Frederick National Laboratory forCancer
Research, Frederick, MD

Standard Operating Procedure

Biopharmaceutical Development Program

Title: Determination of Opalescence of a Sample Relative to a Set of Four Opalescence Reference Standards

SOP Number: 23127 Revision Number: 02

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Originator/Date: [redacted]

Approval/Date:

Approval/Date:

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1.0 Purpose

This procedure determinesthe opalescence of a sample relative to a set of four opalescence reference standards.

2.0 Scope

This Standard Operating Procedure (SOP) applies to Process Analytics/Quality Control (PA/QC) personnel who perform wavelength analysis on the Opalescence Standards using the UV/VIS spectrophotometer in order to ensure proper and consistent utilization.

3.0 Authority and Responsibility

3.1 The Director of Process Analytics/Quality Control (PA/QC) has the authority to define this procedure.

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3.2 PA/QC is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA).

3.3 PA/QC personnel are responsible for the performance of this procedure.

3.4 PA/QC is responsible for reviewing the data and documentation of the results of this procedure.

3.5 BQA is responsible for quality oversight of this procedure.

4.0 Supplies and Reagents

4.1 Beckman Coulter DU800 Spectrophotometer, or Biopharmaceutical Development Program (BDP) approved equivalent.

4.2 Computer Software (proprietary).

4.3 Bovine Serum Albumin Standard, BDP PN 30060, or Bovine Gamma Globulin Standard, BDP PN 30059, or BDP approved equivalent.

4.4 Sample Formulation Buffer, BDP approved.

4.5 Ultrapure water, or BDP approved equivalent.

4.6 Borosilicate glass culture tubes (12 x 75 mm) BDP PN 20143, and (16 x 125 mm) BDP PN 20144 or BDP approved equivalent.

4.7 Volumetric Flasks 100 mL or sized as needed.

4.8 Calibrated Pipettors 20-200 μL and 100-1000 μL.

4.9 Pipette tips 250 μL, BDP PN 21767, and 1000 μL, BDP PN 20769, or BDP approved equivalent.

4.10 Nitrile Gloves, BDP PN 20767 or BDP approved equivalent.

4.11 Quartz Cuvettes (1 cm pathlength).

4.12 Disposable Pasteur pipettes, BDP PN 20843 and BDP PN 21107, or BDP approved equivalent.

4.13 Methanol BDP PN 30853, or BDP approved equivalent.

4.14 Disposable Serological Pipettes 25 mL, BDP PN 20102 and 10 mL BDP PN 20100.

4.15 Primary Opalescence Solution, BDP PN 30927, Primary Opalescent and Turbidity Solution BDP PN 31080 or BDP approved equivalent. Both BDP PN 30927 and BDP PN 31080 are formulated at 4000 NTU (Nephelometric Turbidity Units) i.e. the same concentration of Formazin.

4.16 Hexamethylenetetramine, BDP PN 30923 and Hydrazine Sulfate, BDP PN 30924.

4.17 Kim-wipes EX-L, BDP PN 20091, or BDP approved equivalent.

4.18 Aspirator cuvette cleaner with vacuum line.

4.19 Vortexer.

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5.0 Procedure

5.1 Turn on the spectrophotometer by double clicking on the ***DU 800*** **Spectrophotometer** icon. Turn on the UV and Visible lamps by clicking the Visible and UV buttons in the lower left-hand corner. Refer to **SOP 22941 -** ***Operation of Beckman Coulter DU 800 Spectrophotometer***.

5.2 Allow the instrument to warm up (approximately one hour).

5.3 At the Main Screen, select **Fixed Wavelength** from the drop-down box.

5.4 Select Method **Opalescent Wavelength A350** from the drop-down box.

NOTE: In this Method the Optical Density is measured at two wavelengths (280 nm and 350 nm). Absorbance at 350 nm is used to evaluate opalescence while measurements at 280 nm are used for system suitability in conjunction with NIST traceable solutions of bovine serum albumin or bovine gamma globulin.

5.5 Using the Cuvette

5.5.1 Wear gloves and avoid touching the clear sides (light path) of the cuvette.

5.5.2 Wash cuvettes using the Aspirator cuvette cleaner; first with water, then with Methanol. Make sure to wash the cuvette before placing a new sample in the cuvette.

5.5.3 Wipe the clear sides of the cuvette gently using a Kimwipe before placing into the sample holder.

5.5.4 Make sure the cuvette is filled at least a third (400 μL). If the sample size is small, use a limited volume cuvette (300 μL).

5.5.5 If possible, use the same cuvette for the blank and samples.

5.6 Blanking

5.6.1 Fill the cuvette with the Ultrapure water (see 4.5).

5.6.2 Place the cuvette into sample compartment number 1 (the furthest away from you) with the transparent side of the cuvette facing towards the light path (left and right).

5.6.3 Close the lid before performing the analysis.

5.6.4 Left click the **Blank** icon to blank the spectrophotometer.

5.6.5 Left click on the **Sample ID**. Label the sample “Ultrapure H2O, water blank, etc.”

5.6.6 Left click on **Read Samples**.

5.6.7 Remove the cuvette from the sample compartment and wash (refer to step 5.5.2).

NOTE: Since the instrument was blanked using Ultrapure water, when reading the water, it should read approximately 0.00 au.

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5.7 Standardizing the Instrument

5.7.1 If analyzing antibodies, use Bovine Gamma Globulin (BGG) as the standard (see 4.3). For all other samples, use Bovine Serum Albumin (BSA).

5.7.2 Dilute BSA/BGG to 1 mg/mL (1:2) with Ultrapure water in a glass culture tube (see 4.6) and vortex slightly; make three separate dilutions for three individual readings. For example, add 200 µL of BSA/BGG standard to 200 µL of Ultrapure water in a borosilicate glass culture tube. Vortex.

5.7.3 Left click on **Sample ID** to label the standard/water; include the dilution factor and/or concentration (i.e., BSA 1 mg/mL).

5.7.4 Fill the washed cuvette with standard/water prepared in step 5.7.2.

5.7.5 Place the cuvette in the sample compartment number 1 (refer to step 5.6.2).

5.7.6 Left click on **Read Samples**.

5.7.7 At a 280 nm wavelength, BSA (1 mg/mL) must read 0.7 au ± 10% and BGG (1 mg/mL) must read 1.3 au ± 10%. If the results are not within 10%, prepare fresh standards. If after a second attempt the standards do not meet the criteria, do not proceed further and notify the area supervisor. If the results are within 10% of these values, proceed with the analysis.

5.7.8 Remove the cuvette from the sample compartment and wash (refer to step 5.5.2).

5.7.9 Fill the cuvette with the next standard/water and repeat the reading (2nd and 3rd reading) refer to steps 5.7.4 and 5.7.6.

5.8 Opalescence Standards

5.8.1 Primary Opalescence Solution: Ready to use primary opalescence solution BDP PN 30927 or BDP PN 31080 (See 4.15). If using BDP PN 30927 or BDP PN 31080 primary opalescence solution skip to step 5.8.3.

5.8.2 A primary Opalescence Solution can alternately be prepared in the lab **24 hours before running the assay** by mixing a Hexamethylene-tetramine solution and a Hydrazine Sulphate solution. To make a Hexamethylenetetramine solution, dissolve 2.5 g of hexamethylenetetramine in 25.0 mL of Ultrapure water. To make a Hydrazine Sulphate solution, dissolve 1.0 g of Hydrazine Sulphate in Ultrapure water and dilute to 100.0 mL. **Allow the hydrazine sulfate solution to stand for 4-6 hours at room temperature**. Both solutions are prepared in glass stoppered flasks.

To complete the Primary Opalescence Solution, add 25.0 mL of the hydrazine sulphate solution to the solution of hexamethylenetetramine. **Mix and allow to stand for 24 hours.** This solution is initially clear, but will become a milky white turbid suspension upon standing. This suspension is stable for 2 months in a glass container. The suspension must not adhere to the glass and must be well mixed before each use. Label this solution according to the instructions given in **SOP 22702 - *Solutions Used in Process Analytics***.

5.8.3 Standard of Opalescence: A Standard of Opalescence is prepared daily using the Primary Opalescence Solution from step 5.8.1 or 5.8.2. In a 100 mL volumetric flask (see 4.7) dilute 1.5 mL of the Primary Opalescent Solution to 100 mL with

Ultrapure water. This suspension is freshly prepared and may be stored for 24 hours at room temperature. Smaller volumes may be utilized as needed as long as the ratios remain the same.

5.8.4 Reference Suspensions: Prepare reference suspensions in 100 mL volumetric flasks according to the chart below. The reference suspensions are prepared fresh daily and used immediately after preparation. Mix and shake before each use. Smaller volumes may be utilized as needed providing the ratios are maintained.

Standard Opalescence Level	I	II	III	IV
STD of Opalescence	5.0 mL	10.0 mL	30.0 mL	50.0 mL
Ultrapure water	95.0 mL	90.0 mL	70.0 mL	50.0 mL

5.8.5 Read the four Opalescence Reference Suspension Standards (refer to steps 5.7.3-5.7.11).

5.8.6 A total of three individual readings for each standard are to be taken.

5.8.7 Read the formulation buffer and all samples that need to be measured. Samples are run neat/undiluted. A total of three individual readings are taken for all buffers and samples.

5.8.8 When all samples have been analyzed, standardize the instrument with three freshly prepared standards as listed in step 5.7.7.

5.9 Drift

5.9.1 Repeat the standardization section to ensure the instrument has not drifted (5.7). If the results obtained are not within 5% of the initial standardization, prepare a fresh standard set. If after a second attempt the standards do not meet the criteria, the instrument failed to standardize and the assay is invalid. If the post-run and pre-run results match within 5% analysis is complete.

5.10 Results

5.10.1 After completing the assay, save the data by clicking on the **File** button and then **Headers for Current Run**. Fill in the Department, Operator, Product Name, Component Name, Lot Number, and Comments (if the information is provided on the QC Test Request Form). Click **OK**.

5.10.2 Click the **File** button and **Save as**. From the drop-down menu in the **Save in** window, select the BDP scientific data server folder **(S:)**. Select the **DU 800 Data** folder. Save the data by using the QC test request number and or by other easily traceable nomenclature: Lot number, Project number, etc. Click the **Save** button.

5.10.3 Print out the report by clicking **File**, **Print** and then **OK**. After completing the entire test, click **Systems** from the top menu and then **Exit**. Turn off the Lamps.

5.10.4 Average the $\mathbf{A_{350}}$ sample readings and the four opalescent reference suspension standards. Subtract the corresponding buffer average $\mathbf{A_{350}}$. This average $\mathbf{A_{350}}$ of the sample is compared against the average $\mathbf{A_{350}}$ reading of the reference suspensions to determine the level of opalescence.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

6.0 Documentation

6.1 Record the results from step 5.10.4 on the BQC Test Request Form 22002-01. Attach the $\mathbf{A_{350}}$ printout from step 5.10.3 to the BQC Test Request Form.

6.2 Record the sample preparation on Form 23127-01, Opalescence Standard Preparation Form (Attachment 1). Attach the form to BQC Test Request and submit it for PA/QC and BQA review.

6.3 Record all use of the UV/VIS in the Equipment Logbook, refer to **SOP 21531 - *Equipment Logs***.

7.0 References and Related Documents

7.1 **SOP 21531** *Equipment Logs*

7.2 **SOP 22702** *Solutions Used in Process Analytics*

7.3 **SOP 22941** *Operation of Beckman Coulter DU 800 Spectrophotometer*

7.4 *European Pharmacopoeia 5.0 (2005) 2.21 Clarity and Degree of Opalescence of Liquids.*

7.5 *European Journal of Pharmaceutics and Biopharmaceutics 59 (2005) 407-417- Induction and analysis of aggregates in a liquid IgG1-antibody formulation.*

8.0 Attachments

8.1 **Attachment 1** Form 23127-01, Opalescence Standard Preparation

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

Attachment 1

Form 23127-01, Opalescence Standard Preparation

FNLCR, BDP
Form No.: 23127-01
SOP No.: 23127
Revision 02: MAY 23 2017

QC Number:_____ Analyst:_____ Date:_____

Equipment/MEF #: _____ Calibration Due Date: _____

Bovine Standard: _____ Lot#_____ Exp. Date: _____

System Suitability - Bovine Standard Dilutions

Pre-run Standards	**Post-run Standard**
Dilution: _____	Dilution: _____
Volume of Diluent: _____ µL	Volume of Diluent: _____ µL
Volume of Bovine Std: _____ µL	Volume of Bovine Std: _____ µL
Mean Result: _____ au	Mean Result: _____ au
Acceptable Range: BSA (0.63 – 0.77 au)	**BGG (1.17 – 1.43 au)**
Pre-run Stds: Pass / Fail	**Post-run Stds: Pass / Fail**

Opalescence Standard

Primary Opalescence Solution
BDP PN:_____ Lot#:_____ Exp. Date:_____

Standard of Opalescence (1.5 mL Standard + 98.5 mL H_2O):

Volume of Primary Opalescence Solution:_____mL
Volume of Ultrapure Water:_____mL

STANDARD	**I**	**II**	**III**	**IV**
STD of Opalescence	5.0 mL	10.0 mL	30.0 mL	50.0 mL
Ultrapure Water	95.0 mL	90.0 mL	70.0 mL	50.0 mL
Record actual volumes used:				
STD of Opalescence				
Ultrapure Water				
Average A_{350}				

Performed by:_____ Date: _____

Reviewed by:_____ Date: _____

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Attachment 1 (Continued)

Form 23127-01, Opalescence Standard Preparation

FNLCR, BDP
Form No.: 23127-01
SOP No.: 23127
Revision 02: MAY 23 2017

Sample Analysis

Test Sample #1

Name: _____
Lot #_____
Volume of Test Sample: _____µL
Sample Mean A_{350} Result:_____au
Buffer Name:_____
Buffer Lot:_____
Buffer Mean A_{350} Result:_____au
Buffer Corrected A_{350}:_____au

Result: Less Than Opalescence Standard
Circle: I II III IV

Test Sample #2

Name: _____
Lot #_____
Volume of Test Sample: _____µL
Sample Mean A_{350} Result:_____au
Buffer Name:_____
Buffer Lot:_____
Buffer Mean A_{350} Result:_____au
Buffer Corrected A_{350}:_____au

Result: Less Than Opalescence Standard
Circle: I II III IV

Test Sample #3

Name: _____
Lot #_____
Volume of Test Sample: _____µL
Sample Mean A_{350} Result:_____au
Buffer Name:_____
Buffer Lot:_____
Buffer Mean A_{350} Result:_____au
Buffer Corrected A_{350}:_____au

Result: Less Than Opalescence Standard
Circle: I II III IV

Test Sample #4

Name: _____
Lot #_____
Volume of Test Sample: _____µL
Sample Mean A_{350} Result:_____au
Buffer Name:_____
Buffer Lot:_____
Buffer Mean A_{350} Result:_____au
Buffer Corrected A_{350}:_____au

Result: Less Than Opalescence Standard
Circle: I II III IV

Test Sample #5

Name: _____
Lot #_____
Volume of Test Sample: _____µL
Sample Mean A_{350} Result:_____au
Buffer Name:_____
Buffer Lot:_____
Buffer Mean A_{350} Result:_____au
Buffer Corrected A_{350}:_____au

Result: Less Than Opalescence Standard
Circle: I II III IV

Test Sample #6

Name: _____
Lot #_____
Volume of Test Sample: _____µL
Sample Mean A_{350} Result:_____au
Buffer Name:_____
Buffer Lot:_____
Buffer Mean A_{350} Result:_____au
Buffer Corrected A_{350}:_____au

Result: Less Than Opalescence Standard
Circle: I II III IV

Pipettes Used:

Performed by: _____ Date: _____

Reviewed by: _____Date: _____

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