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

BARC PRO 024 IL-6 BIOMARKER SOP

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Description
IL-6 biomarker SOP for Thrombosis project

Comments for version 1.1 (last major revision)
Initial versionIncludes all comments and edits made by Leidos

Comments for version 1.2 (this revision)
Typos and clarifications

Approval and Periodic Review Signatures

Type	Description	Date	Version	Performed By	Notes
Approval	QA Review	8/20/2018	1.2	ERDuffy Elizabeth Duffy	

Prior History
Initial drafts vetted through Leidos and all comments included in this draft.

Version History

Version	Status	Type	Date Added	Date Effective	Date Retired
1.2	Approved and Current	Minor revision	8/20/2018	8/20/2018	Indefinite
1.1	Retired	Initial version	11/21/2017	2/13/2018	8/20/2018

BOSTON MEDICAL CENTER		Thrombosis in Cancer Patients IL-6 ELISA	
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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 Interleukin-6 (IL-6) in human plasma in the Pathology and Laboratory Medicine Department of Boston Medical Center (BMC).

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 interleukin 6 has been pre-coated onto a microplate. Standards, samples and control are pipetted into the wells and unbound substances are washed away after incubation. A polyclonal antibody specific for IL-6 and conjugated to alkaline phosphatase is added as the detection antibody, followed by washing and addition of substrate. Color developed is proportional to analyte concentration. 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. This is a high sensitivity assay designed to detect low levels of IL-6.

2.2. CLINICAL SIGNIFICANCE: Interleukin 6 (IL-6) is a cytokine involved in a variety of processes, most notably the acute inflammatory response. During chronic inflammatory diseases, like cancer, IL-6 is dysregulated. Because inflammation pathways and the coagulation cascade crossover, there may be a correlation of IL-6 with other pro-thrombotic molecules, which could be helpful toward developing biomarker profiles that report thrombotic risk in cancer patients. Aberrant IL-6 signaling promotes tumorigenesis and metastasis (Chang Q, et al *Sem Immun* 26:48-53, 2014). Increasing IL-6 circulating levels correlate with tumor stage and overall survival in a broad based meta-analysis of >10,000 patients with 23 cancer types (Lippitz BE et al, *Oncoimmunology* 5:e1093722, 2016).

2.3. SPECIMEN REQUIREMENT: Human platelet-poor plasma (citrate or EDTA anticoagulant; heparin plasma is not recommended for use in this assay). A minimum of 400 microliters (400 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 Soure 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 BRG-QM and TPM along with a corrective action description for documentation.

4.0 DEFINITIONS and ACRONYMS

4.1. Acronyms- see Table I.

Table I. Acronyms

Acronym	Name
IL-6	Interleukin 6
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
RT	Room temperature

4.2. Assay Procedure Summary For ELISAs

Prepare all reagents, samples, standards and controls

Add 100 µL Assay Diluent RD1-75 to each well
Add 100 µL each standard, sample, and control to triplicate wells.

Aspirate and Wash for total of 6 washes.

Add 200 µL Human IL-6 HS conjugate.
Incubate 2 hrs, RT on shaker

Aspirate and wash for total of 6 washes.

Add 50 µL of substrate solution.
Incubate 1 hr, RT on the benchtop. Do not wash plate.

Add 50 µL of amplifier solution.
Incubate 30 minutes, RT on the benchtop.

Add 50µL of stop solution to each well.
Read Plate at 490nm within 30 minutes.

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 sample processing, storage, and shipping). Samples can be anticoagulated with citrate or EDTA from blood obtained in standard vacutainer collection tubes. Heparin plasma is not recommended for use in this assay.

6.2. Critical Reagents- see Table II

6.2.1. Quantikine HS human IL-6 immunoassay (R&D System, Inc., Minneapolis, MN 55413 USA). Catalog number HS600B. Store up to 1 month.

6.2.2. Recombinant human IL-6 (10 ug) for IL-6 BMC Control (R&D System, Inc, Minneapolis, MN 55413 USA). Catalog number 206-IL-010. Store stock at -80°C after reconstitution.

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, frozen. Use once, then discard.
Human IL-6 HS Microplate	R&D Systems	892291	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for Human IL-6.
Human IL-6 HS Standard	R&D Systems	892293	Aliquot and store for up to 1 month at ≤I-20 °C in a manual defrost freezer. Avoid repeated freeze-thaw cycles.	Recombinant human IL-6 in a buffered protein base with preservatives; lyophilized. Refer to the vial label for reconstitution volume.
Human IL-6 HS Conjugate	R&D Systems	892292	May be stored for up to 1 month at 2-8 °C.	21 mL/vial of a polyclonal antibody specific for human IL-6 conjugated to alkaline phosphatase with preservatives.
Assay Diluent RD1-75	R&D Systems	895811	May be stored for up to 1 month at 2-8 °C.	11 mL/vial of a buffered animal serum with preservative. May contain a precipitate. Mix well before and

				during use.
Calibrator Diluent RD6-11 Concentrate	R&D Systems	895489	May be stored for up to 1 month at 2-8 °C.	21 mL/vial of a buffered protein base with preservatives. Use undiluted for serum/plasma samples.
Wash Buffer Concentrate	R&D Systems	895188	May be stored for up to 1 month at 2-8 °C.	100 mL/vial of a 10-fold concentrated solution of buffered surfactant with preservative. May turn yellow over time.
Stop Solution	R&D Systems	895032	May be stored for up to 1 month at 2-8°C.	6 mL/vial of 2 N sulfuric acid.
Substrate	R&D Systems	895884	Store in an upright position for up to 1 month at ≤ -20°C. Avoid repeated freeze-thaw cycles.	Lyophilized NADPH with stabilizers.
Substrate Diluent	R&D Systems	895885	Store in an upright position for up to 1 month at ≤ -20°C. Avoid repeated freeze-thaw cycles.	7 mL/vial of buffered solution with stabilizers and preservative.
Amplifier	R&D Systems	895886	Store in an upright position for up to 1 month at ≤ -20°C. Avoid repeated freeze-thaw cycles.	Lyophilized amplifier enzymes with stabilizers.
Amplifier Diluent	R&D Systems	895887	Store in an upright position for up to 1 month at ≤ -20°C. Avoid repeated freeze-thaw cycles.	7 mL/vial of buffered solution containing INT-violet with stabilizer and preservative.

Normal human pooled plasma in 4% trisodium citrate	Sigma-Aldrich	P9523-5ML x3	2-8°C, sterile	Prepare BMC Control
Recombinant IL-6 (10 µg)	R&D Systems	206-IL-010	12 months at -80°C after reconstitution	Prepare BMC Control

6.3. Reagent Comments

6.3.1. The kit should not be used beyond the expiration date on the kit label.

6.3.2. Do not mix or substitute reagents with those from other lots or sources.

6.3.3. Seal unused wells with adhesive tape. Return to the foil pouch containing the desiccant pack. Reseal along the entire edge of the zip-seal. May be stored for up to 1 month at 2-8°C

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

6.3.5. Alkaline phosphatase is detectable in saliva. Take precautionary measures to protect reagents during preparation and use while running the assay.

6.3.6. Inorganic phosphate is a strong competitive inhibitor of alkaline phosphatase; avoid the use of PBS based wash buffers and other sources of inorganic phosphate contamination.

6.3.7. Stop Solution provided is an acid solution. Take precautionary measures to protect personnel when working with this solution.

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	
0.5-mL tubes, O-ring screw cap, conical bottom, sterile	1.5 mL		Sarstedt 72.692.005
Polypropylene tubes, sterile	15 mL		VWR 21008-918
Polypropylene tubes, sterile	50 mL		VWR 21008-951

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	0.5-10 µL			
Multichannel Pipettor	30-300 µL			
Plate Washer		BioTek	ELx50	259186
Plate Reader		Molecular Devices	VersaMax	BNR06440
Graduated Cylinders	100 and 1000			
Microplate orbital shaker				
-80°C Freezer	-80 ± 5 °C			

6.6. Reagent storage and stability

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

6.6.2. All critical reagents are to be labeled with date of receipt and stored under the specified conditions for no longer that the recommended duration.

6.6.2.1. Check dates on all vials and replace any that are expired.

6.6.2.2. Storage conditions and expiration dates for all Critical Reagents are provided on the package inserts.

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

6.6.2.4. 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). Include reference to 96-well plate ID, if applicable.

7.3. Plate Map Preparation

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 standards. A single patient's batched samples should be contained on one 96-well plate, not split over two plates, 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 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.

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

7.4. **Pre-Assay Reagent Preparation**

7.4.1. Prepare BMC IL-6 Control for aliquots and storage.

7.4.1.1. Reconstitute lyophilized recombinant IL-6 protein in 1000 µL sterile HBSS. Let solubilize for 30 minutes at room temperature with occasional gentle swirling to mix. Do not shake. This is the Stock Solution with 10µg/mL concentration.

7.4.1.2. Transfer 2µL of IL-6 Stock Solution to a 15mL conical containing 9.998 mL of HBSS and labeled "IL-6 Working Solution." Vortex briefly to mix. The working solution concentration is 2ng/mL.

7.4.1.3. Reconstitute each vial of lyophilized plasma with 5.0mL DI water. Allow to sit for 15 minutes at room temperature with gentle mixing. Do not shake.

7.4.1.4. Transfer 4.5mL plasma from each vial to one 15mL conical tube labeled "IL-6 Contol". Mix gently by swirling. Total volume will be 13.5 mL of plasma. Transfer 13.5 µL of IL-6 working solution to this tube. Mix by gentle swirling or inverting twice. Do not shake. The final concentration of IL-6 will be 2pg/mL increase over initial levels present in plasma. *Caution: IL-6 levels will vary considerably between lots of commercially available human pooled citrated plasma. Each plasma lot should be assayed along with the BMC control for reference.*

7.4.1.5. Aliquot IL-6 control solution in 400µL aliquots (about 33 aliquots) into 0.5mL cryovials labeled "BMC IL-6 Control."

7.4.1.6. Discard remainder of IL-6 working solution.

7.4.1.7. For remainder of IL-6 stock solution, make 20 µL aliquots (about 50) in screw cap tubes with O-ring. Label and put in -80°C freezer to freeze rapidly.

7.4.1.8. For remainder of normal human pooled plasma, make 100 µL aliquots (about 12-14) in screw cap tubes with O-ring. Label and put in -80°C to freeze rapidly.

7.4.1.9. Store BMC control aliquots frozen at -80°C. Controls are used once and excess is discarded.

7.5. **Reagent Preparation on Assay Day:** All reagents should be at room temperature prior to assay

7.5.1. Assay Diluent

7.5.1.1. Mix well before and during use as precipitate may form.

7.5.2. Wash Buffer

7.5.2.1. Add 100 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 1000 mL of Wash Buffer.

7.5.3. Substrate Solution

7.5.3.1. Reconstitute the lyophilized Substrate with 6.0 mL of Substrate Diluent at least 10 minutes before use. Re-stopper and re-cap the vial, and mix thoroughly.

7.5.4. Amplifier Solution

7.5.4.1. Reconstitute the lyophilized Amplifier with 6.0 mL of Amplifier Diluent at least 10 minutes before use. Re-stopper and re-cap the vial, and mix thoroughly, but gently to avoid foaming.

7.6. **Preparation of Standards (for triplicates on each plate)**

7.6.1. Reconstitute the Human IL-6 HS Standard with Calibrator Diluent RD6-11 Concentrate with the volume specified on the vial. This produces a concentration

of 10 pg/mL IL-6. Allow the standard to sit for a minimum of 15 minutes with inverted mixing prior to making dilutions.

Table V. Preparation of Standards

Standard #	Concentration (pg/mL)	Volume Calibrator Diluent RD6-11 (µL)	Volume IL-6 Standard (µL)	Final concentration in assay (pg/mL)
1	10	----	volume on vial	5
2	5	500	500 of standard stock solution	2.5
3	2.5	500	500 of tube #2	1.25
4	1.25	500	500 of tube #3	0.625
5	0.625	500	500 of tube #4	0.313
6	0.313	500	500 of tube #5	0.156
7	0.156	500	500 of tube #6	0.078
8	0	500	----	0
	(Volume, µL)	(3500)		

7.7. Preparation of Unknowns (plasma samples)

7.7.1. Plasma samples are added undiluted to the plate.

7.8. **Assay Procedure**

7.8.1. In each well of the 96 well plate, add 100µL of assay diluent RD1-75 to each well with a multichannel pipette. Assay diluent may contain a precipitate; mix well before and during use.

7.8.2. Add 100µL of standard, control or plasma sample to the plate. Each is run in triplicate wells. Refer to Plate Map Design.

7.8.3. Cover with adhesive plate sealer and allow to incubate at RT on a horizontal orbital microplate shaker set at 500 ± 50 rpm for 2 hours.

7.8.4. Wash

7.8.4.1. Following incubation with the IL-6 Standards, Samples or control, aspirate the plate using a plate washer. Immediately wash the plate 6 times with 400 µL Wash Buffer, aspirating the plate between each wash and being sure no residual liquid remains. Addition of a 30 second soak period after dispense of the wash buffer is recommended by the manufacturer. Rotating the plate 180 degrees between wash steps may improve assay precision.

7.8.4.2. 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.

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

METHOD	ELx50 Select	ELx405
Number of Cycles:	6	6
Soak/Shake:	Yes	Yes
Soak Time:	30 sec	30 sec
Dispense Volume:	400 µL/well	400 µL/well

7.8.5. Add 200 µL of Human IL-6 HS Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at RT on a horizontal orbital microplate shaker set at 500 ± 50 rpm.

7.8.6. Repeat the wash step for a total of 6 washes. Tap the plate on paper towels to remove residual buffer. Proceed immediately to the next step; do not allow the plate to dry out

7.8.7. Add 50 µL of Substrate Solution to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature on the benchtop. Do not wash the plate.

7.8.8. Add 50 µL of Amplifier Solution to each well. Cover with a new adhesive strip. Incubate for 30 minutes at room temperature on the benchtop.

7.8.9. Add 50 µL of Stop Solution to each well.

7.8.10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 490 nm with wavelength correction set to 650nm or 690nm. Subtract readings at wavelength correction from readings at 490nm.

7.9. Save the resulting readings in IL-6 ASSAY MM/DD/YEAR PLATE X format to a secure computer; recommended to label the file with the date and a unique assay identifier (Plate ID). Print a paper copy of the raw data for inclusion with the Batch Record.

7.10. 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. Signal data is converted to analyte 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 an analyte analysis program which tells the calculation-program the location of samples, standards, controls, the initial dilution and any 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 fluorescence of the “blank wells” from the fluorescence of other wells.

8.1.3. The analyte concentration for each sample is found by calculating the mean of the sample triplicate determinations based on the standard curve.

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 > 3 outlier wells are 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 (CV<30%; comparing plates run on different days).

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.

8.3.1. Obtain average signal of Standards and each sample well groupings.

8.3.2. For each analyte concentration, obtain the ‘signal’ by subtracting the average signal of the background wells from the average signal 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 analyte concentration.

9.0 REFERENCES

1. R&D User's Guide for Human P-Selectin/CD62P Immunoassay.
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	Initial Draft	MPT, DSK, JK, ED	11/20/2017
1.1	Draft	DSK, MPT	02/01/2018
1.2	Minor Clarifications, typos, formatting	BET, DSK, ERD, MPT	08012018

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/donor 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 IL-6 ELISA (Time 0 samples Only)

	1	2	3	4	5	6	7	8	9	10	11	12
A		STDS		S1			S9			S17		
B				S2			S10			S18		
C				S3			S11			S19		
D				S4			S12			S20		
E				S5			S13			S21		
F				S6			S14			S22		
G				S7			S15			S23		
H				S8			S16			BMC CTL	BMC CTL	BMC CTL

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

Complete the table as designated. Be sure the lot numbers on each of the reagents match those cited in the product insert accompanying the reagents. Reagents from one kit **should not** be exchanged with reagents from another.

Reagent Name	Date Received	Lot No	Exp Date
Human IL-6 HS Microplate	/ /		/ /
Human IL-6 HS Standard	/ /		/ /
Human IL-6 HS Conjugate	/ /		/ /
Assay Diluent RD1-75	/ /		/ /
Calibrator Diluent RD6-11 Concentrate	/ /		/ /
Wash Buffer Concentrate	/ /		/ /
Stop Solution	/ /		/ /
Substrate	/ /		/ /
Substrate Diluent	/ /		/ /
Amplifier	/ /		/ /
Amplifier Diluent	/ /		/ /
Normal human pooled plasma in 4% trisodium citrate	/ /		/ /
Recombinant IL-6	/ /		/ /
1x HBSS	/ /		/ /

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	20		
S1					
S2					
S3					
S4					
S5					
S6					
S7					
S8					
S9					
S10					
S11					
S12					
S13					
S14					
S15					
S16					
S17					
S18					
S19					
S20					
S21					
S22					
S23					

3. Plate Incubation: If not applicable, cross out.

a. Add assay diluent, clinical samples, controls, and standards, and conjugate to the 96-well plate, cover plate, and incubate at room temperature for 2 hours. Record below.

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

b. Add conjugate to the 96-well plate, cover plate, and incubate at room temperature for 2 hours. Record below.

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

c. Add substrate to the 96-well plate, cover plate, and incubate at room temperature for 1 hour. Record below.

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

d. Add amplifier to the 96-well plate, cover plate, and incubate at room temperature for 30 minutes. Record below.

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

4. Software:

a. SoftMax ProVersion:_____

b. Name of original SoftMax Pro data file: _____

5. 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
	Plate Washer	BioTek	ELx50	259186
	Plate Reader	Molecular Devices	VersaMax	BNR06440
	Fluorometer	Molecular Devices	Gemini XPS	XPS05453
	Refrigerator (2-8^{o}C)			
	Freezer (-80^{o}C)			

6. Plate Map QC

a. Name of saved Excel data analysis workbook

_____

b. Plate Map Set Up QC

( ) Recommended Plate Map used. Circle one: A1 A2 A3 A4

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

Reason: _____

7. 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. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (Print)

_____ (Sign)

9. Date: _____

APPENDIX 3: Work Process Flow

OVERVIEW OF IMMUNOASSAY SAMPLE PROCESSING

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.

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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 (400 ul) will be aliquoted to a pre-labeled cryovial for IL-6 ELISA.

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BARC PRO 024: Thrombosis in Cancer Patients: Immunoassay of Interleukin-6 in blood sample	• Perform ELISA with clinical samples, IL-6 standards and controls • Using Versa Max Microplate reader, determine relative signal of all samples