SOP Title:	**Determination of Opalescence of a Sample Relative to a Set of Four Opalescence Reference Standards**
SOP Number:	**23127**
Revision:	**03**

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1. PURPOSE

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

2. 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 to ensure proper and consistent utilization.

3. RESPONSIBILITIES

3.1 Director / Process Analytics/Quality Control (PA/QC)

- Defines procedure.

3.2 Personnel / PA/QC

- Trains lab personnel
- Performs procedure.
- Reviews data.
- Documents results.

3.3 Biopharmaceutical Quality Assurance (BQA)

- Provides quality oversight.

4. MATERIALS AND REAGENTS

Part Number	Description	BDP Approved Substitution Permitted?
varies w/ sample under test	Sample Formulation Buffer, received with the sample from production	☒ YES ☐ NO
20091	Kim wipes EX-L	☒ YES ☐ NO
20100	Disposable Serological Pipette10 mL	☐ YES ☒ NO
20102	Disposable Serological Pipette 25 mL	☐ YES ☒ NO
20143	Borosilicate glass culture tubes (12 x 75 mm)	☒ YES ☐ NO
20144	Borosilicate glass culture tubes (16 x 125 mm)	☒ YES ☐ NO
21080	Primary Opalescent and Turbidity Solution*	☒ YES ☐ NO
21107	Disposable Pasteur pipettes	☒ YES ☐ NO
21767	Pipette tips 250 µL	☒ YES ☐ NO
21769	Pipette tips 1000 µL	☒ YES ☐ NO
20767	Nitrile Gloves	☒ YES ☐ NO
20843	Disposable Pasteur pipettes	☒ YES ☐ NO
30060 or 30059	Bovine Serum Albumin Standard or Bovine Gamma Globulin Standard	☒ YES ☐ NO
30853	Methanol	☒ YES ☐ NO
30923	Hexamethylenetetramine	☐ YES ☒ NO
30924	Hydrazine Sulfate	☐ YES ☒ NO
30927	Primary Opalescence Solution *	☒ YES ☐ NO

*** NOTE:** BOTH ARE FORMULATED AT 4000 NTU (NEPHELOMETRIC TURBIDITY UNITS) I.E. THE SAME CONCENTRATION OF FORMAZIN

5. EQUIPMENT

- Beckman Coulter DU800 Spectrophotometer
- Computer Software (proprietary)
- Ultrapure water
- Volumetric Flasks 100 mL or sized as needed.
- Calibrated Pipettors 20-200 μL and 100-1000 μL
- Quartz Cuvettes (1 cm pathlength)
- Aspirator cuvette cleaner with vacuum line
- Vortexer

6. PROCEDURE

6.1 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**.

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

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

6.4 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.

6.5 Using the Cuvette

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

6.5.2 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.

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

6.5.4 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).

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

6.6 Blanking

6.6.1 Fill the cuvette with the Ultrapure water (listed in section 5).

6.6.2 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).

6.6.3 Close the lid before performing the analysis.

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

6.6.5 Left click on the **Sample ID**. Label the sample "Ultrapure H2O, water blank, etc."

6.6.6 Left click on **Read Samples**.

6.6.7 Remove the cuvette from the sample compartment and wash (refer to step 6.5.2).

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

6.7 Standardizing the Instrument

6.7.1 If analyzing antibodies, use Bovine Gamma Globulin (BGG) as the standard (see section 4). For all other samples, use Bovine Serum Albumin (BSA).

6.7.2 Dilute BSA/BGG to 1 mg/mL (1:2) with Ultrapure water in a glass culture tube (see section 4 and 5) 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.

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

6.7.4 Fill the washed cuvette with standard/water prepared in step 6.7.2.

6.7.5 Place the cuvette in the sample compartment number 1 (refer to step 6.6.2).

6.7.6 Left click on **Read Samples**.

6.7.7 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.

6.7.8 Remove the cuvette from the sample compartment and wash (refer to step 6.5.2).

6.7.9 Fill the cuvette with the next standard/water and repeat the reading (2nd and 3rd reading) refer to steps 6.7.4 and 6.7.6.

6.8 Opalescence Standards

6.8.1 Primary Opalescence Solution: Ready to use primary opalescence solution BDP PN 30927 or BDP PN 31080 (See section 4). If using BDP PN 30927 or BDP PN 31080 primary opalescence solution skip to step 6.8.3.

6.8.2 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**.

6.8.3 Standard of Opalescence: A Standard of Opalescence is prepared daily using the Primary Opalescence Solution from step 6.8.1 or 6.8.2. In a 100 mL volumetric flask (see section 5) 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.

6.8.4 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

6.8.5 Read the four Opalescence Reference Suspension Standards (refer to steps 6.7.3-6.7.11).

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

6.8.7 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.

6.8.8 When all samples have been analyzed, standardize the instrument with three freshly prepared standards as listed in step 6.7.7.

6.9 Drift

6.9.1 Repeat the standardization section to ensure the instrument has not drifted 6.7). 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.

6.10 Results

6.10.1 After completing the assay, save the data by clicking on the **File** button and then **Headers for Current Run**

6.10.2 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.

6.10.3 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.

6.10.4 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.

7. DOCUMENTATION AND RECORDS

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

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

8. REFERENCES AND RELATED DOCUMENTS

Document Number	Title
21531	Equipment Logs
22702	Solutions Used in Process Analytics
22941	Operation of Beckman Coulter DU 800 Spectrophotometer
23127-01	Opalescence Standard Preparation
N/A	European Pharmacopoeia 11th Edition (2023) chapter 2.2.2 Clarity and Degree of Opalescence of Liquids