1. PURPOSE

1.1. The purpose of this procedure is to detect and quantitate total protein within a sample using the bicinchoninic acid (BCA) colorimetric assay.

2. SCOPE

2.1. This procedure applies to the Human Papillomavirus (HPV) Serology Laboratory located at the Advanced Technology Research Facility (ATRF), Room C2007.

3. REFERENCES

3.1. F.E. Grubbs, “Procedures for Detecting Outlying Observations in Samples” Technometrics 11:1 pp 1-21 (1969)

3.2. HSL_EQ_001: Biosafety Cabinet (BSC) Use and Maintenance

3.3. HSL_EQ_005: Use and Maintenance of a Molecular Devices M5 Plate Reader in the HPV Serology Laboratory

3.4. HSL_EQ_007: Use and Maintenance of a 2-8°C Refrigerator the HPV Serology Laboratory

3.5. HSL_EQ_012: Use and Maintenance of Pipettes in the HPV Serology Laboratory

3.6. HSL_EQ_017: Use and Maintenance of a Laboratory Convection Oven

3.7. HSL_EQ_023: Use and Maintenance of a Compact Digital MicroPlate Shaker

3.8. HSL_GL_001: Waste Disposal at the Advanced Technology Research Facility

4. RESPONSIBILITIES

4.1. The Research Associate, hereafter referred to as analyst, is responsible for reviewing and following this procedure.

4.2. The Scientific Manager or designee is responsible for training personnel in this procedure and reviewing associated documentation.

4.3. The Quality Assurance Specialist is responsible for quality oversight and approval of this procedure.

5. DEFINITIONS

Term	Definition
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CI	Confidence Interval
DPBS	Dulbecco's PBS
ID	Identification
RT	Room Temperature
SDS	Safety Data Sheets

6. REAGENTS, MATERIALS AND EQUIPMENT

6.1. Reagents

6.1.1. Pierce BCA Protein Assay Kit (VWR, Cat # PI23225 or PI23227)

6.1.2. BSA Standard, 2 mg/mL Concentration, 10 x 1 mL Ampoules (VWR, Cat # PI-23209 or equivalent)

6.1.3. DPBS (Life Technologies, Cat # 14190-235 or equivalent)

6.1.4. BSA_QC1 (Developed in-house)

6.1.5. BSA_QC2 (Developed in-house)

6.2. Consumables

6.2.1. 96-well Flat Bottom Tissue Culture Plate (Thomas Scientific, Cat # 6906A07 or equivalent)

6.2.2. Plate Sealers (Thomas Scientific, Cat # 6980A01 or equivalent)

6.2.3. Microcentrifuge Tubes (VWR, Cat # 10025-726 or equivalent)

6.2.4. Cluster Tubes (VWR, Cat # 29442-612 or equivalent)

6.2.5. Reagent Reservoir (Corning, Cat # 4870 or equivalent)

6.2.6. Pipette Tips

6.2.7. Serological Pipettes

6.3. Equipment

6.3.1. Disposable Ampule Snapper (VWR, Cat # 66009-125, or equivalent)

6.3.2. Convection Oven

6.3.3. Microplate Shaker

6.3.4. Microplate Reader (Molecular Devices M5 or equivalent)

6.3.5. Pipettes (Ranging from 2 µL to 1000 µL)

6.3.6. Serologic Pipettor

6.3.7. Class II Biosafety Cabinet (BSC)

7. HEALTH AND SAFETY CONSIDERATIONS

7.1. Proper safety precautions should be taken while working in a laboratory setting. This includes, but is not limited to, proper protective equipment such as lab coats, safety glasses, closed-toe shoes, and non-latex gloves.

7.2. When possible, needle-resistant gloves should be used when breaking open the BSA ampule.

7.3. Refer to the respective SDS when working with any chemicals.

7.4. Refer to “HSL_GL_001: Waste Disposal at the Advanced Technology Research Facility” regarding waste disposal processes at the ATRF.

8. PROCEDURE PRINCIPLES

8.1. The BCA Protein Assay is used to determine the protein concentration of an unknown sample.

8.2. Cu^{+2} is reduced to Cu^{+1} in the presence of protein when in an alkaline medium and is chelated to BCA, leading to absorbency at a wavelength of 562 nm and demonstrating linear correlation to protein values.

8.3. A known BSA standard curve is used to confirm protein concentrations and to calculate the unknown sample’s protein concentration.

8.4. All work should be performed inside a BSC.

8.5. Process relevant information is recorded on “HSL_LAB_009.01: BCA Data Capture Form.”

8.6. The Data Reference consists of the Logbook Reference number and Page number. For example: Logbook Reference number (LAB2017003) and Page number (001 for page 1) are combined for final Data Reference number LAB2017003001.

9. PROCEDURE

9.1. Label the skirt/face of each 96-Flat Bottom plate with Plate Number, Data Reference, Analyst Initials and Date. See Attachment 1 for where to properly label the plate.

9.2. Standard Curve Preparation

9.2.1. Prepare nine dilution tubes and label each tube with vial letter (see Table 1) (may use cluster tubes if desired).

9.2.2. Prepare the standard curve dilutions in DPBS.

9.2.2.1. Carefully open an ampule of the BSA standard. Use needle-resistant gloves to break the lid of the ampule on the line toward the top of the vial and dispose of the glass top in a plastic sharps container, or use ampule snapper.

Note: Ensure the BSA is at the bottom of the ampule prior to opening it.

9.2.2.2. Prepare the BSA standard curve dilutions per Table 1.

Table 1: BSA Standard Curve Dilutions

Vial	Volume of DPBS (µL)	Volume and Source of Stock (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of Vial B Dilution	750
E	325	325 of Vial C Dilution	500
F	325	325 of Vial E Dilution	250
G	325	325 of Vial F Dilution	125
H	400	100 of vial G Dilution	25
I	400	0	0 (Blank)

9.3. Sample Preparation

9.3.1. Thaw sample at room temperature prior to use.

9.3.2. Dilute each sample so the expected protein concentration falls within the standard curve.

9.3.2.1. A minimum of 100 µL total volume will be required for each sample, as it will be plated in triplicate.

9.3.2.2. Three separate dilution factors will be prepared for each sample.

9.3.3. Initial sample dilutions are recommended in Table 2, but may be adjusted based on the expected protein concentration.

Table 2: Recommended Initial Sample Dilutions

Description	Starting Dilution Factor	Sample Volume	DPBS
Dilution 1	1:2	100 µL	100 µL
Dilution 2	1:4	100 µL of Dilution 1	100 µL
Dilution 3	1:8	100 µL of Dilution 2	100 µL

9.4. Preparation and Addition of the Working Reagent (WR)

Note: A volume of 200 µL of WR is required per well, including standards and controls. To test one plate, 25 mL total WR is required.

9.4.1. Mix 50 parts BCA Reagent A with 1 part BCA Reagent B from kit to make the WR. For example, combine 25 mL of Reagent A with 500 µL Reagent B for a total of 25.5 mL WR. The WR should be a clear green color when both reagents are mixed.

9.4.2. Add 25 µL of the standards, BSA_QC1, BSA_QC2, and samples to the plate in triplicate. Refer to Attachment 1 for plate layout.

Note: Unused sample wells remain empty throughout the procedure.

9.4.3. Add 200 µL of WR to all wells of the 96-well plate, being careful not to touch the pipette tip to the liquid already present in the plate.

9.4.4. Once all standards, controls, and samples have been added to the plate, cover it with a plate sealer and mix on a plate shaker at 250-350 rpm for approximately 30 seconds per “HSL_EQ_023: Use and Maintenance of a Compact Digital MicroPlate Shaker.”

9.4.5. Incubate the plate at 37 ± 2°C for 30 ± 5 minutes in the convection oven per “HSL_EQ_017: Use and Maintenance of a Laboratory Convection Oven.’”

Note: Do not use CO_2 incubator.

9.4.6. Remove plate from the oven and allow the plate to equilibrate to room temperature for 5 ± 1 minutes.

9.5. Plate Analysis

9.5.1. During the room temperature incubation (step 9.4.6), turn on the M5 Plate Reader and open the "BCA Template" file [redacted]

9.5.2. Enter Sample IDs (HPV-Type, Sample Description and Data Reference when applicable), Dilution Factors, and background information into the template.

9.5.3. Once the room temperature incubation has completed, remove the plate sealer, place the plate into the M5 plate reader and select "Read" on the screen.

9.5.4. Name the data file as follows:

"Data Reference_BCA_DDMMMYYAnalyst Initials"
(LB12345001_BCA_20MAY17ABC)

9.5.5. Save the data file [redacted]

9.5.6. Print data file and store in the Raw Data binder.

10. SYSTEM SUITABILITY

10.1. The percent Coefficient of Variance (CV) between standard replicates 1500 µg/mL to 125 µg/mL must be ≤ 10% for the data to be considered valid.

10.1.1. One whole data point may be masked within this range if it does not meet the percent CV criteria. See Attachment 2 for outlier assessment to indicate which Optical Density (OD) value between triplicates is masked for calculation.

10.2. The percent CV for the standard replicates 2000 µg/mL and 25 µg/mL must be ≤15 % for the data to be considered valid.

10.2.1. One whole data point may be masked within this range if it does not meet the percent CV criteria. See Attachment 2 for outlier assessment to indicate which Optical Density (OD) value between triplicates is masked for calculation.

10.3. The blanks must have an average absorbance (abs) reading below the 25 µg/mL standard. Up to one well may be masked if considered contaminated.

10.4. The BSA_QC1 and BSA_QC2 controls must fall within the established range [redacted] and have a percent CV of ≤ 20%.

11. DATA ANALYSIS

11.1. At least one of the sample dilutions must fall within the BCA Standard Curve at concentrations of 1500 µg/mL to 125 µg/mL for the results to be valid. Otherwise, repeat the sample at a different set of dilutions, where the protein concentration for at least one of the sample dilutions falls within the standard curve at those ranges.

11.2. The percent CV within the triplicates of each sample dilution must be ≤ 20% for the data to be considered valid. If any triplicates have a percent CV of >20%, see Attachment 2 for outlier assessment to indicate which OD value between triplicates is masked for calculation.

11.3. If more than one acceptable sample dilution is within the BCA standard curve, the percent CV between each accepted sample dilution must be ≤ 20%.

11.4. If any of these criteria are not met, repeat the sample test.

12. ATTACHMENTS

12.1. Attachment 1: Plate Layout

12.2. Attachment 2: Outlier Test: Grubb's Test for Triplicates

12.3. Attachment 3: 96-Well Plate Skirt Label

12.4. Attachment 4: HSL_LAB_009.01: BCA Data Capture Form

Attachment 1: Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	2000 µg/mL			0 µg/mL (Blank)			BSA_QC1			BSA_QC2		
B	1500 µg/mL			Sample 1, Dilution 1			Sample 3, Dilution 1			Sample 5, Dilution 1		
C	1000 µg/mL			Sample 1, Dilution 2			Sample 3, Dilution 2			Sample 5, Dilution 2		
D	750 µg/mL			Sample 1, Dilution 3			Sample 3, Dilution 3			Sample 5, Dilution 3		
E	500 µg/mL			Sample 2, Dilution 1			Sample 4, Dilution 1			Sample 6, Dilution 1		
F	250 µg/mL			Sample 2, Dilution 2			Sample 4, Dilution 2			Sample 6, Dilution 2		
G	125 µg/mL			Sample 2, Dilution 3			Sample 4, Dilution 3			Sample 6, Dilution 3		
H	25 µg/mL			Sample 7, Dilution 1			Sample 7, Dilution 2			Sample 7, Dilution 3		

Attachment 2: Outlier Test: Grubb's Test for Triplicates (Standard Deviation Method)

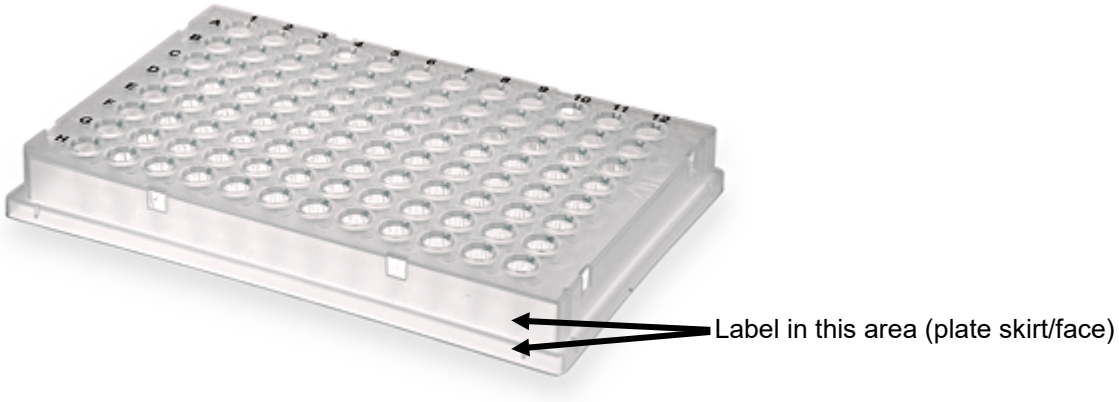
1. Rank the three values from lowest to highest: X1, X2, X3.
2. Calculate the Mean (M) and Standard Deviation (SD).
    a. $M = (X1 + X2 + X3) / 3$
    b. $SD = \sqrt{((X1-M)^2 + (X2-M)^2 + (X3-M)^2) / 3)}$
3. Calculate the Grubb's Test (GT) value using calculation below if the HIGHEST value is the suspected outlier.

    $GT = (X3-M) / SD$
4. Calculate the GT value using calculation below if the LOWEST value is the suspected outlier.

    $GT = (M-X1) / SD$
5. If the GT is GREATER THAN the value in the table below, the suspected value IS an outlier.

N # replicates	95% CI	97.5% CI	99% CI
3	1.15	1.15	1.15

Attachment 3: 96-Well Plate Skirt Label



Attachment 4: HSL_LAB_009.01: BCA Data Capture Form

Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute		HPV Serology Laboratory Standard Operating Procedure Form	
Form Title: BCA Data Capture Form			
Document ID: HSL_LAB_009		Version:	3.0
Associated SOP: HSL_LAB_009.01		Effective Date:	
Supersedes Version:	2.0	**Page 1 of 5**	

Equipment

Equipment Description	Equipment ID	Calibration Due Date
Convection Oven	☐ HSL_025 ☐ Other:	
Microplate Shaker	☐ HSL_030 ☐ HSL_031 ☐ HSL_032 ☐ Other:	
M5 Microplate Reader	☐ HSL_018 ☐ Other:	
☐ N/A Pipette: µL	PIP_	
☐ N/A Pipette: µL	PIP_	
☐ N/A Pipette: µL	PIP_	
☐ N/A Pipette: µL	PIP_	
☐ N/A Pipette: µL	PIP_	
☐ N/A Pipette: µL	PIP_	

Reagents

Reagent	Lot Number	Expiration Date
DPBS		
BCA Kit		
BSA Standard, 2 mg/mL		
BSA_QC1		
BSA_QC2		

Comments:

☐ N/A

Performed by/date:	
Reviewed by/date:	

Verify current version prior to use. Use of a superseded or obsolete document is prohibited.

Frederick National Laboratory for Cancer Research
sponsored by the National Cancer Institute

HPV Serology Laboratory
Standard Operating Procedure
Form

Form Title: BCA Data Capture Form

Document ID: HSL_LAB_009		Version:	3.0
Associated SOP: HSL_LAB_009.01		Effective Date:	
Supersedes Version:	2.0	**Page 2 of 5**	

Sample Identification

Sample Number	HPV Type	Sample Description	Data Reference/Unique Identifier
example	*HPV-16*	*Pooled fractions 3-5, T225*	*PDN2017099001*
1 ☐ N/A			
2 ☐ N/A			
3 ☐ N/A			
4 ☐ N/A			
5 ☐ N/A			
6 ☐ N/A			
7 ☐ N/A			

Comments:

☐ N/A

Performed by/date:	
Reviewed by/date:	

Verify current version prior to use. Use of a superseded or obsolete document is prohibited.

Frederick National Laboratory for Cancer Research
sponsored by the National Cancer Institute

HPV Serology Laboratory
Standard Operating Procedure
Form

Form Title: BCA Data Capture Form

Document ID: HSL_LAB_009		Version:	3.0
Associated SOP: HSL_LAB_009.01		Effective Date:	
Supersedes Version:	2.0	**Page 3 of 5**	

Sample Preparation

Sample Number	Starting Dilution Factor	Sample Volume (µL)	DPBS Volume (µL)
1 ☐ N/A	1.		
	2.	µL of Dilution 1	
	3.	µL of Dilution 2	
2 ☐ N/A	1.		
	2.	µL of Dilution 1	
	3.	µL of Dilution 2	
3 ☐ N/A	1.		
	2.	µL of Dilution 1	
	3.	µL of Dilution 2	
4 ☐ N/A	1.		
	2.	µL of Dilution 1	
	3.	µL of Dilution 2	
5 ☐ N/A	1.		
	2.	µL of Dilution 1	
	3.	µL of Dilution 2	
6 ☐ N/A	1.		
	2.	µL of Dilution 1	
	3.	µL of Dilution 2	
7 ☐ N/A	1.		
	2.	µL of Dilution 1	
	3.	µL of Dilution 2	

Performed by/date:	
Reviewed by/date:	

Frederick National Laboratory for Cancer Research
sponsored by the National Cancer Institute

HPV Serology Laboratory
Standard Operating Procedure
Form

Form Title: BCA Data Capture Form

Document ID: HSL_LAB_009		Version:	3.0
Associated SOP: HSL_LAB_009.01		Effective Date:	
Supersedes Version:	2.0	**Page 4 of 5**	

Incubation Times

Condition	Start Time	End Time	Total Time
37°C 30±5 minutes			
RT Equilibration 5±1 minutes		Read Start Time:	

Data File Name: _____

System Suitability Results

Curve	Range	Result	Pass, Fail, FIO, N/A
2000 µg/mL	% CV ≤ 15%		□ Pass □ Fail □ FIO □ N/A
1500 µg/mL	% CV ≤ 10%		□ Pass □ Fail □ FIO □ N/A
1000 µg/mL	% CV ≤ 10%		□ Pass □ Fail □ FIO □ N/A
750 µg/mL	% CV ≤ 10%		□ Pass □ Fail □ FIO □ N/A
500 µg/mL	% CV ≤10%		□ Pass □ Fail □ FIO □ N/A
250 µg/mL	% CV ≤ 10%		□ Pass □ Fail □ FIO □ N/A
125 µg/mL	% CV ≤ 10%		□ Pass □ Fail □ FIO □ N/A
25 µg/mL	% CV ≤ 15%		□ Pass □ Fail □ FIO □ N/A
0 µg/mL (Blank)	Abs Value < 25 µg/mL STD	□ Yes □ No	□ Pass □ Fail □ FIO □ N/A
BSA_QC1	% CV ≤ 20%		□ Pass □ Fail □ FIO □ N/A
	Conc. Range: (µg/mL)		□ Pass □ Fail □ FIO □ N/A
BSA_QC2	% CV ≤ 20%		□ Pass □ Fail □ FIO □ N/A
	Conc. Range: (µg/mL)		□ Pass □ Fail □ FIO □ N/A

Performed by/date:	
Reviewed by/date:	

Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute		HPV Serology Laboratory Standard Operating Procedure Form	
Form Title: BCA Data Capture Form			
Document ID: HSL_LAB_009		Version:	3.0
Associated SOP: HSL_LAB_009.01		Effective Date:	
Supersedes Version:	2.0	**Page 5 of 5**	

Sample Results

Sample Number	Reported Result (µg/mL)*	% CV of Reported Results *(Range ≤ 20%)*	Pass, Fail, FIO, N/A
1 ☐ N/A			☐ Pass ☐ Fail ☐ FIO ☐ N/A
2 ☐ N/A			☐ Pass ☐ Fail ☐ FIO ☐ N/A
3 ☐ N/A			☐ Pass ☐ Fail ☐ FIO ☐ N/A
4 ☐ N/A			☐ Pass ☐ Fail ☐ FIO ☐ N/A
5 ☐ N/A			☐ Pass ☐ Fail ☐ FIO ☐ N/A
6 ☐ N/A			☐ Pass ☐ Fail ☐ FIO ☐ N/A
7 ☐ N/A			☐ Pass ☐ Fail ☐ FIO ☐ N/A

*only for values within range of curve, that pass % CV criteria

Comments:

☐ N/A

Performed by/date:	
Reviewed by/date:	