.9. Adding TMB Substrate

7.8.9.1. Calculate the required amount needed before beginning the experiment. One 96-well plate needs 10 ml of the Substrate. No dilution is required. Use a sterile volumetric pipette to transfer the needed volume of TMB Substrate to a reagent reservoir.

7.8.9.2. Add 90 µL of TMB Substrate to each well using a multichannel pipettor. Cover the plate with a new plate sealer.

7.8.9.3. Incubate for 15 minutes at 37°C; start timer after addition of substrate to the last column. Protect the plate from light. Record the date, start time, and incubation temperature in the Batch Record (Appendix 2, Section 3D).

7.8.10. Adding Stop Solution

7.8.10.1. Calculate the required amount needed before beginning the experiment. One 96-well plate needs 5 ml of the stop solution. No dilution is required. Transfer the needed volume of Stop Solution to a reagent reservoir.

7.8.10.2. Add 50 µL of Stop Solution to each well using a multichannel pipettor. The Stop Solution should be added to the wells in the same order and timing as the TMB Substrate solution.

7.8.10.3. The blue color will change to yellow immediately. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.

7.8.11. Determine Optical Density (O.D.)

7.8.11.1. Determine the optical density of the wells within 30 minutes using a microplate reader set to 450 nm with wavelength correction set to 620 nm to correct for imperfections in the plate.

7.8.11.2. Save the resulting readings to a secure computer; recommended to label the file with the date and a unique assay identifier (Plate ID): PF ELISA MM/DD/YEAR PLATEX format (e.g., PF ELISA 03062017 PLATE1). Record the file name in the Batch Record (Appendix 2, Section 4B).Print a paper copy of the raw data for inclusion with the Batch Record.

7.8.12. Review and finalize the Batch Records (Appendix 2) and obtain required signature. Document ANY and ALL deviations from this SOP in the Batch Record (Appendix 2, Section 7).

8.0 **DATA ANALYSIS**

8.1. **PRINCIPLE:**

8.1.1. Optical density data is converted to antigen (PF1+2) concentration with a computer program, SoftMax Pro. Acceptable results are obtained with computer programs using a standardized curve-fitting four parameter logistic method, or a logistic/log regression analysis.

8.1.2. The Protocol calls for a “PF ELISA Analysis”, which tells the calculation-program the location of samples, standards, QC, the initial dilution and serial dilutions. Wells designated as Diluent Only in the Plate Map (Appendix 1) should be labeled as “blank wells” in the template. The program should subtract the average OD of the “blank wells” from the OD of other wells.

8.1.3. The PF1+2 concentration for each sample is found from the standard curve using the mean of the sample triplicate determinations.

8.2. DATA INSPECTION RULES

8.2.1. Blanks: the signal of blank wells should be less than 0.2 units for all assay plates. If any blank wells are >0.2, the assay should be examined for inappropriate results and should be re-assayed if no apparent causes are found.

8.2.2. Triplicates: If the coefficient of variation (CV) of triplicate wells is >15% and two wells have a CV of ≤ 10%, then the outlier well value can be excluded from the calculation. This has to be documented in Appendix 2, section 7. If > 1 outlier well is observed, the assay should be examined for cause and re-assayed if no apparent causes are found.

8.2.3. Standards: The slope of the linear portion of the reference standard curve (e.g., OD 0.1 to 2.0) should be near 1.0 (0.9 – 1.1) when the log of the OD signal is graphed against the log of the standard concentration.

8.2.4. Sensitivity: Calculate the lower detection limit for the assay and confirm that the detection limit is within in the established range.

8.2.5. Quality Control: Control sample values must be within the established range for intra-assay variability (CV<15%; plates run on the same day) and inter-assay variability comparing plates run on different days (CV<30%).

8.2.6. If a sample has readings greater than the highest standard used in the assay, the sample should be re-assayed after additional dilution.

8.2.6.1. If an unknown value is high and is diluted more than that defined in the assay procedure, then new controls should be made with normal human pooled plasma using the same dilution factor to replicate the amount of plasma in all the samples.

8.2.7. If the analyte concentration of the sample was calculated by averaging the data from multiple dilutions and the CV of the concentration exceeds 30%, then the data should be examined for inappropriate results and should be re-assayed if no apparent causes are found.

8.2.8. If the lower limit of detection is equal to or less than the lowest standard concentration and a sample has undetectable analyte concentration, report one half of the established assay lower limit as the concentration for the sample. If the lower limit of detection is more than the established value and a sample has undetectable

analyte concentration, do not report the result for the sample and re-analyze the sample.

8.3. **DATA ANALYSIS:** Most software analysis packages, including SoftMax Pro, will perform curve fitting and data analysis to obtain concentrations. Multiply by any dilution factor to obtain the final PF1+2 concentrations. Perform the following if manual calculations are needed:

8.3.1. Obtain average OD of serially diluted Standards and each sample well groupings.

8.3.2. For each PF1+2 concentration, obtain the 'signal' by subtracting the average OD value of the zero standard wells (primary and secondary antibody) from the average OD value of the corresponding wells that contain standards or unknowns.

8.3.3. Plot the background corrected signal values on the Y-axis and the logarithm of standard concentration on the X-axis to obtain the standard curve.

8.3.4. Obtain unknown concentrations from the standard curve. Multiply by any dilution to obtain the final PF1+2 concentration.

9.0 **REFERENCE**

9.1. LSBio User Manual for Human Prothrombin Fragment 1+2 ELISA Kit.

9.2. National Clinical Target Validation Laboratory, Applied/Developmental Research Directorate, Leidos Biomedical Research, Inc. by Frederick National Laboratory for Cancer Research.

10.0 **ATTACHMENTS**

INITIATION/REVISION HISTORY			
REV #	**DESCRIPTION OF CHANGE**	**AUTHOR**	**EFFECTIVE DATE**
1.0	Draft	John Kim	
1.1	Draft	DSK, JK	3/15/2017
1.2	Draft	DSK	5/5/2017
1.3	Draft	DSK, MT	5/18/2017
1.4	Draft; minor clarifications	DSK, MT	8/16/2017
2.0	Change of standard diluent to conform to manufacture instructions and actual practice, as well as a few other modification to location for clarity	ERD	10/04/2017
2.1	Minor Clarifications, typos, formatting	BET,DSK,ERD,MPT	8/1/2018

APPENDIX 1: PLATE MAP DESIGN: Patient samples from Module I and II may be assayed on the same plate (same design), but the pre-analytic variable grouping for each patient must be included on the same plate.

- When only 1 or 2 patient samples (S) are run, the Plate Map Design can be adjusted, so long as triplicate wells are used for samples, standards and controls.
- Blank wells are loaded with Reagent Diluent only (no sample).
- Document the sample/patient IDs and other pertinent information in the Sample Calculation Table in the Batch Record (Appendix 2).

A1.1 Module I Plate Design (Time to Centrifuge): 37°C Assay

	1	2	3	4	5	6	7	8	9	10	11	12
A		STDS		Blank (Diluent)								
B				S1T 1			S7T1			S13T1		
C				S2T 2			S8T2			S14T2		
D				S3T 4			S9T4			S15T4		
E				S4T 1			S10T 1			BMC CTL	BMC CTL	BMC CTL
F				S5T 2			S11T 2					
G				S6T 4			S12T 4			Blank (Diluent)		
H				Blank (Diluent)								

A1.2 Module I Plate Design (Freeze-Thaw Cycles): 37°C Assay

	1	2	3	4	5	6	7	8	9	10	11	12
A		STDS		Blank (Diluent)								
B				S1C 1			S7C1			S13C1		
C				S2C 2			S8C2			S14C2		
D				S3C 3			S9C3			S15C3		
E				S4C 1			S10C 1			BMC CTL	BMC CTL	BMC CTL

F				S5C 2	S11C 2	
G				S6C 3	S12C 3	Blank (Diluent)
H				Blank (Diluent)		

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: _____

Date Immunoassay Run: _____

Plate ID (optional): _____

1. Critical Reagents

The critical reagents are listed below; complete the table as designated. Be sure the lot numbers on each of the reagents match those cited in the product insert with the kit. Reagents from one kit **should not** be exchanged with reagents from another.

Table 1: Critical Reagents

Reagent Name	Date Received	Lot No	Provided Reagent	Dilution/Conc for working solution	Exp Date
Pooled plasma	/ /		commercial or donors		/ /
Coated 96-well plate	/ /		N/A	N/A	/ /
PF1+2 Standards	/ /		Lyophilized powder		/ /
Sample Diluent	/ /		1 Vial x 20mL		/ /
Biotinylated Detection Antibody (100X)	/ /		1 Vial x 120µL		/ /
Biotinylated Detection Antibody Diluent	/ /		1 Vial x 10mL		/ /
HRP Conjugate (100X)	/ /		1 Vial x 120µL		/ /
HRP Conjugate Diluent			1 Vial x 10mL		
Wash Buffer (25X)	/ /		1 Vial x 30mL		/ /
TMB Substrate	/ /		1 Vial x 10mL		/ /
Stop Solution			1 Vial x 10mL		/ /

2. **Unknown Samples**: The first line gives an example with sample/patient ID, Module with Pre-analytic variable (PAV) - and plasma dilution.

Sample No	Sample/Patient ID	Module/ PAV	Dilution (X)		
S Ex	TCP_0001	I / T2	10		
S1					
S2					
S3					
S4					
S5					
S6					
S7					
S8					
S9					
S10					
S11					
S12					
S13					
S14					
S15					
S16					
S17					
S18					
S19					
S20					

3. Plate Incubation

A. Add clinical samples, controls, or blank to the 96-well plate, cover plate, and incubate at 37°C for 90 minutes.

Date	Start	Stop	Incubation Temp (°C)
/ /	**:**	**:**	

B. Add 1X Biotinylated Detection Antibody, cover plate, and incubate at 37°C for 1 hour.

Date	Start	Stop	Incubation Temp (°C)
/ /	:	:	

C. Add 1X HRP Conjugate, cover plate, and incubate at 37°C for 30 minutes.

Date	Start	Stop	Incubation Temp (°C)
/ /	:	:	

D. Add TMB Substrate, and incubate at 37°C for 15 minutes.

Date	Start	Stop	Incubation Temp (°C)
/ /	:	:	

4.0 Software:

4.1. SoftMax Pro Version:_____

4.2. Name of original SoftMax Pro data file: _____

5.0 **Equipment :** Standard equipment is listed below. Check if used for the biomarker assay. If different equipment was used, document in Appendix 2, Section 7.

Check if used	Equipment	Manufacturer	Model	Serial No
	Microplate Washer	BioTek		ELx50
	Microplate Reader	Molecular Devices		VersaMax
	Spectrofluorometer	Molecular Devices	Gemini XPS	XPS05453
	Refrigerator (2-8^{o}C)			
	Freezer (-80^{o}C)			
	Incubator (37^{o}C)			

6.0 Plate Map QC

6.1. Name of saved PF1+2 Excel data analysis workbook

_____

6.2. Plate Map Set Up QC

( ) Recommended PF1+2 Plate Map used.

( ) Alternative plate map used; cells copy and pasted individually to the Plate Layout QC worksheet.
Reason: _____

7.0 Notes, including any deviations from the SOP:

If assay fails QC, state the specific reason for assay failure and notify the Laboratory Director/Supervisor.

8.0 Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (Print)

_____ (Sign)

9.0 Date: _____

APPENDIX 3: Work Process Flow

BARC PRO 012: Thrombosis in Cancer Patients: Blood sample Collection SOP	• Properly collect blood at all BSSs for the the Thrombosis in Cancer Patients Pre-Analytical Factors (TCP) study. • Immediately invert the tube slowly and gently. • Transport to blood processing laboratory.

↓

BARC PRO 023: Thrombosis in Cancer Patients: Blood sample Processing, Storage and Shipping	• Instruction to biospecimen source sites for blood sample processing, storage and shipping. • Blood will be processed for the preparation of blood derivatives from all study donors for downstream marker analyses. • Collected Plasma will be aliquoted to a pre-labeled cryovial for PF1+2 ELISA.

↓

BARC PRO 015: Thrombosis in Cancer Patients: Immunoassay of human Prothrombin Fragment 1+2 in blood sample	• Perform ELISA with clinical samples and Prothrombin Fragment 1+2. • Using Versa Max Microplate reader, determine relative signal and concentration of all samples