Protocol for Validation of UV Disinfection Reactors Using Lagrangian Actinometry

Web Report #4112

Subject Area: Water Quality
PROTOCOL FOR VALIDATION OF UV DISINFECTION REACTORS USING LAGRANGIAN ACTINOMETRY
PROTOCOL FOR VALIDATION OF UV DISINFECTION REACTORS
USING LAGRANGIAN ACTINOMETRY

Prepared by:
HDR|HydroQual, Inc.
Purdue University

Jointly sponsored by:
**Water Research Foundation**
6666 West Quincy Avenue, Denver CO  80235

And

**New York State Energy Research and Development Authority**
17 Columbia Circle, Albany NY  12203

Published by:
**Water Research Foundation**
DISCLAIMER

This report was prepared by HDR|HydroQual in the course of performing work contracted for and sponsored by the New York State Energy Research and Development Authority and the Water Research Foundation (hereafter the “Sponsors”). The opinions expressed in this report do not necessarily reflect those of the Sponsors of the State of New York, and reference to any specific product, service, process, or method does not constitute an implied or expressed recommendation or endorsement of it. Further, the Sponsors and the State of New York make no warranties or representations, expressed or implied, as to the fitness for particular purpose or merchantability of any product, apparatus, or service of the usefulness, completeness, or accuracy of any processes, methods, or other information contained, described, disclosed, or referred to in this report.
CONTENTS

LIST OF TABLES ........................................................................................................................ vii
LIST OF FIGURES ....................................................................................................................... ix
GLOSSARY .................................................................................................................................. xi
LIST OF UNITS, ABBREVIATIONS, AND ACRONYMS ...................................................... xix
FOREWORD ............................................................................................................................... xxi
ACKNOWLEDGMENTS ......................................................................................................... xxiii
EXECUTIVE SUMMARY ........................................................................................................ xxv

Objectives ....................................................................................................................... xxv
Background ..................................................................................................................... xxvi
Approach ........................................................................................................................ xxvi
Results/Conclusions ...................................................................................................... xxvii
Applications/Recommendations ................................................................................... xxvii
Multimedia .................................................................................................................... xxvii
Research Partners ......................................................................................................... xxviii
Participants ................................................................................................................... xxviii

CHAPTER 1 - INTRODUCTION .................................................................................................. 1

Objectives of this Document............................................................................................... 1
Overview of UV disinfection Reactor Validation Using Lagrangian actinometry ....... 1
Summary Description of Lagrangian Actinometry ....................................................... 1
Lagrangian Actinometry as an Alternate UV Reactor Validation Method ............ 5
Organization of this Document ....................................................................................... 6
Regulations Summary ...................................................................................................... 6

CHAPTER 2 - GENERAL REQUIREMENTS FOR VALIDATION OF UV REACTORS BY
LAGRANGIAN ACTINOMETRY ................................................................................................ 7

Minimum Requirements for Validation Testing ................................................................. 7
Overview of the alternate Validation Protocols ............................................................... 8
Key Steps in Recommended Validation Protocol ............................................................ 8
Alternative Validation Protocols .................................................................................. 14
Third Party Oversight .................................................................................................... 14
Emerging Methods ......................................................................................................... 14

CHAPTER 3 - VALIDATION AND EQUIPMENT SPECIFICATIONS ................................... 17

Dyed Microspheres ........................................................................................................... 17
Characteristics to Consider When Selecting the Dyed Microspheres ................. 17
Equipment Needs for Full-scale Reactor Testing ..................................................... 24
Accuracy of Measurement Equipment .................................................................... 27
Identifying Test Conditions ......................................................................................... 28
Quality-Control Samples for Lagrangian Actinometry Validation .................. 32

CHAPTER 4 - GUIDELINES FOR CONDUCTING EXPERIMENTAL DYED-MICROSPHERES TESTS ................................................................. 35

PREPARATION OF THE DYED MICROSPHERES FOR FIELD TESTS .................... 35
Pre-Irradiation of Dyed Microspheres ............................................................... 35
Full-scale UV Reactor Testing ............................................................................... 36
DMS Field Tests and Sample Collection ................................................................. 36
Collimated Beam Testing for the Dyed Microspheres ..................................................... 38
Collimated Beam Apparatus Design and Operation....................................................... 39
Accuracy of Monitoring Equipment Used for the Collimated Beam Tests................. 40
Collimated Beam Exposures for Dyed Microspheres..................................................... 41
Collimated Beam UV Dose Calculation...................................................................... 43
Analysis of Dyed Microspheres Samples by Flow Cytometry........................................ 43
Principles of Flow Cytometry....................................................................................... 44
Flow Cytometer Specifications and Settings............................................................... 44
Flow Cytometry Data Analysis..................................................................................... 45
CHAPTER 5 - VALIDATION: DATA REDUCTION AND ANALYSIS .................................. 55
Overview...................................................................................................................... 55
Deconvolution and Microbial Inactivation Predictions: Basic Principles ...................... 55
Numerical Interpolation of UV Dose-Response Data .................................................. 56
Deconvolution Algorithm.............................................................................................. 57
Deconvolution Program............................................................................................... 59
Constraints on the deconvolution algorithm............................................................... 60
Numerical starting seed for the deconvolution algorithm........................................... 60
Dose-Response Matrix Optimization.......................................................................... 61
Estimation of Microbial (Log) Inactivation.................................................................... 62
Case Study for Application of LA ................................................................................ 65
Case Study Background: ............................................................................................. 66
Development of Log Inactivation for Setpoint and Calculated Approaches................ 80
Determining the Maximum Log Inactivation for the UV Intensity Setpoint Approach... 80
Developing a Log Inactivation Equation for the Calculated LI Control Approach.... 80
Deriving the Validation Factor (VF)............................................................................. 81
Uncertainty of Validation (UVal).................................................................................. 82
Determining the Validated Log Inactivation................................................................. 84
Determining the Validated LI and Operating Conditions for the UV Intensity Setpoint Approach................................................................. 84
Determining the Validated LI and Operating Conditions for the Calculated LI Approach........................................................................................................ 85
REFERENCES ............................................................................................................ 87
# LIST OF TABLES

Table 2-1. Summary of LT2SWTR Validation Requirements (Excerpted from UVDGM Table 5.1, Chapter 5) ................................................................. 8
Table 3-1. Factors to be considered in validation test design ............................................................... 29
Table 3-2. Minimum Test Conditions for the UV Intensity Setpoint Approach ..................... 31
Table 5-1. Summary of Example Reactor Operating Conditions ................................................ 66
Table 5-2. UV dose requirements for inactivation of *C. parvum*, *G. lamblia* and Adenovirus (from *UV Disinfection Guidance Manual*, EPA, 2006) .............. 80
LIST OF FIGURES

Figure ES-1. Representation of the Dyed-microspheres Lagrangian Actinometry Process ... xxvi
Figure 1-1. Basic UV photochemistry of $S$ in aqueous solution (adapted from Bergstrom et al., 1982) .................................................................................................................................................. 3
Figure 1-2. Schematic illustration of the application of Lagrangian actinometry ......................... 4
Figure 2-1. Step 1 and Step 2 in Lagrangian actinometry validation process ............................... 10
Figure 2-2. Step 3 in the Lagrangian actinometry validation process .......................................... 12
Figure 2-3. Step 4 and 5 in the Lagrangian actinometry validation process ............................... 13
Figure 3-1. Spectral sensitivity of DMS, B. Subtilis spores, and coliphage MS2 to germicidal UV radiation (from Shen et al., 2009, and Mamane-Gravetz et al., 2005) ........................................................................................................... 18
Figure 3-2. Rotational devices used for mixing of microsphere suspensions to accomplish conjugation of biotinylated-$S$ to avidin-coated microspheres. The devices typically used to promote mixing in these applications are rotary mixers (a) and clinical rotators (b) ........................................................................ 23
Figure 4-1. Collimated Beam Apparatus ..................................................................................... 39
Figure 4-2. Typical results of a DMS UV dose-response experiment. Labels in the legend refer to the nominal UV$_{254}$ dose delivered to each sample in the experiment, in units of mJ/cm$^2$ ................................................................................................................. 42
Figure 4-3. Forward scatter and side scatter signals of a DMS sample detected by flow cytometry (The cytogram was generated using the freeware, WinMDI provided by the Cytometry Lab at Purdue University, Indiana)........................................... 47
Figure 4-4. DMS FI versus forward scatter signal of a DMS sample detected by flow cytometry (The cytogram was generated using the freeware, WinMDI provided by the Cytometry Lab at Purdue University, Indiana)........................................... 48
Figure 4-5. Application of threshold of 10 to the cytometric data set shown in Figure 4-3,... 49
Figure 4-6. Definition of a gate based on the size and granularity of DMS using a sample with limited background particles. This sample is usually prepared from the direct dilution of a DMS stock, which is the same DMS source for the reactor validation test ...................................................................................................................... 50
Figure 4-7. Application of the gate R1, defined in Figure 4-6, to a DMS sample collected from a UV validation challenge test. Left upper panel is before applying gate R1, which shows no clear separation between DMS and background particles. The right upper panel overlays R1 on the raw data set. Then with the application of R1 (eliminating particles outside of gate), the lower panel illustrates a gated particle population with the same size and granularity as DMS ........................................................................................................................................ 51
Figure 4-8. Definition of gate R2 based on the fact that the DMS possess higher fluorescence than background particles with the similar size and granularity .................................................. 52
Figure 4-9. Upper panel shows a DMS group defined by the intersection of the two gates R1 and R2, and the lower panel is a histogram representing the DMS group’s particle abundance at different FI levels ...................................................................................................................... 53
PROTOCOL FOR VALIDATION OF UV DISINFECTION REACTORS USING LAGRANGIAN ACTINOMETRY

Figure 5-1. Relative sum-of-squares residuals as function of the number of columns in the dose response matrix \([\gamma]\). A demonstration of the effect of the optimizing algorithm for the dose response matrix \([\gamma]\). .......................................................... 62

Figure 5-2. Hypothetical UV dose distribution delivered by a reactor......................................................... 63

Figure 5-3. Comparison of predicted challenge organism inactivation (using DMS actinometry, y axis) with measured inactivation responses for the same organism in validation experiments conducted on an LPHO reactor .......................... 65

Figure 5-4. Example FI histogram from flow cytometry software. Notice the units of abundance on the y axis. ....................................................................................... 67

Figure 5-5. Example FI distribution after normalization; notice the change in vertical axis scale....................................................................................................................... 68

Figure 5-6. Example of four-parameter Weibull distribution fitted to a FI distribution.  
The black line represents the normalized FI distribution for a sample that was subjected to a UV dose of 60 mJ/cm² under a collimated beam, while the red line represents a non-linear fit of the four-parameter Weibull distribution to the data ........................................................................................................................................... 69

Figure 5-7. Four-parameter Weibull distribution parameters fitted with linear splines as a function of UV dose (see equations 5-23 and 5-24) ............................................... 70

Figure 5-8. Example output of FI distribution predictions for doses of 0, 100, and 400 mJ/cm² from a DMS dose-response experiment.......................................................... 72

Figure 5-9. Combination of FI Distributions from Flow-through Replicate Samples for Noise Reduction .................................................................................................................. 72

Figure 5-10. Generation of a known dose distribution through a convolution experiment...... 74

Figure 5-11. Dose distribution of mixed sample in convolution experiment.............................................. 75

Figure 5-12. Measurements of FI from convolution experiment. FI distributions based on measurements of sample developed from equal-volume mixture of subsamples that had been exposed to known doses under collimated beam (dose mixture) were compared with calculated FI distribution based on convolution of data from individual doses (convolution) (from Blatchley et al., 2006) ........................................ 75

Figure 5-13. Known FI distribution information for an effluent sample from LPHO UV reactor (left: dose response matrix, right: FI distribution for effluent DMS sample from a continuous-flow reactor) .................................................................................................................. 76

Figure 5-14. Example of initial numerical seed to start deconvolution algorithm .......................... 77

Figure 5-15. Solution for deconvolution algorithm, an estimated dose distribution for an LPHO reactor (top panel: deconvolved dose distribution (PDF and CDF); bottom panel: FI distribution from LPHO UV reactor (dotted blue) fitted with the convolution (solid red) of the dose response matrix \([\gamma]\) with the deconvolved dose distribution vector \([\alpha]\)). ........................................................... 78
GLOSSARY

The following definitions are the union of new terms relevant to Lagrangian actinometry and terms that were excerpted from the glossary in the UVDGM, 2006. As stated in the UVDGM, these definitions are derived from existing UV literature, standard physics textbooks, and/or industry standards and conventions. Some concepts have more than one acceptable term or definition, but for consistency within the document, only one term is used.

**Absorption (Absorbance)** – the transformation of UV radiation to other forms of energy as it passes through a substance.

**A254 (UV Absorbance at 254 nm)** – a measure of the amount of UV radiation that is absorbed by a substance at 254 nm.

**Actinometry** – see Chemical Actinometry

**Action Spectrum** – the relative efficiency of UV energy frequencies at inactivating microorganisms. Each microorganism has a unique action spectrum.

**Bacteriophage** – a virus that infects bacterial cells and can be used a microbial surrogate during validation testing.

**Ballast** – an electrical device that provide the proper voltage and current required to initiate and maintain the gas discharge within the UV lamp.

**Batch Reactor** – A batch reactor is a volume where the reactants and the catalyst are placed in the reactor which is then closed to transport of matter and the reaction is allowed to proceed for a given time whereupon the mixture of unreacted material together with the products is withdrawn.

**Bioassay** – in the context of this document, an empirical assessment of the inactivation response of a specific microorganism to a controlled dose of UV light, usually in UV reactors. Bioassay has been used in the UV disinfection literature in the same context as “biodosimetry” (see Biodosimetry).

**Biodosimetry** – a procedure used to determine the reduction equivalent dose (RED) of a UV reactor. Biodosimetry involves measuring the inactivation of a challenge microorganism after exposure to UV light in a UV reactor and comparing the results to the known UV dose-response curve of the challenge microorganism (determined via bench-scale collimated beam testing).

**Calculated Dose Approach** – See Dose-monitoring Strategy.

**Centrifuge** – a machine using centrifugal force for separating substances of different densities, for removing moisture, or for simulating gravitational effects.

**Centrifugal Pump** – a pump that applies energy to a fluid by a moving rotor.
Challenge Microorganism – a non-pathogenic microorganism used in validation testing of UV reactors.

Chemical Actinometry (Actinometry) – a chemical system that undergoes a light-induced reaction (at a certain wavelength) for which the quantum yield is known.

Coliphage – a bacteriophage that infects the bacterium *E. Coli*.

Collimated Beam – a device used to collimate UV radiation so that the UV dose-response of a challenge microorganism can be quantified. Both time and UV light intensity are directly measured; the UV dose is calculated using the intensity of the incident UV light, UV absorbance of the water, and exposure time.

Convolution – a mathematical operation that combines two signals, \( f \) and \( g \), to get a third signal \( y \).

Cytidine -- A white crystalline nucleoside, \( C_9H_{13}N_3O_5 \), composed of one molecule each of cytosine and ribose.

Cytograph – The population density plot that is a result of a flow cytometry analysis that is used for numerical gating in the Lagrangian actinometry method. A cytograph can display densities of multiple parameters that are measured from a flow cytometer.

Deconvolution – a mathematical term used to describe the inverse process of a convolution of recorded or measured data (see Convolution).

Design UVT – The minimum UVT that will typically occur at the design flow of the UV facility. The design UVT and design flow are typically used by the UV manufacturer to determine the appropriate UV equipment for a target pathogen inactivation. The design UVT may not necessarily be the minimum operating UVT (see Minimum Operating UVT).

Dose Distribution – see UV Dose.

Dose-monitoring Strategy – the method by which a UV reactor maintains the required dose at or near some specified value by monitoring UV dose delivery. Such strategies must include, at a minimum, flow rate and UV intensity (measured via duty UV sensor[s]) and lamp status. They sometimes include UVT and lamp power. Two common Dose-monitoring Strategies that are discussed in this manual are the UV Intensity Setpoint Approach and the Calculated Dose Approach.

- **The UV Intensity Setpoint Approach** relies on one or more “setpoints” for UV intensity that are established during validation testing to determine UV dose. During operations, the UV intensity as measured by the UV sensors must meet or exceed the setpoint(s) to ensure delivery of the required dose. Reactors must also be operated within validated operation conditions for flow rates and lamp status [40 CFR 141.720(d)(2)]. In the UV Intensity Setpoint Approach, UVT does not need to be monitored separately. Instead, the intensity
readings by the sensors account for changes in UVT. The operating strategy can be with either a single setpoint (one UV intensity setpoint is used for all validated flow rates) or a variable setpoint (the UV intensity setpoint is determined using a lookup table or equation for a range of flow rates).

- **The Calculated Dose Approach** uses a dose-monitoring equation to estimate the UV dose based on operating conditions (typically flow rate, UV intensity, and UVT). The dose-monitoring equation may be developed by the UV manufacturers using numerical methods; however, EPA recommends that systems use an empirical dose-monitoring equation developed through validation testing. During reactor operations, the UV reactor control system inputs the measured parameters into the dose-monitoring equation to produce a calculated dose. The system operator divides the calculated dose by the Validation Factor (see Chapter 5 for more details on the Validation Factor) and compares the resulting value to the required dose for the target pathogen and log inactivation level.

**Duty UV Sensor (or Duty Sensor)** – the duty (on-line) UV sensor installed in the UV reactor that monitors UV intensity during UV equipment operations.

**Dyed Microspheres (DMS)** – polystyrene microspheres that are coated with a dye that is derived from cytidine. Dyed microspheres are used in Lagrangian actinometry to measure a UV dose distribution.

**Emission Spectrum** – the relative power emitted by a lamp at different wavelengths.

**Flow Cytometer** – an optical analytical instrument that is capable of measuring the optical characteristics of individual particles (or cells) at relatively high particle counts per second.

**Flow Cytometry** -- is a technique for counting and examining microscopic particles, such as cells and chromosomes, by suspending them in a stream of fluid and passing them by an electronic detection apparatus. Flow cytometry provides information on size, granularity, and fluorescence intensity (FI) of particles in an aqueous suspension, such as the DMS.

**Fluorescence** – the emission of radiation, especially of visible light, by a substance during exposure to external radiation, as light or x-rays.

**Fluorescence Intensity (FI)** – the intensity of fluorescent light emitted from an excited particle (cell or molecule). In Lagrangian actinometry, fluorescence intensity refers to the intensity of fluorescent light emitted from the surface of a dyed microsphere during excitation in the flow cytometry analysis.

**Gas Discharge** – a mixture of non-excited atoms, excited atoms, cations, and free electrons formed when a sufficiently high voltage is applied across a volume of gas. Most commercial UV lamps use mercury gas discharges to generate UV light.

**Gating** -- is a data post-processing method in flow cytometry to differentiate a population space based on physical/chemical characteristics of target particles.
**Germicidal Effectiveness** – the relative inactivation efficiency of each UV wavelength in an emission spectrum. This value is usually approximated by the relative absorbance of DNA at each wavelength.

**Germicidal Range** – the range of UV wavelengths responsible for microbial inactivation in water (200 to 300 nm).

**Germicidal Sensor** – A UV sensor with a spectral response that peaks between 250 and 280 nm and has less than 10 percent of its total measurement due to light above 300 nm when mounted on the UV reactor and viewing the UV lamps through the water that will be treated at the water treatment plant.

**Inactivation** – in the context of UV disinfection, a process by which a microorganism is rendered unable to reproduce, thereby rendering it unable to infect a host.

**Irradiance** -- The density of radiation incident on a given surface usually expressed in Watts per square centimeter or square meter.

**Lagrangian** – A term used refer to a frame of reference for a calculation or measurement that is particle-specific. In the case of Lagrangian actinometry, the term Lagrangian refers to UV dose being measured at the level of an individual dyed microsphere.

**Lagrangian Actinometry (LA)** – The application of chemical actinometry at the level of an individual particle (dyed microsphere) to measure a UV dose distribution within a UV reactor.

**Lamp Envelope** – the exterior surface of the UV lamp, which is typically made of quartz.

**Lamp Sleeve** – the quartz tube or thimble that surrounds and protects the UV lamp. The exterior is in direct contact with the water being treated. There is typically an air gap (approximately 1 cm) between the lamp envelope and the quartz sleeve.

**Lignin Sulfonate** – a commercially available liquid lignin mixture (typically procured from paper mills) used to adjust the UV transmittance of natural waters during validation testing.

**Low-pressure (LP) Lamp** – a mercury-vapor lamp that operates at an internal pressure of 0.13 to 1.3 Pa (2 x 10-5 to 2 x 10-4 psi) and electrical input of 0.5 watts per centimeter (W/cm). This results in essentially monochromatic light output at 254 nm.

**Low-pressure high-output (LPHO) Lamp** – a low-pressure mercury-vapor lamp that operates under increased electrical input (1.5 to 10 W/cm), resulting in a higher UV intensity than low-pressure lamps. It also has essentially monochromatic light output at 254 nm.

**Medium-pressure (MP) Lamp** – a mercury vapor lamp that operates at an internal pressure of 1.3 and 13,000 Pa (2 to 200 psi) and electrical input of 50 to 150 W/cm. This results in a
polychromatic (or broad spectrum) output of UV and visible light at multiple wavelengths, including wavelengths in the germicidal range.

**Microspheres** – are small spherical particles ranging in diameters from 1 micron to 1,000 microns. Microspheres can be fabricated from materials ranging from polystyrene to magnetic metals.

**Minimum Operating UVT**: The lowest UVT expected to occur during lifetime of the UV facility. Understanding the minimum UVT is critical because the UV reactor should be designed and validated for the range of UVT and flow rate combinations expected at the WTP to avoid off-specification operation.

**Monochromatic** – light output at only one wavelength, such as UV light generated by low-pressure and low-pressure high-output lamps.

**MS-2 Bacteriophage** – a non-pathogenic bacteriophage commonly used as a challenge organism in UV reactor validation testing.

**Non-germicidal Sensor** – A UV sensor with a spectral response that is not restricted to the germicidal range (see “Germicidal Sensor” for more details).

**Operating Strategy** – the strategy used by the PWS to operate the UV equipment with the UV Intensity Setpoint Approach. Typically, single setpoint or variable setpoint operation is used.

**Peristaltic Pump** – a pump that uses positive displacement to move a variety of fluids.

**Petri Factor** – a ratio used in collimated beam testing that is equal to the average intensity measured across the surface of a suspension in a petri dish divided by the intensity at the center of a petri dish.

**Photoisomerization** -- Photochemical process leading to an isomerization of the substrate, either by bond rotation, skeletal rearrangement or atom- or group- transfer.

**Polychromatic** – light energy output at several wavelengths such as with MP lamps.

**Polychromatic Bias** – a potential bias in validation test data resulting from polychromatic differences between validation and operation of a UV reactor at a water system. Polychromatic bias can occur in MP reactors when non-germicidal sensors are used.

**Pre-irradiation** – exposure of the newly prepared dyed microsphere stock suspension to UV radiation under a collimated beam before validation testing. This allows for the dyed microspheres to have an initial fluorescence so that differentiation against ambient particles is possible during numerical gating.

**Quartz Sleeve** – see lamp sleeve.
Quantum Yield – is defined as the the number of events per photon absorbed. Specifically in photochemistry, the quantum yield ($\Phi_\lambda$) is the amount of reactant consumed (or product formed) per absorbed photon.

Radiometer – an instrument used to measure UV irradiance.

Reduction Equivalent Dose (RED) – see UV Dose.

Reduction Equivalent Dose (RED) Bias – a correction that accounts for the difference between the UV dose measured with a surrogate microorganism and the UV dose that would be delivered to a target pathogen due to differences in the microorganisms’ inactivation kinetics.

Reference UV Sensor (or Reference Sensor) – a calibrated, off-line UV sensor used to monitor duty UV sensor calibration and to determine UV sensor uncertainty.

Required Dose – the UV dose required for a certain level of log inactivation. Required doses are set forth by the LT2ESWTR.

Setpoint (also called “operational setpoint”) – a specific value for a critical parameter, such as UV intensity, that is related to UV dose. Setpoints are established during validation testing. During operations, the PWS compares the measured parameter to the setpoint to confirm performance.

Spectral Response – A measure of the output of the UV sensor as a function of wavelength.

State – the agency of the state or Tribal government that has jurisdiction over public water systems. During any period when a state or Tribal government does not have primary enforcement responsibility pursuant to section 1413 of the Act, the term “state” means the Regional Administrator, U.S. Environmental Protection Agency.

Streptavadin – A protein from the bacterium *Streptomyces avidinii* that has an extraordinarily high affinity with biotin.

Target Log Inactivation - For the target pathogen, the specific log inactivation the PWS wants to achieve using UV disinfection. The target log inactivation is driven by requirements of the SWTR, LT1ESWTR, IESWTR, and LT2ESWTR.

Target Pathogen (also called “target microorganism”) – For the purposes of this manual, the target pathogen is defined as the microorganism for which a PWS wants to obtain inactivation credit using UV disinfection.

UV Absorbance (A) – a measure of the amount of UV light that is absorbed by a substance (e.g., water, microbial DNA, lamp envelope, quartz sleeve) at a specific wavelength (e.g., 254 nm). This measurement accounts for absorption and scattering in the medium (e.g., water). Standard Method 5910B details this measurement method. However, for UV disinfection applications, the sample should not be filtered or adjusted for pH as described in Standard Methods.
UV Dose – the UV energy per unit area incident on a surface, typically reported in units of mJ/cm² or J/m². The UV dose received by a waterborne microorganism in a reactor vessel accounts for the effects on UV intensity of the absorbance of the water, absorbance of the quartz sleeves, reflection and refraction of light from the water surface and reactor walls, and the germicidal effectiveness of the UV wavelengths transmitted. This guidance manual also uses the following terms related to UV dose:

- **UV dose distribution** – the probability distribution of delivered UV doses that microorganisms receive in a flow-through UV reactor; typically shown as a histogram.

- **Reduction Equivalent Dose (RED)** – The UV dose derived by entering the log inactivation measured during full-scale reactor testing into the UV dose-response curve that was derived through collimated beam testing. RED values are always specific to the challenge microorganism used during experimental testing and the validation test conditions for full-scale reactor testing.

- **Required Dose (Dreq)** – The UV dose in units of mJ/cm² req needed to achieve the target log inactivation for the target pathogen. The required dose is specified in the LT2ESWTR and presented in Table 1.4 of this guidance manual.

- **Validated Dose (Dval)** – The UV dose in units of mJ/cm² delivered by the UV reactor as determined through validation testing. The validated dose is compared to the Required Dose (Dreq) to determine log inactivation credit.

- **Calculated Dose** - the RED calculated using the dose-monitoring equation that was developed through validation testing.

UV Dose-Response – the relationship indicating the level of inactivation of a microorganism as a function of UV dose.

UV Equipment – the UV reactor and related components of the UV disinfection process, including (but not limited to) UV reactor appurtenances, ballasts, and control panels.

UV Facility – all of the components of the UV disinfection process, including (but not limited to) UV reactors, control systems, piping, valves, and building (if applicable).

UV Intensity – the power passing through a unit area perpendicular to the direction of propagation. UV intensity is used in this guidance manual to describe the magnitude of UV light measured by UV sensors in a reactor and with a radiometer in bench-scale UV experiments.


UV Irradiance – the power per unit area incident to the direction of light propagation at all angles, including normal.

UV Radiation – light emitted with wavelengths from 200 to 400 nm.
UV Reactor – the vessel or chamber where exposure to UV light takes place, consisting of UV lamps, quartz sleeves, UV sensors, quartz sleeve cleaning systems, and baffles or other hydraulic controls. The UV reactor also includes additional hardware for monitoring UV dose delivery; typically comprised of (but not limited to): UV sensors and UVT monitors.

UV Reactor Validation – Experimental testing to determine the operating conditions under which a UV reactor delivers the dose required for inactivation credit of Cryptosporidium, Giardia lamblia, and viruses.

UV Sensitivity – the resistance of a microorganism to inactivation by UV light, expressed as mJ/cm² per log inactivation.

UV Sensor – a photosensitive detector used to measure the UV intensity at a point within the UV reactor that converts the signal to units of milliamps (mA).

UV Transmittance (UVT) – a measure of the fraction of incident light transmitted through a material (e.g., water sample or quartz). The UVT is usually reported for a wavelength of 254 nm and a pathlength of 1-cm. If an alternate pathlength is used, it should be specified or converted to units of cm⁻¹. UVT is often represented as a percentage and is related to the UV absorbance (A254) by the following equation (for a 1-cm path length): % UVT = 100 x 10⁻A.

Validated Dose – see UV Dose.

Validation Factor – an uncertainty term that accounts for the bias and uncertainty associated with validation testing.

Validation Uncertainty – an uncertainty term that accounts for error in measurements made during validation testing to develop the UV intensity setpoint(s) (for the UV Intensity Setpoint Approach) or dose-monitoring equation (for the Calculated Dose Approach).

Visible Light – Wavelengths of light in the visible range (380 – 720 nm).
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda )</td>
<td>wavelength</td>
</tr>
<tr>
<td>( \mu g )</td>
<td>microgram</td>
</tr>
<tr>
<td>( \mu g/mL )</td>
<td>microgram per milliliter</td>
</tr>
<tr>
<td>( \mu m )</td>
<td>micrometer, micron</td>
</tr>
<tr>
<td>A254</td>
<td>ultraviolet light absorbance at 254 nanometers</td>
</tr>
<tr>
<td>AIAA</td>
<td>American Institute of Aeronautics and Astronautics</td>
</tr>
<tr>
<td>APHA</td>
<td>American Public Health Association</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>B_{poly}</td>
<td>polychromatic bias</td>
</tr>
<tr>
<td>°C</td>
<td>degree Centigrade</td>
</tr>
<tr>
<td>CDF</td>
<td>cumulative distribution function</td>
</tr>
<tr>
<td>CFD</td>
<td>computational fluid dynamics</td>
</tr>
<tr>
<td>CFD-I</td>
<td>computational fluid dynamics with intensity field model</td>
</tr>
<tr>
<td>CFR</td>
<td>Code of Federal Regulations</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>DMS</td>
<td>dyed microspheres</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DReq</td>
<td>required UV dose</td>
</tr>
<tr>
<td>EOLL</td>
<td>end-of-lamp-life</td>
</tr>
<tr>
<td>EPA</td>
<td>U.S. Environmental Protection Agency</td>
</tr>
<tr>
<td>E_s</td>
<td>Average incident irradiance</td>
</tr>
<tr>
<td>°F</td>
<td>degree Fahrenheit</td>
</tr>
<tr>
<td>FI</td>
<td>fluorescence intensity</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward Scatter</td>
</tr>
<tr>
<td>FTR</td>
<td>flow-through reactor</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g/L</td>
<td>gram per liter</td>
</tr>
<tr>
<td>g/mL</td>
<td>gram per milliliter</td>
</tr>
<tr>
<td>GAC</td>
<td>granular activated carbon</td>
</tr>
<tr>
<td>gpm</td>
<td>gallon per minute</td>
</tr>
<tr>
<td>GUI</td>
<td>graphical user interface</td>
</tr>
<tr>
<td>I</td>
<td>intensity field</td>
</tr>
<tr>
<td>J</td>
<td>joule</td>
</tr>
<tr>
<td>kVA</td>
<td>kilovolt ampere</td>
</tr>
<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>LA</td>
<td>Lagrangian actinometry</td>
</tr>
<tr>
<td>log I, LI</td>
<td>log Inactivation</td>
</tr>
<tr>
<td>LP</td>
<td>low pressure</td>
</tr>
<tr>
<td>LPHO</td>
<td>low pressure high output</td>
</tr>
<tr>
<td>LSA</td>
<td>lignin sulfonic acid</td>
</tr>
<tr>
<td>LT1ESWTR</td>
<td>Long Term 1 Enhanced Surface Water Treatment Rule</td>
</tr>
<tr>
<td>LT2ESWTR</td>
<td>Long Term 2 Enhanced Surface Water Treatment Rule</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
</tbody>
</table>
PROTOCOL FOR VALIDATION OF UV DISINFECTION REACTORS USING LAGRANGIAN ACTINOMETRY

mM  millimolar
mA  milliampere
mg/L  milligram per liter
mgd  million gallon per day
mJ  millijoule
mJ/cm^2  millijoule per centimeter squared
mL  milliliter
MP  medium pressure
MS2  male-specific-2 bacteriophage
MW  molecular weight
mW/cm^2  milliwatt per centimeter squared
N  microbial concentration after UV exposure
N0  initial microbial concentration (prior to UV exposure)
NIST  National Institute of Standards and Technology
nm  nanometer
NWRI  National Water Research Institute
NYSERDA  New York State Energy Research and Development Authority
O&M  operation and maintenance
P  fluorescent chromophore, 3-\(\beta\)-D-ribofuranosyl-2,7-dioxopyrido[2,3-d]pyrimidine
Pa  pascal
pdf  probability density function
Pf  Petri factor
pfu/mL  plaque forming units per milliliter
PLC  programmable logic controller
PWS  public water system
QA/QC  quality assurance/quality control
RED  reduction equivalent dose
RNA  ribonucleic acid
s  second
S  Sensor intensity reading at a power level not equal to 100%
S0  Sensor intensity reading at 100% power level and maximum UVT
S  non-fluorescent chromophore, (E)-5-[2-(methoxycarbonyl)ethenyl]cytidine
t  time
T1  bacteriophage T1
UV  ultraviolet
UVDGM  Ultraviolet Disinfection Guidance Manual
UVaL  Uncertainty in Validation
UDR  Uncertainty of the Dose-response Fit
UIN  Uncertainty in Interpolation
US  Uncertainty in UV Sensor Measurements
USP  Uncertainty in the Setpoint Value
UVT  ultraviolet transmittance
VF  validation factor
W  watt
WEF  Water Environment Federation
WTP  water treatment plant
FOREWORD

The Water Research Foundation (WRF) is a nonprofit corporation dedicated to the development and implementation of scientifically sound research designed to help drinking water utilities respond to regulatory requirements and address high-priority concerns. WRF’s research agenda is developed through a process of consultation with WRF subscribers and other drinking water professionals. WRF’s Board of Trustees and other professional volunteers help prioritize and select research projects for funding based upon current and future industry needs, applicability, and past work. WRF sponsors research projects through the Focus Area, Emerging Opportunities, and Tailored Collaboration programs, as well as various joint research efforts with organizations such as the U.S. Environmental Protection Agency and the U.S. Bureau of Reclamation.

This publication is a result of a research project fully funded or funded in part by WRF subscribers. WRF’s subscription program provides a cost-effective and collaborative method for funding research in the public interest. The research investment that underpins this report will intrinsically increase in value as the findings are applied in communities throughout the world. WRF research projects are managed closely from their inception to the final report by the staff and a large cadre of volunteers who willingly contribute their time and expertise. WRF provides planning, management, and technical oversight and awards contracts to other institutions such as water utilities, universities, and engineering firms to conduct the research.

A broad spectrum of water supply issues is addressed by WRF's research agenda, including resources, treatment and operations, distribution and storage, water quality and analysis, toxicology, economics, and management. The ultimate purpose of the coordinated effort is to assist water suppliers to provide a reliable supply of safe and affordable drinking water to consumers. The true benefits of WRF’s research are realized when the results are implemented at the utility level. WRF’s staff and Board of Trustees are pleased to offer this publication as a contribution toward that end.

Denise L. Kruger
Chair, Board of Trustees
Water Research Foundation

Robert C. Renner, P.E.
Executive Director
Water Research Foundation
ACKNOWLEDGMENTS

The authors of this report are Dr. Chengyue Shen, HDR|HydroQual, Dr. Ernest Blatchley, Purdue University, Dr. Eric Cox, HDR|HydroQual and Mr. Karl Scheible, HDR|HydroQual. The authors wish to acknowledge the New York State Energy Research and Development Authority and the Water Research Foundation for their financial and technical support. We thank the organizations respective Project Officers, Kathleen O’Connor, P.E. and Hsiao-Wen Chen for their insight and patience.

This Protocol development has its genesis in the work at Purdue University, led by Dr. Blatchley, and we thank him and his team for their leadership in developing this unique technology. A special thanks also to Dr. Dominic DiToro, University of Delaware, for his insights and help in developing the deconvolution methods.

We appreciate the time and technical input of the Technical Advisory Committee that joined in its review of the protocol development as it progressed:
- James Malley, University of New Hampshire
- Richard Sakaji, East Bay Municipal Utilities District
- Brian Bernados, California Department of Public Health
- Betsy Lichti, California Department of Public Health
- Paul Zambratto, USEPA, Region 2
- Michael Montysko, NYS Department of Health
- Christine Cotton, Malcolm Pirnie/Arcadis
- Matthew Valade, Hazen and Sawyer
- Steven Via, AWWA
- William Sullivan, Boston MWRA
- Ramesh Kashinkunti, Greater Cincinnati Water Works
- Jason Fleming, Greater Cincinnati Water Works
- Brian Petri, Trojan Technologies
- Phyllis Posy, Atlantium LTD
- Michael Newberry, ITT Wedeco
- Samuel Hayes, USEPA-Cincinnati
- Daniel Leprade
- Keith Bircher, Calgon Carbon
- Thomas Hargy, Clancy Environmental
- Shawn Wagner, Newark OH Water Treatment Plant
- Po-Shun Chan, Purdue University
EXECUTIVE SUMMARY

OBJECTIVES

The *Ultraviolet Disinfection Guidance Manual* (UVDGM) was issued by the USEPA (November 2006) in support of the Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR). It provides guidance in the evaluation, design, implementation and operation of UV disinfection systems at water treatment plants. A key element of the UVDGM is a protocol for validating the dose-delivery performance of the UV system, a specification that is required at full-scale by the LT2ESWTR in order to gain credit for *Cryptosporidium*, *Giardia* and/or virus inactivation. The UVDGM accounts for uncertainties associated with using challenge organisms for validation, and assumes a worst-case dose distribution because biodosimetry methods are unable to estimate a UV system’s dose distribution. To account for these uncertainties, the reactor RED performance is significantly downgraded through the use of the *RED bias*.

Lagrangian actinometry (LA) circumvents this penalty by directly measuring the UV dose distribution within a UV system and eliminates the use of surrogate challenge organisms for RED predictions. This document provides a protocol for using LA to validate UV systems by directly measuring their dose distribution. The intent of this document is to provide a standardized protocol for validation of UV systems using LA. This protocol is designed to be a supplement to the UVDGM. It is written in a format similar to the UVDGM (Chapter 5), and relies on the general protocols provided by the UVDGM that are common to both of the validation processes.

BACKGROUND

Lagrangian actinometry (LA) was developed as a method for measurement of the UV dose distribution delivered by a UV reactor for a given set of operating conditions. The name of the method – Lagrangian actinometry – indicates that actinometry is being applied for UV dose measurement from a Lagrangian frame of reference. In other words, the method allows for dose measurement at the level of an individual particle.

In LA, a compound \( S \) is first biotinylated, then conjugated to the surface of a streptavidin-coated polystyrene microsphere through a streptavidin-biotin linkage. This molecule \( S \) (non-fluorescent) is converted to a molecule \( P \) (fluorescent) when exposed to UV radiation. The \( S \rightarrow P \) phototransformation that takes place on the surface of the microsphere results in the microsphere becoming fluorescent, with the fluorescence intensity (FI) of an individual microsphere being a function of the dose of UV radiation to which it has been subjected (Figure iii-1).
The application of LA for measurement of the dose distribution delivered by a UV reactor involves several steps. This procedure is repeated over a range of operating conditions, thereby allowing quantification of the UV dose distribution delivered by the reactor as a function of operating conditions. In turn, the dose distribution measurements can be integrated with UV dose-response behavior for one or more target microorganisms, using a mathematical analog of the segregated-flow model, to allow definition of the performance of the reactor (relative to the desired antimicrobial endpoint[s]) as a function of operating conditions.

**APPROACH**

This document is constructed in essentially the same format as the UVDGM (Chapter 5) and certain aspects of UVDGM Appendices A, B, C and D. The Table of Contents is similar to that of the UVDGM Chapter 5, and some sections of this document simply refer to the UVDGM. This similarity is intentional – although LA represents a new validation method, its objectives, general applications approach and results are similar to those articulated for biodosimetry in the UVDGM. By putting it into the context of the UVDGM validation process, it is anticipated that the transition from current validation practices (i.e., biodosimetry) will be facilitated, particularly with respect to the analysis of data and the determination of credited log inactivation and RED.

The main tasks that are necessary to accomplish in the development of this protocol are the following: (1) Formalization of the experimental and numerical methods in LA so that UV reactor validation experiments can be accurately and repetitively conducted using LA. (2) Standardization of the multi-step data analysis. (3) Formulation and development of statistical methods used to assess uncertainty in the LA method. (4) Development of a user-friendly graphical user interface (GUI) to assist in the reduction of LA data from their raw form to UV dose distributions.
RESULTS/CONCLUSIONS

This project resulted in a UV validation protocol that is based on the LA method. The protocol was constructed in essentially the same format as the UVDGM (Chapter 5) and certain aspects of UVDGM Appendices A, B, C and D. Experimental methods in LA were standardized along with numerical deconvolution steps and statistical methods. For the facilitation of the data analysis, a GUI was written that will allow an analyst to reduce the LA data from FI distributions to dose distributions. The GUI runs natively in MATLAB, and will significantly reduce the time-to-solution in the LA method. A main conclusion that resulted from the production of this document is that LA is a viable method for the validation of UV systems.

APPLICATIONS/RECOMMENDATIONS

This document has also been developed so as to describe the benefits and limitations of the method. Overall, the objective of this document is to describe LA in sufficient detail to allow other organizations to apply the method and reach consistent, repeatable results. Moreover, the document is intended to provide guidance to state regulatory agencies regarding the application and interpretation of LA as a UV reactor validation protocol. As such, the procedures within this document can be followed by any utility or validation facility that wishes to use LA as a supplement to traditional biodosimetry testing or as an alternative to that approach.

Validation by LA eliminates the significant downgrade in reactor performance via the UVDGM RED Bias. This will result in a positive economic benefit for utilities. Utilities can realize this benefit by supplementing the UVDGM biodosimetry-based validation method with LA-based validation. Further, by quantifying the dose-distribution of a reactor across its prescribed operating envelope, one can assess the performance of the system for any microbe now or in the future, once that microbe’s dose-response behavior is understood. It is recommended that further development in the numerical deconvolution solver for low-dose applications/validation and the non-phosphate form of the dye used in polychromatic systems.

MULTIMEDIA

The numerical deconvolution is accomplished through a GUI-based program developed in MATLAB. This program is currently in its draft version as an executable, which is provided as a beta version with the Lagrangian actinometry protocol that had been referenced earlier, and forms the basis for this validation analysis. This software is designed to facilitate the data reduction from raw FI data to UV dose distributions estimated by LA. Documentation on usage can be provided with the software itself.
RESEARCH PARTNERS

The Purdue University team, led by Dr. Blatchley partnered with HDR|HydroQual in this effort. Dr. Eric Cox and Dr. Po-Shun Chan assisted Dr. Blatchley in these efforts.

PARTICIPANTS

Key participants in this project, in addition to the Water Research Foundation, included:
- New York State Energy Research and Development Authority
- State University at Albany Medical Research Department
- Gloversville-Johnstown Joint Wastewater Treatment Facility
- Newark OH Water Department
- Trojan Technologies
- ITT Wedeco
- Atlantium LTD
OBJECTIVES OF THIS DOCUMENT

The Ultraviolet Disinfection Guidance Manual (UVDGM) was issued by the USEPA (November 2006) in support of the Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR). It provides guidance in the evaluation, design, implementation and operation of UV disinfection systems at water treatment plants. A key element of the UVDGM is a protocol for validating the dose-delivery performance of the UV system, a specification that is required at full-scale by the LT2ESWTR in order to gain credit for Cryptosporidium, Giardia and/or virus inactivation. The protocol is based on biodosimetry, using microbial surrogates to challenge the reactor across its operating envelope.

This document provides a protocol for using Lagrangian actinometry (LA) to validate UV reactors for dose-distribution performance, from which the inactivation of targeted microbes can be determined directly, including pathogens of concern. The intent is to provide this document as a resource for validating UV reactors, and as a supplement to the UVDGM. It is written in a format similar to the UVDGM (Chapter 5), and relies on the general protocols provided by the UVDGM that are common to both of the validation processes.

OVERVIEW OF UV DISINFECTION REACTOR VALIDATION USING LAGRANGIAN ACTINOMETRY

Summary Description of Lagrangian Actinometry

Ultraviolet (UV) dose represents the master variable in UV disinfection systems. Although many interpretations of the term “dose” (aka fluence) are used, the definition based on the so-called “Lagrangian” (or particle-centered) perspective may be the most fundamental. This “particle-centered” dose is formally defined as the time-integral of the UV intensity history that a particle is subjected to:

\[
Dose = \int_{0}^{\tau} I(t) \cdot dt \quad \text{Equation 1-1}
\]

where,
- \(Dose\) = UV dose received by an individual particle (mJ/cm²)
- \(I(t)\) = UV intensity history, or intensity of UV radiation received by a particle (mW/cm²)
- \(t\) = time (s)
- \(\tau\) = period of exposure (s)

All UV reactors used in treatment settings are characterized by strong spatial gradients in the UV intensity field, as well as spatial and temporal gradients in velocity. Moreover, these
systems almost always operate in the turbulent regime. Together, these aspects of UV reactor behavior dictate that some particles (microorganisms) will receive small UV doses, while other particles will receive larger doses. More generally, it can be asserted that all UV reactors that are used for water and wastewater treatment in practical applications are characterized by a UV dose distribution for any given operating condition.

Accurate predictions of UV reactor performance can be developed by integrating the UV dose distribution with the intrinsic kinetics of the reaction(s) of interest (aka, UV dose-response behavior). However, the validity of any such prediction relies on the validity of the dose distribution estimate, as well as the validity of the dose-response information.

Until recently, the only tools available for estimation of UV dose distribution delivery by a UV reactor relied on simulations of fluid mechanics and radiation intensity fields. The original work in this area relied on the use of physical measurements of fluid flow to develop a so-called “random walk” simulation of individual particle trajectories (Chiu et al., 1999a, 1999b). These simulated trajectories were then integrated with intensity field simulations to allow prediction of particle-specific doses. By repeating these calculations for a large number of particles, it was possible to build an estimate of the UV dose distribution. In turn, the UV dose distribution estimates could be combined with UV dose-response behavior for target microorganisms using a segregated-flow model approach to simulate reactor performance, as defined by microbial inactivation. Although this method was shown to yield accurate predictions of reactor performance, it required extensive, time-consuming measurements of fluid flow in the system.

Purely numerical simulations were a natural evolution of this modeling approach. These simulations involve combined applications of computational fluid dynamics (CFD) with intensity field (I) models. CFD-I models have been used successfully to simulate the dose-distribution in a UV reactor by following either a Lagrangian or an Eulerian approach. Indeed, CFD-I models have evolved to the point where, in some cases, they now form the basis for design of new reactors. Manufacturers of UV systems have found that numerical prototyping is less expensive than physical prototyping, particularly as a means of optimizing reactor performance for a given application.

While numerical models, such as CFD-I, represent important tools for analysis of UV reactors, they have not evolved to the point where they can be used for reactor validation. Several issues can be identified that prevent the application of CFD-I models for validation. First, there is no uniform standard for their application. Second, it is common to expect considerable uncertainty in the values of some important input variables (e.g., lamp output power). Third, the models themselves may ignore or incompletely account for some relevant physical behavior (e.g., reflection and refraction of UV radiation). Collectively, these and other factors mean that CFD-I models are developing, but need a basis for verification before they receive full acceptance in the UV owner and regulatory communities.

Lagrangian actinometry (LA) was developed as a method for measurement of the UV dose distribution delivered by a UV reactor for a given set of operating conditions. The name of the method – Lagrangian actinometry – indicates that actinometry is being applied for UV dose
measurement from a Lagrangian frame of reference. In other words, the method allows for dose measurement at the level of an individual particle.

Like all methods of actinometry, LA involves a well-defined photochemical reaction that yields a measurable endpoint. To date, successful applications of LA have involved (E)-5-[2- (methoxycarbonyl)ethenyl]cytidine (hereafter referred to as S) as the actinometer. The reaction is illustrated in Figure 1-1. When subjected to germicidal UV radiation, S undergoes a photoisomerization reaction to yield an unstable intermediate, which spontaneously reacts to yield 3-β-D-ribofuranosyl-2,7-dioxopyrido[2,3-d]pyrimidine (hereafter referred to as P) as a stable photoproduct (Bergstrom et al., 1982). The starting material for this reaction (S) is non-fluorescent, whereas the photoproduct (P) is brightly fluorescent, with excitation and emission maxima at 330 nm and 385 nm, respectively. Moreover, the quantum yield for the S→P phototransformation is high across the germicidal UV spectrum (Shen et al., 2005).

While it is true that all successful applications of LA to date have relied on S as the particle-associated actinometer, it is conceivable that other actinometers could be developed to serve this function. This document has been written to address the specific form of LA that has been successfully applied and for which details of its application are available. However, this document is also intended to be sufficiently general so as to allow for the application of other forms of LA, perhaps involving other chemical actinometers.

![Figure 1-1. Basic UV photochemistry of S in aqueous solution (adapted from Bergstrom et al., 1982)](image)

In LA, the compound S is first biotinylated, then conjugated to the surface of a streptavidin-coated polystyrene microsphere through a streptavidin-biotin linkage. As a result, the photochemistry defined above is constrained to the surfaces of individual microspheres. The S→P phototransformation that takes place on the surface of the microsphere results in the microsphere becoming fluorescent, with the fluorescence intensity (FI) of an individual microsphere being a function of the dose of UV radiation to which it has been subjected (see Figure 1-2). Although it is possible to develop Lagrangian actinometers based on other photochemistry, all successful applications of this method, to date, have relied on the S→P phototransformation. If other LA methods are developed, the particle-associated actinometer should allow for measurements of UV dose received by individual particles.
The application of LA for measurement of the dose distribution delivered by a UV reactor involves several steps. First, the dose-response behavior of the microspheres is quantified. This is accomplished by subjecting small volumes of aqueous microsphere suspension to well-defined UV doses, usually using a shallow, well-mixed batch reactor and a collimated UV source. The FI distribution of microspheres within the population from each sample are measured by flow cytometry. Next, a population of the same microspheres is imposed on the reactor of interest at a fixed, steady-state operating condition. As with the samples from the dose-response experiments, the FI distributions of microspheres from these samples are measured by flow cytometry. In all cases, the FI responses of microsphere samples (populations) will be in the form of a FI distribution (i.e., histogram). The UV dose distribution for a given operating condition is calculated from these distributions using a numerical deconvolution algorithm. This procedure is repeated over a range of operating conditions, thereby allowing quantification of the UV dose distribution delivered by the reactor as a function of operating conditions. In turn, the dose distribution measurements can be integrated with UV dose-response behavior for one or more target microorganisms, using a mathematical analog of the segregated-flow model, to allow definition of the performance of the reactor (relative to the desired antimicrobial endpoint[s]) as a function of operating conditions.

Figure 1-2. Schematic illustration of the application of Lagrangian actinometry

Lagrangian Actinometry as an Alternate UV Reactor Validation Method

The primary advantage of LA characterization is that the method allows for direct measurement of the UV dose distribution delivered by a reactor for a fixed set of operating conditions.
conditions. By comparison, biodosimetry cannot yield a measure of the dose distribution; rather, it provides a measure of challenge organism inactivation for a given operating condition, which in turn can be translated into a Reduction Equivalent Dose (RED). The RED is specific to the challenge microbe and cannot be used to make quantitatively accurate predictions of the inactivation response of any other (target) microorganism, unless the UV dose-response behaviors of the challenge and target organisms are identical. As a result, validation of systems on the basis of biodosimetry is accompanied by factors to account for the uncertainty that results from lack of information regarding the UV dose distribution, as well as other sources of uncertainty.

CFD-I models yield an estimate of the UV dose distribution. However, these models are constrained by the need for accurate measurements of input parameters; for some of these parameters, it is difficult to obtain these values. Moreover, no “standard” method for CFD-I model implementation has been developed, and some models do not account for relevant physical behavior. Again, by comparison, LA avoids these shortcomings because it allows for actual measurement of the UV dose distribution.

Ideally, UV reactor validation (and characterization) procedures should incorporate all three methods of reactor analysis: biodosimetry, CFD-I, and LA. By following this three-pronged approach, it is possible to compare and cross-validate all three methods. This approach allows for a more comprehensive understanding of the behavior of a UV system than can be accomplished by any of these methods alone, or any other combination of these methods.

In a typical validation effort, a matrix of operating conditions will be defined that spans the range of relevant operating conditions for the anticipated application. This matrix will be defined as a set of process variables (e.g., flow rate, UV transmittance, lamp power) with multiple “levels” for each operating variable being used to define the matrix. This protocol suggests that LA be applied at all operating conditions within such a matrix, while biodosimetry is applied to a limited number of operating conditions within this matrix.

Validation of a CFD-I model against LA allows for verification of the ability of the model to accurately represent the UV dose distribution delivered by the reactor. Validation of a CFD-I model against biodosimetry provides an indication of the ability of the model to predict microbial inactivation. If these cross-validations are successful, then the CFD-I model should be applicable for interpolation of reactor behavior among the matrix of validated operating conditions. This is an important benefit for large systems, or for systems where treatment objectives (e.g., target microorganism) may change.

This document is intended to provide a detailed description of LA, so that appropriately qualified organizations may apply the method for reactor validation. It is intended as a supplement to the UVDGM, and has been intentionally formatted in a manner that is consistent with the UVDGM. Included in this report are detailed descriptions of the theoretical basis for the method, the methods for preparation of microspheres, conduct of experiments (dose-response and reactor characterization), microsphere sample preparation, sample analysis, data reduction and interpretation, and QA/QC.

The document has also been developed so as to describe the benefits and limitations of the method. Overall, the objective of this document is to describe LA in sufficient detail to allow other
organizations to apply the method and reach consistent, repeatable results. Moreover, the document is intended to provide guidance to state regulatory agencies regarding the application and interpretation of LA as a UV reactor validation protocol.

ORGANIZATION OF THIS DOCUMENT

This document is constructed in essentially the same format as the UVDGM (Chapter 5) and certain aspects of UVDGM Appendices A, B, C and D. The Table of Contents is similar to that of the UVDGM Chapter 5, and some sections of this document simply refer to the UVDGM. This similarity is intentional – although LA represents a new validation method, its objectives, general applications approach and results are similar to those articulated for biodosimetry in the UVDGM. By putting it into the context of the UVDGM validation process, it is anticipated that the transition from current validation practices (i.e., biodosimetry) will be facilitated, particularly with respect to the analysis of data and the determination of credited log inactivation and RED.

Differences from the biodosimetric approaches are noted in the appropriate sections; among these, the ability of the LA method to yield a dose-distribution measurement is critical. In turn, this allows implementation of a method of data interpretation that is, in certain aspects, fundamentally different than the UVDGM’s interpretation of biodosimetric data. Still, the objective of providing a quantitative assessment of reactor performance remains.

REGULATIONS SUMMARY

Reference is made to the UVDGM (Chapter 1, Sections 1.3 and 1.4) for a comprehensive discussion of the relevant regulatory requirements regarding disinfection goals and associated UV reactor dose-delivery performance. The intention is to have the LA protocol clearly defined in a manner that it can be used by qualified organizations, and recognized as an accepted validation process, such that a utility will receive appropriate disinfection credit.
CHAPTER 2
GENERAL REQUIREMENTS FOR VALIDATION OF UV REACTORS BY LAGRANGIAN ACTINOMETRY

This section explains the key steps recommended for validation of UV disinfection systems using Lagrangian actinometry. As stated earlier, the protocol is formatted in the same manner as used by the UVDGM for biodosimetry, including recommendations for selecting test conditions, quality assurance/quality control (QA/QC) steps, and data analysis procedures.

Where appropriate, the relevant UVDGM sections are cited, and text used by the UVDGM is sometimes repeated within this document. This was not done with the intent of plagiarism, but to recognize the strong body of work that resulted in the UVDGM, and to clearly put the LA method in the context of the Guidance Manual. This approach was reviewed with and encouraged by those involved in the preparation of this document and the UVDGM.

MINIMUM REQUIREMENTS FOR VALIDATION TESTING

Reference is made to the discussions in Section 5.1 of the UVDGM. In order to receive treatment credit for inactivating Cryptosporidium, Giardia or viruses using UV radiation, the LT2ESWTR requires water systems to use UV reactors that have undergone validation testing. Table 2.1 is excerpted from the UVDGM (Table 5.1 in the UVDGM), summarizing the requirements for UV reactor validation.

The UVDGM (see Table 2-1) cites the LT2ESWTR requirements (40 CFR 141.720). Although the rule states that “validation testing must include inactivation of a test microorganism whose dose-response characteristics have been quantified with a low-pressure mercury vapor lamp,” it also notes that the “state may approve an alternative approach to validation testing.” Importantly, the rule specifically requires that the method “account for UV dose distributions arising from the velocity profiles through the reactors.” In the first instance, the LA method is counter to the biodosimetric method, while in the second, it actually resolves the measurement of dose-distribution. The UVDGM addresses the inability of the biodosimetric method to measure a UV dose-distribution by incorporating a bias factor (RED Bias) that assumes a relatively poor dose distribution by default (which, interestingly, can penalize an efficient reactor, but also reward an inefficient reactor). By incorporating a limited degree of biodosimetric testing with a comprehensive measurement of UV dose-distribution, this protocol can more directly meet the requirements articulated in Table 2.1 than by using biodosimetry alone.
Table 2-1. Summary of LT2SWTR Validation Requirements (Excerpted from UVDGM, USEPA 2006 Table 5.1, Chapter 5)

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Conditions</th>
<th>Citation</th>
</tr>
</thead>
</table>
| Validated operating conditions must include | • Flow rate  
• UV intensity as measured by a UV sensor  
• UV lamp status | 40 CFR 141.720 (d)(2) |
| Validation testing must include | • Full-scale testing of a reactor that conforms uniformly to the UV reactors used by the water system  
• Inactivation of a test microorganism whose dose-response characteristics have been quantified with a low-pressure mercury vapor lamp | 40 CFR 141.720 (d)(2)(ii) |
| Validation testing must account for | • UV absorbance of the water  
• Lamp fouling and aging  
• Measurement uncertainty of on-line sensors  
• UV dose distributions arising from the velocity profiles through the reactor  
• Failure of UV lamps or other critical components  
• Inlet and outlet piping or channel configurations of the UV reactor | 40 CFR 141.720 (d)(2)(i) |

1 The state may approve an alternative approach to validation testing.

OVERVIEW OF THE ALTERNATE VALIDATION PROTOCOLS

Lagrangian actinometry using dyed microspheres (DMS) can be used for UV system validation as an alternative to, or in conjunction with, biodosimetry. The method has been demonstrated as an effective and practical validation tool. Its direct output is the dose-distribution behavior of a reactor within its operating envelope. The ability to measure a UV dose distribution is an important advantage; by doing so, credited RED or log inactivation of a target pathogen(s) can be determined for a reactor under a given set of operating conditions without being decreased by the RED bias. This is especially the case for those reactors that are effectively designed and will exhibit dose-distributions that are more efficient than the default dose-distributions used by the UVDGM. Moreover, by measuring the dose distribution delivered by a reactor, as opposed to assuming a worst-case scenario, LA gives appropriate credit to reactor design. Well-designed reactors will be recognized as such, and will not be penalized, as they may be if validation is conducted using biodosimetry with significantly less sensitive surrogates than the targeted pathogen. Most importantly, poorly designed reactors will be characterized and will not be masked by the default RED bias when, in fact, the actual dose distribution is worse than the default dose-distributions used by the UVDGM. Knowledge of UV dose-distribution delivery will be increasingly important as system size increases, because the ability to define the dose distribution can greatly diminish uncertainty in predictions of reactor performance.

Key Steps in Recommended Validation Protocol

Following the general approach described in the UVDGM (Section 5.2.1 and Figure 5.1), the protocol for validation of UV disinfection systems by Lagrangian actinometry can be described in five main steps. These are illustrated in Figure 2-1, Figure 2-2 and Figure 2-3:

**Step 1: Planning**
The planning step establishes the procedures to be followed for validation of a UV system, and third-party testing organization protocols with respect to personnel and oversight. The test plan developed for the validation will include definitions of the operating range to be validated, incorporating water quality (UVT, turbidity, temperature, etc.) and the system variables such as flow rate, power input, sensor output and the number and configuration of the UV lamps.

A Validation Test Plan in compliance with this LA validation protocol and the UVDGM is developed by the designated testing organization. The test plan will include detailed site and equipment specifications, procedures for testing (including documentation for conformity to the generic protocol and UVDGM), and a quality assurance project plan. In some cases, the test plan may be submitted to the appropriate State regulators for approval prior to conducting the field tests.

**Step 2: Conduct Experimental Tests Using Dyed Microspheres**

The testing step involves the actual assembly, installation, and operation of the test stand, collection of the targeted samples, and completion of all analyses required under the test plan. UV reactor validation by LA is addressed in two experimental steps, similar to the biodosimetric method (see Figure 2-1).

2a. **Bench-scale testing using a collimated beam apparatus.** Collimated beam testing characterizes the UV dose-response relationship of the DMS over a range of doses. In these experiments, UV radiation is directed through a collimating device to allow uniform irradiation of a sample of DMS contained in a well-mixed, shallow, batch reactor. The collimating device should house one or more UV sources that emit (essentially) monochromatic UV radiation at a characteristic wavelength of 254 nm. After a specified exposure time, selected to achieve delivery of a pre-defined UV dose to the microspheres in the sample, the sample is collected and prepared for analysis of microsphere fluorescence intensity (FI) by flow cytometry. By analysis of DMS samples exposed to a range of UV doses using this method, the change in FI distribution among the DMS group is obtained as a function of UV dose.
The UV dose delivered to the aqueous sample is calculated based on the UV intensity, exposure time, and other experimental factors. The procedures for the collimated-beam test, including the design and assembly of the collimated beam apparatus and the equation to compute the UV dose, are the same as those described by the UVDGM-Appendix C. UV doses selected for inclusion in the collimated beam testing should span the anticipated range of doses to be delivered by the flow-through reactor, so as to allow interpolation of results in numerical deconvolution, as opposed to extrapolation (see discussion of deconvolution below).

2b. Full-scale reactor testing. In these experiments, the DMS are injected upstream of the UV reactor. Effluent samples are collected and analyzed to quantify the FI distribution among a representative population of DMS for each prescribed test condition (combinations of flow rate, UVT, lamp status, power, etc.). Full-scale reactor testing can be performed on-site at the commissioned water treatment plant or off-site at a test facility.

As a QA measure, this protocol suggests that a limited number of biodosimetric tests should be performed. These tests may include two different challenge organisms – for example coliphages MS2 and T1, following the UVDGM guidance for biodosimetry. Having these points and their respective dose-response kinetics allows one to verify the predicted log inactivation (or RED) for active and measurable microbes. This would generally not be possible to do with a live
pathogen. With time and experience, one can expect that the need for use of biodosimetry as a check of the LA method will diminish.

Five to ten percent of the total DMS testing conditions should be selected for biodosimetric tests for QA purposes. Two different challenge microorganisms with different UV sensitivities are preferred (e.g., MS2 and T1). It is recommended to run these QA bioassays at conditions near the edges of the operation envelop defined by the full matrix of DMS testing conditions. Ideally, at least a portion of the QA test points should involve simultaneous injection and measurement of two surrogate microbes and DMS, so that the dose distribution measured by DMS can be verified using the results from both challenge microorganisms under the same operating condition.

2c. Analysis by Flow Cytometer. The samples generated by the collimated beam and reactor tests are analyzed by a flow cytometer. The raw data from the flow cytometer will include optical measurements corresponding to DMS, as well as other (ambient) particles that are present in the water sample. The data to be used in estimation of the dose distribution are only those measurements that correspond to the microspheres themselves. Therefore, it is necessary to isolate the DMS measurements from measurements of other particles in the sample. The process used to accomplish DMS discrimination against other particles in the sample is known as “gating”. In gating, the known optical behaviors of the particles are used to identify regions of the cytogram that correspond with the particles of interest. Optical characteristics that can be measured by flow cytometry include fluorescence intensity, size, and granularity. For each parameter, it is possible to define a gate to segregate the area of the cytogram where the particles of interest are known to provide a signal. By defining one or more gates, the signals of all other particles in the cytogram will be minimized.

Step 3. Numerical Deconvolution of FI Distributions to Yield UV Dose Distribution Estimate

Sample analysis by flow cytometry will yield measurements of the FI distribution among the populations of DMS that comprise each sample. For the samples from the bench-scale, collimated-beam tests, the fact that one measures a distribution instead of a discrete dose is attributable to the variations in the DMS population, in the form of variations in microsphere size, dye surface loading, shape, and other factors, and the inherent variation (error) in the flow cytometry method of FI measurement.

The samples collected from the flow-through reactor will be influenced by these same sources of variance, but will also include variance that is attributable to the UV dose distribution in the reactor itself. Since these sources of error can be assumed to be independent, their effects are additive (Blatchley et al., 2006). As such, it is possible to separate the effects of the dose distribution in the samples from the flow-through reactor from the variance that is attributable to the microspheres and flow cytometry.

Numerical deconvolution of the FI distributions that result from analysis of the DMS samples from collimated beam and flow-through reactor testing is then conducted to develop an estimate of the dose distribution delivered by a UV reactor for the operating conditions under which the DMS sample was collected. The logic associated with deconvolution is presented in Figure 2-2, Step 3.
Step 4: Determine the Log Inactivation and RED for the Targeted Microbe.

The dose distributions delivered by the UV system are measured at the prescribed validation test conditions. The measured dose distributions can then be integrated with the dose-response behavior of the selected target pathogen(s) or microbial surrogate(s) using a mathematical analog of the segregated-flow model to calculate its (log) inactivation. The reduction equivalent dose (RED) can also be estimated for the targeted microbe by using the same dose-response relationship. Although the ability of LA to yield a prediction of (log) inactivation for a target pathogen may eliminate the need for RED calculation, there may be some circumstances where translation of LA results to an RED is desirable. The logic behind Steps 4 and 5 is illustrated in Figure 2-3.

### Step 3. Deconvolution for Dose Distributions

Deconvolve the measured effluent FI in Step 2b from the dose response curves in Step 2a for the dose distributions.

\[
[\beta] = [\gamma] \times [\alpha] \quad ; \quad [\gamma]^{-1} \times [\beta] = [\alpha]
\]

\[
[\alpha] = \begin{bmatrix}
\alpha_0 \\
\alpha_1 \\
\vdots \\
\alpha_m
\end{bmatrix}
\]

is the dose distribution. Parameter “m” depends on the range of dose and the increment of the dose bin. For example, if the dose range is 300, and the dose bin increment is 2, then \( m = 300/2 = 150 \);

\[
[\beta] = \begin{bmatrix}
\beta_0 \\
\beta_1 \\
\vdots \\
\beta_{1023}
\end{bmatrix}
\]

is the effluent sample FI distribution, as a 1024×1 matrix. The rows of the matrix commonly used for typical flow cytometers;

\[
[\gamma] = \begin{bmatrix}
\gamma_{0,0} & \gamma_{0,1} & \cdots & \gamma_{0,m} \\
\gamma_{1,0} & \gamma_{1,1} & \cdots & \gamma_{1,m} \\
\vdots & \vdots & \ddots & \vdots \\
\gamma_{1023,0} & \gamma_{1023,1} & \cdots & \gamma_{1023,m}
\end{bmatrix}
\]

is the dose response matrix, 1024×m.

![Figure 2-2. Step 3 in the Lagrangian actinometry validation process](image)

Step 5: Adjust for Uncertainty to Calculate the Validated Log Inactivation and Dose for the Targeted Microbe

The UVDGM uses a “validation factor” to account for experimental uncertainties and system biases to adjust the biodosimetrically-determined log inactivation and RED. This same
approach is applied to the actinometric method. Because the dose-distribution is known, the $B_{RED}$ term found with the biodosimetric analysis is eliminated,

**Step 4. Determine the Target Log Inactivation (LI) or Reduction Equivalent Dose (RED) for Designed Operation Conditions**

Deconvolute the measured effluent FI in Step 2b from the dose response curves in Step 2a for the dose distribution at each designed operation condition.

Integrate the dose distribution with the dose-response curve of the pathogen (e.g., *Cryptosporidium*, *Giardia*, and adenovirus) in concern to estimate the log inactivation for such pathogen directly without any biological bias.

**Step 5. Adjust for Uncertainty to Calculate the Validated LI or RED**

$$\text{Validated LI (or, RED)} = \frac{\text{Calculated LI (or, RED)}}{\text{VF}}$$

Where, $\text{VF}$ = validation factor that accounts for biases and experimental uncertainties.

For Lagrangian actinometry (DMS) testing,

$$\text{VF} = \left( 1 + \frac{U_{Val}}{100} \right)$$

$$U_{Val} = (U_{SP}^2, \text{ or, } U_{IN}^2 + U_S^2)^{1/2}$$

$U_{SP}, U_{IN}$ = set point or interpolation uncertainties as described in UVDGM; $U_S$ = sensor uncertainty as in UVDGM.

**Figure 2-3. Step 4 and 5 in the Lagrangian actinometry validation process**
Alternative Validation Protocols

In the context of the UVDGM, this Lagrangian actinometry method using dyed microspheres is in itself an alternative validation protocol. Refer to 5.2.2 of the UVDGM for other protocols that have some level of acceptance under the LT2ESWTR rule. As described earlier, although this document addresses the LA protocol within specific bounds of dye and microsphere selection, researchers may develop variations that would be acceptable and possibly improve on this LA technique.

Third Party Oversight

The USEPA recommends an independent third party provide oversight to ensure that validation testing and data analyses are conducted in a technically sound manner and without bias. A person independent of the UV reactor manufacturer should oversee validation testing. Individuals qualified for such oversight include engineers experienced in testing and evaluating UV reactors and scientists experienced in chemical actinometry. Appropriate individuals should have no real or apparent conflicts of interest regarding the ultimate use of the UV reactor being tested.

At a minimum, independent oversight should include observing validation testing to verify that the individuals performing the validation follow the documented protocol, and reviewing the report for accurate data and results. The independent third party should review the validation report before its release. When appropriate, the third party should rely on additional outside experts to review various aspects of UV validation testing, such as lamp physics, optics, hydraulics, microbiology, and electronics. The third-party should be familiar with Lagrangian actinometry theory and experimental procedures, analysis of the dyed microspheres (i.e., flow cytometry), and the analysis of the DMS data.

Emerging Methods

CFD-I modeling and LA are both noted as emerging validation methods by the UVDGM. Potential model-based approaches use computational fluid dynamics (CFD) to predict microorganism trajectories through a UV reactor. When coupled with UV intensity field models, the CFD-I modeling calculation can estimate the UV dose delivered to each microorganism, from which the dose distribution can be determined. A possible approach for verifying and validating hydraulic CFD models is outlined in the AIAA CFD Guide (1998). No analogous document is known to exist for intensity-field models; more generally, no standardized protocol has been developed for the application of CFD-I models for simulation of UV reactor performance. Although model simulation approaches clearly have potential for use in validating UV reactors, certain difficulties still exist when dealing with free surface situations. Some input parameters (e.g., lamp output power) are difficult to measure, particularly for large, complex systems. Lastly, I-field models generally do not provide a full accounting for the optical processes that are known to be relevant in these systems, including reflection and refraction.

The direct measurement of dose distribution by Lagrangian actinometry provides a valuable resource to the CFD-I modeling approach. The dose-distribution information can be used...
to calibrate and/or verify the CFD-I model, enhancing the modeling approach’s credibility from a regulatory perspective, and yielding a stronger tool for optimizing the design of UV reactors. Similarly, biodosimetry serves to verify LA results. One can consider the use of all three methods as a ‘three-pronged’ approach to the validation of UV reactors.
CHAPTER 3
VALIDATION AND EQUIPMENT SPECIFICATIONS

DYED MICROSPHERES

Reference is made to the UVDGM (Section 5.3) regarding the evaluation and selection of microbiological surrogates. The following discussions relate only to dyed microspheres.

Characteristics to Consider When Selecting the Dyed Microspheres

To date, successful, quantitative applications of DMS for purposes of measuring UV dose distributions have all involved the use of polystyrene microspheres conjugated with the compound (E)-5-[2-(methoxycarbonyl)-ethenyl]cytidine through an avidin-biotin linkage. These microspheres have been demonstrated to be effective for quantification of UV254 doses ranging from 0 to 600 mJ/cm².

According to the UVDGM, the difference in the UV sensitivity of a challenge microorganism from that of the target pathogen of concern needs to be accounted for by a biological bias factor, defined as RED bias (BRED). However, the UV sensitivity of DMS relative to challenge or target organisms, as well as the microbial bias (RED bias) discussed in the context of biodosimetry, are not of concern for Lagrangian actinometry since UV dose distributions are measured directly through this approach. The action spectrum of the DMS is similar to those of MS2 and B. subtilis spores, as shown in Figure 3-1; however, a correction factor must be included if target microbes have action spectra that differ significantly from that of the DMS. Reference is made to Appendix D of the UVDGM, which addresses the use of polychromatic (medium pressure) lamps and the impacts of the polychromatic bias and the surrogate action spectrum relative to that of the targeted pathogen.

The physical characteristics of DMS that are critical to their application in Lagrangian actinometry include size, specific gravity, and avidin surface loading. The relevance of these characteristics to LA applications is described below.

Size and Specific Gravity

Microspheres selected for application in LA should have size and specific gravity attributes that allow the microspheres to mimic the trajectories of microorganisms in a UV system. Waterborne microorganisms often have a size on the order of a few micrometers (µm) or smaller, and a specific gravity of slightly more than one. Therefore, microspheres that are nearly neutrally-buoyant in water, with diameter ranging from a few µm to tens of µm in diameter, are appropriate for LA applications.
Figure 3-1. Spectral sensitivity of DMS, *B. Subtilis* spores, and coliphage MS2 to germicidal UV radiation (adapted from Shen et al., 2009, and Mamane-Gravetz et al., 2005*).


For perspective, it is important to recognize that small, nearly-neutrally buoyant particles are used commonly in experimental fluid mechanics to allow for non-invasive measurements of fluid motion. For measurements of this type involving liquid water, particles with sizes up to tens of μm that are nearly neutrally-buoyant have been demonstrated to provide accurate measurements of local fluid motion. This is because particles with these characteristics do not have sufficient inertia to deviate significantly from the trajectories of the surrounding fluid. Based on this argument, it is reasonable to expect that microspheres with size of less than 10-20 μm and that are nearly neutrally-buoyant will follow trajectories that accurately simulate those of waterborne microorganisms. Moreover, it is reasonable to expect that microspheres with these characteristics will also accurately mimic the trajectories of smaller particles (*e.g.*, viruses).

The microspheres used to date in successful applications of LA have been roughly 5 to 6 μm in diameter, with a specific gravity of 1.05. However, it should also be possible to use larger microspheres in these applications. Ongoing research (as of the date of publication of this protocol) based on the application of microspheres with diameters as large as 15 μm (with similar specific gravity) has been promising.
From an experimental standpoint, flow cytometry is the analytical method of choice for measurement of the optical characteristics of microspheres. Flow cytometry allows measurement of one or more optical properties of particles at high throughput rates, often on the order of $10^4$ particles per minute. Particles with sizes ranging from roughly 1 to 40 $\mu$m work well in flow cytometry.

### Avidin Surface Loading

Avidin is a glycoprotein found in raw egg white and in the tissues of some animals. It contains four identical subunits, each of which has the ability to bind to one molecule of biotin. Avidin has a strong affinity for biotin with a $K_D$ (dissociation constant) of approximately $10^{-15}$ M$^{-1}$. This affinity approaches the strength of a covalent bond and allows for essentially irreversible binding of avidin to biotin. Because of its well-defined stoichiometry and small dissociation constant, the avidin-biotin linkage represents a method whereby biotinylated molecules can be irreversibly and predictably conjugated to microsphere surfaces.

Avidin-coated microspheres are commercially-available. In selecting microspheres for application in LA, at least three characteristics can be identified that influence the ultimate surface loading of the biotinylated form of $S$. These include: the surface loading of avidin per unit area of microsphere, the surface area of each microsphere, and the type of avidin group.

Avidin surface loading is performed by microsphere manufacturers. The nature of the manufacturing process dictates that monolayer coverage of avidin on the microsphere surface results in microspheres with the greatest degree of uniformity, in terms of avidin surface-loading. Therefore, monolayer avidin coverage represents the standard for this process variable. Given that microspheres are essentially spherical in shape, their surface area per particle can be accurately characterized on the basis of their size (diameter). The external surface area of microspheres (per microsphere) increases with the square of particle diameter. Therefore, if monolayer avidin coverage is maintained, avidin loading per particle should also increase with the square of microsphere diameter.

Several forms of avidin are available commercially. The extent to which non-specific binding occurs is variable among these various avidin groups, and as such, the behavior of avidin-coated microspheres may vary depending on the avidin group.

Collectively, these parameters (avidin surface loading, microsphere size, and avidin type) determine the binding capacity (per particle) of microspheres for biotinylated $S$. For applications in which the goal is validation of reactors that deliver UV dose distributions that are defined by high doses, it may be desirable to select microspheres with high surface loadings of $S$. For conventional drinking water applications, microspheres with diameter of approximately 6 $\mu$m, a specific gravity of 1.05, and a streptavidin surface loading of 0.04 $\mu$g/mL work well. However, as described above, applications based on other microsphere types may also provide satisfactory performance.
Alternative Dyed Microspheres

The development of new, alternative microspheres should not be precluded by this set of guidelines. The knowledge gained from the work that has been conducted to-date serves as a useful guide in the development of alternative microsphere formulations.

As described above, particles selected for use in LA should have size and specific gravity characteristics that allow them to simulate the trajectories of individual microorganisms. Ideally, the chromophore selected for conjugation to the particles should have a large quantum yield (greater than 0.5 mol/Einstein) and an action spectrum (from 200 nm to 300 nm) that mimics those of microorganisms (Shen et al. 2005). In addition, the photochemical product(s) that result from UV exposure should be quantifiable using optical techniques, thereby allowing relatively rapid quantitation of the extent of UV exposure (i.e., UV dose) received at the level of an individual particle. Lastly, the optical characteristic that results from UV exposure should increase with UV exposure. This approach improves sensitivity to low doses, and is helpful in quantification of the low end of the UV dose distribution, which often limits the overall performance of a UV reactor.

Similarly, this protocol should not preclude the development of alternative methods of reactor validation. However, alternative methods should meet or exceed the capabilities of existing methods, in terms of measuring reactor performance and protecting public health.

Microspheres

The microspheres that have been used in reported studies were sourced from Polysciences, Warrington, PA (www.polysciences.com) and their subsidiary Bangs Laboratories, Inc., Fishers, IN (http://www.bangslabs.com/company/index.php). Specifically, the product is catalog number 24158, Streptavidin Coated Carboxylated Microspheres, 6.1 micron. These are supplied at a concentration of approximately $10^8$ particles per mL. They are very stable, capable of being stored under refrigerated conditions for greater than a year. Freezing is not acceptable. Availability is by special order for large quantities.

Microsphere Specifications

Size and specific gravity should be chosen to allow microspheres to mimic microbial trajectories. As particle size decreases and specific gravity approaches neutral buoyancy, particle trajectories become identical to the surrounding fluid. However, it is also important to consider the effects of particle size on analysis (i.e., flow cytometry). Balancing these issues, it appears that selection of particles with density = 1.05 g/cm$^3$ and size between 1 µm and 30 µm will yield particles that satisfy these constraints.

Surface loading of the binding group should be developed with the objective of providing a sufficient number of binding sites per particle, and maintaining tight quality control on the microspheres. For avidin-based binding materials, this translates to monolayer coverage of the avidin group.
The mean diameter of the polystyrene beads available from Polysciences (as stated earlier) is 6.1 μm nominal. The specific gravity is 1.05, and the surface loading of binding group (protein) is greater than 0.04 μg/mL.

Handling and Storage

Streptavidin coated microspheres before conjugation with the biotinylated dye are not photosensitive, but they may be thermosensitive. Avidin and related compounds are glycoproteins; in essence, they are large molecules that include protein and carbohydrate groups. As such, they are readily used as substrates for microbial growth. Therefore, storage and transport conditions should be maintained to minimize biological activity. However, when microspheres are suspended in water, they are structurally incapable of withstanding the stresses brought about by freezing. Therefore, microspheres should be stored in the dark at 4°C. For transport, an appropriate cooler with a sufficient amount of ice packs should be used. For temporary handling (i.e., a few hours), room temperature (20°C) is acceptable.

Quality Control for the Microspheres

For long-term storage, microspheres should be checked for quality before usage. Visual inspection can be used as the preliminary check for quality changes in microsphere stock based on physical properties, such as color and suspension. For instrumental analysis, a sample of the microsphere stock should be analyzed by flow cytometry to determine if their size and granularity are consistent with those of a fresh sample. With the same instrument settings, a primary group of microspheres with the similar size and granularity shall be detected by the flow cytometer. In addition, the percentage of the primary group among the total population detected by the cytometer flow cell shall not be lower than 70%. Other particles that may appear in a cytogram include microsphere debris, duplets, or triplets, which will not impact the overall quality of the microsphere stock as long as their percentage among the total population is under a 30% limit. Based on our experience, as long as microspheres or pre-dyed microspheres are correctly stored under refrigerated conditions, extended shelf life can be expected. For microspheres that have had the biotinylated form of S conjugated to their surfaces, a dose-response experiment should be performed periodically to ensure stability (see specifications in section 3.1.2.1). This simply requires judgment to the consistency of the dose-response behavior. If bleaching, or lack of proportional response occurs, the dyed microspheres should not be used.

Biotinylated Dye

Biotinylated Dye Specifications


S is biotinylated so that it can be conjugated to the microsphere’s surface through a streptavidin-biotin linkage. Two types of biotinylated S have been developed. The first was originally developed by Dr. Bergstrom’s research group at the Department of Medicinal Chemistry and Molecular Pharmacology at Purdue University. In this case, biotin and S are linked through a
silyl acetal group. This form of the dye is referred to as the silyl-linked dye. The second form of the dye was developed by TriLink BioTechnologies, and uses a phosphate group to link biotin and S, and it is referred to as the phosphate-linked dye.

Both types of biotinyllated dyes were tested through collimated beam dose-response studies and field validation testing. Both types of dyes work successfully with monochromatic UV radiation at 254 nm. However, only the silyl-linked dye can be used for medium pressure systems. This is an important consideration when designing a validation test for either a low-pressure or medium pressure reactor. Additionally, dyes from different production runs should be tested separately and not combined for a testing run.

Handling and Storage

The dye can be stored in the dark at approximately -78°C. It may be stored as a dry powder or in aqueous solution under these conditions. When present in the crystalline form, the dye can be stored at 4°C with an appropriate opaque cover to prevent potential exposure to ambient light. It has been observed that the shelf-life under these storage conditions is on the order of years or longer. The dye in crystalline form is less sensitive to ambient light than when dissolved in aqueous solution. However, the dye in crystal form is a strong water absorber. Therefore, it is recommended that the dye be stored in a gas-tight container and with a desiccant.

When present in solution, the dye can also be stored at 4°C with an appropriate opaque cover for short-term storage. For long-term storage, the solution should be kept at -78°C in an air-tight, opaque container. It is recommended by the manufacturer (TriLink) to limit the freeze-thawing process for the dye solution to no more than three freeze-thaw cycles. If an aliquot of dye is expected to be used over a long-term with multiple applications, the solution (or crystals) should be split into multiple small containers for separate storage.

Quality Control for the Dye

The manufacturer has defined its own QC criteria for the synthesized dye, based on HPLC and NMR analyses and should provide QC data with each purchase of the dye. When received the end-user should conjugate a small amount of the dye to an appropriate number of microspheres; the quality of the dye can be assessed from quality of the dyed microspheres. Quality control for the dyed microsphere is addressed in Section 3.1.4.

Preparing Dyed Microspheres

Coating or Conjugation of the Microspheres

The dye is attached to the surface of the microspheres through a streptavidin-biotin linkage. Microspheres, with a streptavidin surface loading of 0.04 μg/mL, are rinsed with TTL buffer (100 mM Tris-Cl or Tris-HCl, 0.1% Tween 20, and 1 M LiCl) two or three times before the conjugation process, allowing removal of microsphere debris from the suspension. With the cleaned microspheres re-suspended in TTL buffer, the biotinylated dye is added in excess of stoichiometric amounts (as defined below) to the microsphere suspension, followed by overnight rotation to facilitate conjugation. The rotation speed should be well controlled so that the rotation can generate enough mixing for thorough contact between microspheres and the dye, but will not
impact the stability of the newly formed conjugation. There are two generally used types of rotators. For rotators shown in Figure 3-1 (a), the rotation speed should be controlled within the range of 20–30 rpm; for rotators shown as Figure 3-1 (b), the rotation speed should be controlled at approximately 60 rpm. During this extended process, the temperature of the microsphere suspensions should be kept at approximately 20°C or less in order to assure the quality of the microspheres. After rotational mixing period, the microspheres are then sequentially rinsed in TTL buffer and DI water two to three times each to remove the non-reacted biotinyllated dye from the aqueous suspension. The resulting dyed-microsphere suspension is then diluted to the desired concentration for later applications.

Figure 3-2. Rotational devices used for mixing of microsphere suspensions to accomplish conjugation of biotinyllated-S to avidin-coated microspheres. The devices typically used to promote mixing in these applications are rotary mixers (a) and clinical rotators (b)

The molecular weights of the biotinyllated dye and the streptavidin that coats the microspheres are 944.5 g/mol (for silyl-linked, but 773 g/mol if phosphate-linked) and 633 g/mol, respectively; this mass ratio of 1.528 (biotinyllated-S:streptavidin) (for silyl-linked, but 1.221 if phosphate-linked) is equivalent to a 1:1 molar ratio, as defined by the stoichiometry of the reaction between biotinyllated S and streptavidin. Therefore, the mass of dye required for complete conjugation to the microsphere surface can be determined as:

\[ W_{dye} = \frac{SL \times 1.528 \times V \times ODF}{P} \]

Where,
- **SL** = surface loading of streptavidin-coated microspheres, μg/mL;
- **V** = volume of microsphere stock, mL;
- **ODF** = overdosing factor, a minimum value of 3 is usually recommended based on previous experience;
- **P** = purity of biotinyllated-S, usually 0.7 to 0.9.

The value of SL is provided by the microsphere manufacturer as the result of a standardized assay. The purity of the biotinyllated-S is provided by the dye manufacturer.
The TTL buffer can be prepared based on the following formula: 10 mL of Tris-Cl (pH=7.4, 1.0 M) or Tris-HCl (pH=8.0, 1.0 M), 0.1 mL Tween 20, and 4.3 g LiCl. These are mixed together and diluted with DI water to 100 mL. The solution is then autoclaved to avoid biological contamination to the DMS stock suspensions. After preparation, the TTL buffer is stored at 4°C.

Storage and Handling of the Dyed Microspheres

Dyed microspheres are photosensitive and thermosensitive. Except for short periods of transfer, dyed microsphere suspensions should always be kept in covered, opaque containers. Exposure to ambient light should be avoided or minimized, especially any direct sunlight. Dyed microspheres should always be stored at 4°C. Freezing is not acceptable. For transport, an appropriate cooler with a sufficient amount of ice packs should be used. For temporary handling (i.e., a few hours), room temperature (20°C) is acceptable.

Quality Control for Dyed Microspheres

After long-term storage, microspheres should be checked for quality before usage. Visual inspection can be used as the preliminary check for quality changes in microsphere stock based on physical properties, such as color and suspension. For accurate quality assessment, a dose-response study of the DMS stock should be conducted. A group of DMS samples are taken and exposed to a series of UV254 doses. DMS samples after UV exposure are then subjected to flow cytometry analysis. The size and granularity of all DMS samples should meet the same criteria as defined in Section 3.1.3.3. In addition, the fluorescence intensity (FI) of DMS samples should increase with the UV dose applied. In general, the mean FI of DMS samples should follow a first-order kinetics as a function of the UV dose applied. The equation can be generally illustrated as below.

\[
\text{MeanFI} = F_{I_0} + a \times [1 - \exp(-b \times \text{Dose})]
\]

\textbf{Equation 3-2}

In addition, the FI distribution for each DMS sample from the DR test shall demonstrate a regular, consistent trend in the change of the shape of the distribution, such that this change of distribution shape can be described by a probability distribution function, such as a Weibull distribution, as described in Section 5.

Equipment Needs for Full-scale Reactor Testing

As noted in Section 2.2, reactor validation can occur on-site at a water treatment facility or off-site at a third-party validation test center, at a UV manufacturer’s facility, or perhaps at another location. In all cases, the testing site shall meet the minimum requirements described below to be able to set up the reactor train and efficiently conduct field-scale UV validation testing. In keeping with the UVDGM requirements, the UV system must be tested at full-scale.

The next several sections point to critical elements of the test stand. Detailed recommendations are provided in the UVDGM regarding the water source, the UV-absorbing chemical to be used to simulate reduced UVT, mixing, sampling ports, configuration of inlet/outlet piping, accounting for non-uniform lamp aging, lamp positioning, UV sensors, and UV sensor port...
windows. Except for selected specifics, the following text reiterates these key elements or refers to the discussions contained in the UVDGM as the primary source of information.

**Water Source**

Reference is made to the UVDGM Section 5.4.1 with respect to dechlorination and water quality. When considering the use of LA, ambient particles can represent a source of interference in the analysis of dyed microspheres by flow cytometry. In particular, ambient particles that have optical behavior that is similar to the microspheres will be indistinguishable from microspheres in cytometric analysis. As such, it is important to define the extent to which this form of interference may be present in a water supply, and to take measures to limit or avoid this form of interference. For example, recycling the test water during the validation test should not be practiced.

An important step in characterizing potential interference from ambient particles is measurement of their optical behavior. To address this need, water samples are collected from the source water that is to be used in validation testing. Samples should be collected in a manner that is similar to the collection process that is anticipated for the actual validation work, and should be subjected to the same sample work-up procedures (i.e., membrane filtration, centrifugation, and re-suspension in an appropriate aqueous buffer). The samples should then be subjected to analysis by flow cytometry following the same analytical methods that are to be used for microsphere samples from validation tests. If possible, these tests should be repeated on samples collected on several different days, under different operating conditions for the facility that produces the water, so as to provide information regarding the variability to be expected in the background (ambient) particles and their optical characteristics.

The information gained from these analyses will allow for definition of the extent to which ambient particles may interfere with DMS measurements in LA. This information will also be helpful in defining an empirical limit on operating conditions that define an acceptable background, by correlation of the results of these tests with bulk parameters, such as turbidity, that can be measured at the test facility.

The data from these preliminary tests can also be used to define a gating strategy for analysis of DMS samples. Gating is a post-processing numerical procedure that is applied to the cytometry data for a sample, and which can be used to reduce the magnitude of this interference. A gate will define a range of acceptable values for optical parameters. By gating around areas of known interference, it is possible to reduce or eliminate this interference from a data set.

The data from these preliminary tests will also be useful in defining a pre-treatment strategy for the DMS. In particular, pre-irradiation of DMS under a collimated beam has been used as a pre-treatment strategy to increase the fluorescence intensity (FI) of the DMS prior to introduction to the reactor. Pre-irradiation of microspheres can improve microsphere differentiation against background particles by increasing their FI. When used in combination with an appropriate gate (on the FI signal), it is possible to reduce or eliminate interference from ambient particles. It has been observed that a minimum pre-irradiation dose of 200 mJ/cm² can raise the DMS initial FI to
a sufficient level to allow discrimination against background particles, while maintaining enough
sensitivity for DMS to respond to UV doses up to 600 mJ/cm².

**UV Absorbing Chemical**

Selection of a UV absorbing chemical for LA is no different than already used with biodosimetry. Refer to the UVDGM for a discussion of absorbers. If polychromatic lamps are used, full UVT spectral scans should be conducted for each UVT value used in the validation. The ideal UVT adjustment agent should yield test water with an absorbance spectrum similar to the background filtered effluent/water used in the actual application. Lignin sulfonic acid (LSA), coffee, and humic acids, such as those derived from leonardite shales, are candidate UV-absorbing chemicals typically used during validation. Studies have been conducted to compare the dose-response behavior of DMS suspensions prepared with DI water against the dose-response behavior of DMS suspensions prepared with addition of a UVT adjustment chemical. It has been demonstrated that neither LSA nor coffee has any detectable interference to the DMS photochemical reactions. When humic acids are considered for UVT adjustment, a dose-response study is recommended to evaluate potential impacts on the performance of DMS.

**Mixing**

Reference is made to the UVDGM (Section 5.4.3) for a discussion of mixing. The effects of mixing are equally important when using biodosimetry or LA. Test stands should conform to the guidance suggested by the UVDGM, and confirm adequate mixing via the methods provided in the UVDGM.

**Sampling Ports**

Refer to the UVDGM (Section 5.4.4). As with biodosimetry, the sampling ports for DMS should be located far enough from any UV radiation sources to avoid any effect from extraneous irradiation. The sample ports installed for DMS should be sized for collecting a large volume (10 to 20 L) within a relatively short collection time (typically less than 30 seconds, but dependent on the flow rate through the testing unit). This may require larger diameter ports (3/4 to 1” diameter) than are typically used for biodosimetry sampling. The ports should allow a delivery rate of 5 to 10 gpm for systems under pressure. In gravity flow systems, one can consider using a sampling pump. Note that at low feed-forward flows the sampling rate should not be so high that it affects the upstream (for effluent sample) or downstream (for influent sample) hydraulic behavior in the vicinity of the reactor. In pressure systems, this can be accommodated by maintaining a delivery rate that does not affect the pressure with the reactor during sample collection.

**Configuration of Inlet and Outlet Piping**

The test stand’s inlet and outlet piping configuration is important. The discussions provided in the UVDGM (Section 3.6 and 5.4.5) are equally relevant to the application of dyed microspheres.

If the UV system is validated off-site, the inlet and outlet piping configuration at the water treatment plant should result in a UV dose distribution that is statistically indistinguishable from
the dose distribution delivered at the validation test facility. As such, the UVDGM recommends either mimicking the commissioned facility piping, or configuring the inlet piping as a “worst case” hydraulic condition. If Lagrangian actinometry is used for validation, dose distributions for the commissioned operating conditions (UVT and power, or UV intensity) at maximum and minimum flow rates should be measured as part of the validation. These data can then be used to calibrate and verify CFD-I modeling for the validated system. When commissioning, the same operating conditions can be assessed by the verified modeling approach. Alternatively, DMS testing can be tested at a limited number of points to verify that the resulting dose distributions are equivalent or better than observed in the validation.

**Accounting for Non-uniform Lamp Aging**

Reference is made to the UVDGM regarding lamp aging (Section 5.4.6). Validation testing of full-scale UV systems should account for decreased UV output caused by sleeve fouling, sleeve aging, lamp aging, and UV sensor window fouling. In the absence of documented direct testing, the default lamp aging factor is 0.5 for all variations of mercury lamps. Similarly, the default sleeve fouling factor is 0.80. If alternate lamp aging or sleeve fouling factors are to be used, it is the manufacturers’ responsibility to document and justify such choices.

**Lamp Positioning to Address Lamp Variability**

Refer to the UVDGM (Section 5.4.7) to follow proper practice regarding lamp-sensor positions in systems that have less than one sensor per lamp. These protocols are equally applicable to the validations that rely on Lagrangian actinometry.

**UV Sensors**

UV sensors used in disinfection applications should be germicidal, defined by the UVDGM as having a spectral response that peaks between 250 and 280 nm, and less than 10 percent of its total measurement is due to radiation above 300 nm when mounted on the UV reactor and viewing the UV lamps through the water that will be treated. Reference is made to the UVDGM (Section 5.4.8) for protocols dealing with sensors during validation. These are equally applicable to validations based on Lagrangian actinometry.

**UV Sensor Port Windows**

Here, reference is made to the UVDGM (Section 5.4.9) regarding the UV sensor port windows.

**Accuracy of Measurement Equipment**

During validation testing, all equipment should be carefully selected and calibrated to minimize uncertainty. All measurements of flow rate, electrical power consumption, and head loss should be traceable to an independent standard. Moreover, because they are key parameters that affect UV dose delivery, measurements of UVT and UV intensity should be NIST-traceable (or equivalent) with a known measurement uncertainty.
**Flow Meters**

Refer to the UVDGM (Section 5.5.1). During validation testing, the uncertainty of flow rate measurements should be less than or equal to 5 percent. The measurement uncertainty of the flow meter can be verified by comparing measured flow rate to a second, calibrated flow meter or a calibrated pitometer. Alternatively, the flow meters can be calibrated at the testing facility using the approach based on water volume withdrawal/buildup over a period of time.

**UV Spectrophotometers**

Refer to the UVDGM (Section 5.5.2) for a detailed discussion of criteria pertaining to spectrophotometric measurements and calibration. Spectrophotometer measurements of \( A_{254} \) should be verified using NIST-traceable potassium dichromate UV absorbance standards and holmium oxide UV wavelength standards. Many UV spectrophotometers have their own internal QA/QC procedures to verify calibration. UV absorbance of solutions used to zero the spectrophotometer should be verified using reagent grade organic-free water certified by the supplier to have zero UV absorbance.

**Power Measurements**

Refer to the UVDGM (Section 5.5.3). Voltmeters, ammeters, and power meters used to measure (1) ballast and UV equipment input voltage, and (2) consumed current and power, should bear evidence of being in calibration (e.g., have a tag showing that it was calibrated). The accuracy of the measurements can be verified using a second instrument or a standard measurement. Power meters should provide a measure of true power as opposed to apparent power in units of kilovolt ampere (kVA).

**UV Sensors**

Refer to the UVDGM (Section 5.5.4) for procedures to check the uncertainty of the duty and reference UV sensors used during validation. During validation testing, duty UV sensor measurements should be within 10 percent of the average of two or more (preferably three) reference sensor measurements. Reference sensors should be recently calibrated and should agree within the calibration certificate-specified measurement uncertainty. If the reference sensors are the same type as the duty sensors, the measurement from each individual reference sensor should be within 10 percent of the average of all reference sensor measurements as well.

**Identifying Test Conditions**

Numerous combinations of experimental tests can be performed to validate a UV reactor. The number of tests could range from a few tests to a complex matrix spanning a range of UV dose, flow rate, UVT, ballast power, and lamp status combinations. The test design (i.e., number of tests and test conditions) depends on several factors, as summarized in Table 3-1.
Refer to discussions provided in the UVDGM (Section 5.6) regarding the design of the operating test matrix for validation. In effect, the test design for DMS is the same as for biodosimetric testing. The intent is to generate sufficient information within the operating envelope for a specific system. Typically, the matrix is designed to cover at least three points in the range of each variable: the minimum, maximum and an intermediate point. Thus if there are three variables, the test would be designed as a 3 x 3 x 3, or 27-point matrix.

Table 3-1. Factors to be considered in validation test design

<table>
<thead>
<tr>
<th>Factor</th>
<th>Examples</th>
<th>Section with Additional Guidance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Purpose of validation testing</td>
<td>Validation vs. confirmation of existing validation, or system optimization based on dose distributions</td>
<td>UVDGM 3.6 and 5.6. <em>LA provides a direct measurement of the UV dose-distribution – and variations that may occur across a reactor’s operating range. Approach is applicable to all validation objectives.</em></td>
</tr>
<tr>
<td>2. Dose-monitoring strategy of the UV reactor</td>
<td>Intensity Setpoint Approach vs. Calculated Dose Approach</td>
<td>UVDGM 3.5.2. <em>Manufacturers establish the operating strategy. DMS is applicable to both.</em></td>
</tr>
<tr>
<td>3. Operational strategy (for Intensity Setpoint Approach only)</td>
<td>Single-setpoint vs. variable-setpoint</td>
<td>UVDGM 3.5.2. <em>DMS is applicable.</em></td>
</tr>
<tr>
<td>4. Lamp aging and fouling</td>
<td>Default aging factor is 0.5, and default fouling factor is 0.8.</td>
<td>UVDGM 3.4 and 5.4.6. <em>No difference with DMS approach.</em></td>
</tr>
<tr>
<td>5. Target microbe and target log inactivation</td>
<td>Pathogen log inactivation or microbial indicator log inactivation</td>
<td>UVDGM 3.1. <em>Note that DMS uses dose-response relationship developed for targeted pathogens or alternate surrogates. This means that dose-distribution can be used for current and future microbes without repeated biodosimetric testing.</em></td>
</tr>
<tr>
<td>6. Full operating range of flow rate and UVT</td>
<td>Range of flow 3 – 12 MGD, range of UVT 55 – 65%</td>
<td>UVDGM 3.4. <em>Same application to DMS</em></td>
</tr>
</tbody>
</table>
Test Conditions for the UV Intensity Setpoint Approach

This method remains as presented in the UVDGM, Section 5.6.1, and is excerpted here. DMS are used as the surrogate and the resulting dose-distribution is used to determine the log-inactivation (or RED) for the targeted microbe or microbes based on microbe-specific dose-response data are used for this purpose.

For the UV Intensity Setpoint Approach, the purpose of validation testing is to determine the validated LI (or RED) corresponding to the UV intensity setpoint for a reactor at a particular flow rate. Typically, the manufacturer determines the UV intensity setpoint for their reactor. If the manufacturer does not establish the UV intensity setpoint for their reactor, an intensity setpoint can be established through the following procedure:

- Record the UV intensity measurement at conditions of maximum UVT and 100 percent power (S₀).
- Reduce the lamp power until the measured UV intensity results in the following relative sensor intensity (S/S₀).
  (1) If aged lamps are used during validation testing, the relative sensor intensity should be equal to the fouling factor.
  (2) If new lamps are used during validation testing, the relative sensor intensity should be equal to the EOLL factor, which is the fouling factor multiplied by the aging factor.
- Reduce the UVT of the water to the minimum UVT to be used during validation.
- Record the UV Intensity at reduced power and reduced UVT conditions. This intensity is the UV intensity setpoint.

The UV Intensity Setpoint approach uses two validation test conditions, as specified in the UVDGM. The first involves reducing the UVT at full power until the UV intensity measured by the UV sensor is equal to the UV intensity setpoint. The second involves testing at high UVT but reducing input power until the UV intensity measured by the sensor is equal to the UV intensity setpoint. Table 3-2 summarizes only the minimum test conditions for single-setpoint operations; additional test conditions should be conducted at different flow rates for variable setpoint operations, if this encompasses the system’s operating strategy.

The dose-distribution is determined with DMS at each of the two conditions on Table 3-2. The condition that yields the lowest log-inactivation prediction for the targeted microorganism is used as the equivalent setpoint performance for the UV unit. Multiple DMS effluent samples (e.g., six) are recommended for collection at each of the two test conditions to lower testing uncertainty. This is discussed in a later section.
Table 3-2. Minimum Test Conditions for the UV Intensity Setpoint Approach

<table>
<thead>
<tr>
<th>Test ID</th>
<th>Flow Rate</th>
<th>UVT</th>
<th>Lamp Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Design (maximum)</td>
<td>Lowered to give the intensity setpoint</td>
<td>Maximum (100%)</td>
</tr>
<tr>
<td>2</td>
<td>Design (maximum)</td>
<td>Maximum (in operation)</td>
<td>Lowered to give the intensity setpoint</td>
</tr>
</tbody>
</table>

Test Conditions for the Calculated Target Pathogen Inactivation Approach

This method remains as presented in the UVDGM, Section 5.6.2, except that DMS are used as the surrogate. Reference is made to the UVDGM (Section 5.6.2). The resulting dose-distribution is used to determine the log-inactivation for the targeted microbe or microbes, based on their respective dose-response relationships. Although RED is also a product of this analysis, emphasizing log inactivation can be considered a more appropriate method of representing reactor behavior than “dose”. By developing a relationship to predict pathogen inactivation as a function of operating conditions (rather than “dose”), the validation provides a clearer representation of what the reactor actually does. By retaining the dose distribution measurement as a characteristic of a reactor (for a given set of operating conditions), LA also allows for estimation of the performance of the reactor in terms of inactivation of any microorganism for which accurate UV dose-response behavior is available. Moreover, much of the ambiguity associated with the term “dose” is mitigated, since one must carefully define the RED by the microbe used to develop it.

For the Calculated Target Pathogen Inactivation Approach, the purpose of validation testing is to develop a monitoring equation relating pathogen log inactivation to operating parameters such as flow rate, UVT, lamp power (often quantified as a relative sensor value), and, in some cases, lamp status (variations in row and/or banks of lamp within a reactor). For each operating parameter used in the equation, at least three conditions (emphasis by the UVDGM) should be evaluated during validation testing. Three data points are needed for interpolation of results because the relationships between LI (and RED) and operating parameters such as flow rate and UVT are typically non-linear.

In many cases, three operating parameters (e.g., UVT, flow rate, and lamp power) are used to develop the dose-monitoring equation, resulting in a minimum of 27 test conditions ($3 \times 3 \times 3$). Fewer test conditions are needed when the dose monitoring equation is based on fewer than three parameters, such as when a minimum UVT is assumed for all operating conditions. More than 27 test conditions may be needed if lamp status will be a variable as well. Refer to the UVDGM (Section 5.6) for a discussion of test matrix design.

Biodosimetric QA Sampling for Selected Test Points

This QA task will demonstrate the log-inactivation predicted for the biodosimetric surrogate based on the DMS results, as compared to the log inactivation directly measured. The biodosimetric protocols articulated by the UVDGM would be followed for this experimental element, with simultaneous injection of the DMS and microbiological surrogate.
When considering the calculated log-inactivation strategy, the entire test matrix designed for the UV unit validation will be addressed with DMS, from which a known dose-response relationship for the targeted pathogen will be applied against the measured dose-distribution to determine the predicted log inactivation for the target pathogen. It is recommended that approximately 5 to 10% of the test points used for DMS validation include a microbiological surrogate, such as the bacteriophage discussed in the UVDGM. Depending on the expected operating range for the UV unit, two different challenge microorganisms with substantially different UV sensitivities are preferred, e.g., MS2 and T1. These QA bioassays should be at test points at the edges of the operating envelop establish for the unit.

If validating for an intensity-setpoint control strategy, as discussed above, at least one of the test points in Table 3-2 should include a microbiological surrogate, such as the bacteriophage discussed in the UVDGM. The objective is to allow a comparison of the results of the LA testing with another physical experiment.

**Test Conditions for Confirming an Existing Validation Equation**

Reference is made to the UVDGM (Section 5.6.3). If on-site validation testing is conducted to show that the hydraulic conditions at the water treatment plant result in the same or a better UV dose distribution than had been validated at the off-site test facility, test conditions should generally span the range of operating conditions expected at the treatment plant (e.g., minimum and maximum UVT, minimum and maximum flow rate). The dose distribution can illustrate the combined effect of the intensity field and hydraulic conditions inside the UV system. Also, as stated in the UVDGM, on-site and off-site testing is usually conducted under different hydraulic conditions and may produce different results. Therefore, combining the datasets from on-site testing and off-site validation to develop a new dose-monitoring equation is not recommended.

**Quality-Control Samples for Lagrangian Actinometry Validation**

As discussed in Section 3.4.3, the current LA protocol recommends that biodosimetric samples should be collected for a limited set of test conditions to verify the log-inactivation predicted by the dose distribution. As such, it is reasonable to keep the same set of QC criteria outlined by the UVDGM for biodosimetry. The LA DMS QC criteria are added to these.

Reference is made to the UVDGM (Section 5.6.4) for a description of the QC samples and associated criteria for the microbiological surrogates used for validation. The QC samples and criteria recommended for the DMS used for Lagrangian actinometry validation are as follows:

- **Reactor Controls** – These are influent and effluent water samples taken with the reactor UV lamps turned off while adding DMS. The samples are analyzed as by standard protocols and the dose-distribution is integrated with the targeted microbe dose-response equation. The resulting log inactivation should be not greater than 3% of the lowest log inactivation observed in the overall validation matrix. At least one set of reactor controls (one influent and one effluent) is required for each UV system tested.
• **Reactor Blanks** – The presence of dyed microspheres in the test water is not expected. However, with time and use, some DMS may be present in the reservoir or piping. If this is the case and there is a measurable fluorescence level carried by these microspheres, interference could be introduced. An effluent sample should be collected when there is no injection of DMS to the flow stream, and the UV lamps are at full power. Analysis should show negligible presence of DMS based on fluorescence intensity signal. The concentration of particles that will appear as DMS in the blank sample should not represent more than 3% of the lowest concentration of DMS used for testing.

• **Operation Controls** – A sample of the stock, pre-irradiated DMS should accompany the stock DMS during transport and handling at the test facility. At the end of a testing day, a sample of the DMS stock should be collected as well. The mean fluorescence of the stock and that of the influent samples collected during the day should not differ over a FI change greater than 3% of the minimum mean FI increase achieved within such testing day.

• **Stability Samples** – Selected dose-response samples taken during validation testing are used to assess the stability of the DMS fluorescence over the time period from sample collection in the test water to completion of sample analysis. These samples should be analyzed at the same time with other field test samples (day 1), and analyzed weekly for fluorescence in the subsequent three weeks (for example day 8, 15 and 22, or longer). The mean FI measured at each day should not differ from the average mean FI over the stability check period by more than 3% of the minimum mean FI increase achieved among all regular field test samples. Stability samples should be collected when operating with and without addition of UVT adjustment chemicals. Stability of the DMS is independent of any particular UV system; as such, the stability tests can be conducted for a given facility/test water. In the case of an off-site facility with the same water supply year round, one set of stability tests is recommended every six months.

• **Method Blank** – The DI water used for sample preparation, such as the suspension liquid for elution from the filters, will be checked for possible contamination particles. One DI water sample shall be collected on each testing day for analysis through the flow cytometer. The percentage of particles in the blank sample with similar size and granularity to those of DMS, at or above the pre-irradiated fluorescence level, relative to the average DMS particle count analyzed during the injected test runs should be less than 0.1%.
CHAPTER 4
GUIDELINES FOR CONDUCTING EXPERIMENTAL DYED-MICROSPHERES TESTS

This section provides general guidelines for preparing dyed microspheres for testing, recommendations for conducting reactor validation testing, and detailed discussions regarding collimated beam test methods for LA. The analysis of the dyed microspheres samples by flow cytometry is also presented.

PREPARATION OF THE DYED MICROSPHERES FOR FIELD TESTS

Pre-Irradiation of Dyed Microspheres

Background (ambient) particles may elicit fluorescence (and other optical) signals that mimic DMS. Therefore, it is necessary to take steps to maximize the ability to discriminate microspheres from ambient particles. Pre-irradiation of DMS is used to increase their FI as a means of improving discrimination against background particles. However, pre-irradiation also reduces the ultimate dose range over which the microspheres can be applied. Therefore, the pre-irradiation UV dose should be selected to satisfy these two constraints.

For testing at dose levels less than 300 mJ/cm², dyed microspheres should be subjected to a pre-irradiation process with a UV dose of 250 to 300 mJ/cm². To avoid saturation, for very high-dose applications (greater than 300 mJ/cm²) one should reduce the pre-irradiation dose to 150 mJ/cm². Pre-irradiation should be performed in a shallow, well-mixed batch reactor (e.g., Petri dish) under a collimated UV²⁵⁴ source. The DMS concentration in suspension for pre-irradiation is usually around 2×10⁶/mL; the UVT of this DMS suspension (in DI water) is about 40% through a 1.0 cm optical path. This concentration is selected to balance the suspension UVT and the total time required to pre-irradiate a large volume of DMS. The absorbance of the DMS suspension has to be accounted for when calculating the delivered collimated dose. The suspension depth in the Petri dish should be chosen so as to allow a sufficient number of microspheres to be irradiated, while also ensuring uniform irradiation under the collimated beam.

Handling and Storage of Pre-Irradiated Dyed Microspheres

The handling and storage of DMS after pre-irradiation should follow the same protocol as described in Section 3.1.4.2. However, long-term storage of DMS after pre-irradiation is not recommended. It is suggested that pre-irradiation should be conducted on DMS stock sufficient for use within two weeks.

Quality Control for Pre-Irradiation

DMS stock suspensions used within two weeks of pre-irradiation will not require QC checks, as long as the appropriate storage and handling protocol is followed. Pre-irradiated DMS stock suspensions may be used after more than two weeks storage if they are subjected to QC checks; however, use of such suspensions should be avoided, if possible. The protocol described in Section 3.1.4.3 should be followed for pre-irradiated DMS QC checks.
FULL-SCALE UV REACTOR TESTING

Three key steps comprise full-scale reactor testing: (1) verifying reactor properties, (2) installing the reactor, and (3) conducting the tests. Reference is made to the UVDGM and to Section 3 of this document for guidance regarding documentation of the test reactor and setup of the validation test stand. The following discussions relate to the use of the DMS for field tests and sampling. This protocol is focused on DMS testing; any biodosimetric testing should be in compliance with relevant UVDGM guidance.

DMS Field Tests and Sample Collection

During full-scale testing, the reactor is operated at each of the test conditions (established as a function of key operating variables) in accordance with the Validation Test Plan (Section 3). The following steps and guidance should be taken to ensure good results:

- Confirm hydraulic steady-state conditions before injecting DMS by monitoring the UV sensor measurements and the UVT. UVT should be within ± 1.0% of the target UVT. Ensure that a minimum of three volume exchanges have occurred (volume of pipe and reactor from point of injection to point of effluent sampling) at a constant flow rate prior to initiating injection of the DMS suspension.
- Inject the DMS into the flow upstream of an in-line mixer located upstream of the reactor at a rate that will yield approximately 15,000 to 20,000 DMS/L in the feedwater to the UV reactor. The in-line mixer is not needed if it is demonstrated that sufficient mixing occurs between the point of injection and entry into the reactor.
- After initiating the injection of the DMS suspension, allow an additional three volume exchanges before collecting samples.
- For Lagrangian actinometry, at least one influent sample and three effluent samples should be collected for each test condition. Typically, 10,000 dyed microspheres are recommended to be measured from each valid flow test sample when analyzed by flow cytometer. Based on experience, the DMS recovery rate is usually between 5 ~ 10% of what is injected into the test stream. The majority of injected DMS is lost before reaching the point of cytometry analysis, due to the attachment to ambient particles, the filtration, elution and centrifuge process, and debris or multiplets present in the DMS stock. Based on the initial injection rate and the expected DMS recovery in flow cytometric analysis, the volume of samples to be collected can be calculated. DMS samples collected from the field tests can be temporarily stored at room temperature, protected from ambient light. The containers should be opaque, or wrapped in an opaque plastic under indoor conditions for a limited time period, with controlled room temperature and absence of direct sunlight.
- Sample taps should be fully open at least 30 seconds before the DMS sample collection. The flow rate through sample ports should be sufficient to collect the total volume within a short period of time, while not influencing the hydraulic conditions inside the test line. Samples should be collected in large containers (e.g., minimum 10-L polypropylene carboys) that have been thoroughly cleaned. Sample containers should be opaque to UV radiation, or covered with a UV-opaque material.
- On the same day the sample is collected, each sample shall be filtered through a 3.0-µm membrane filter. The intent is to retain greater than 2×10^5 microspheres on each filter; the
sample volume required to reach this target will be determined from the injected DMS concentration. After filtration is completed, the filtrate is discarded and the filter is transferred to a sterile 50-mL centrifuge tube. 30 mL of DI water can be added to the tube to keep the filter completely submerged. The sealed tube should then be wrapped in foil to avoid exposure to ambient light. The tubes should be stored at 4°C until further sample work-up and analysis.

- Separate influent samples at each test condition are required for UVT measurement within 24 hours of collection.
- Measure and record the flow rate through the reactor, all UV sensor measurements, on-line UVT measurements, and any PLC-calculated UV RED values both before and after the samples are recorded.
- Measure and record the electrical power consumed by the lamp ballasts, lamp output, lamp amperage/voltage, power conditioning, ambient air temperature, and water temperature for each flow test.
- Repeat the test if the flow rate, UV intensity, lamp power, or UVT changes by more than the recommended error of the measurement over the course of collecting the influent/effluent sample sets.

Filter holders (4.7 cm diameter) with nitro-cellulose membrane filters (3.0 µm pore size, 4.7 cm diameter) can be used for sample filtrations. A sufficient volume of sample needs to be filtered to capture an adequate DMS population. Target filtration volumes should be established with the goal of allowing capture of roughly 2×10^5 microspheres from each sample. The volume of water required from filtration will depend on the concentration of DMS in the flow. A convenient method for filtering the samples uses an in-line filter holder; a pump is used to pass the sample through the filter, with the filtrate going to drain. A multi-head peristaltic pump can be used, allowing for the processing of several samples (e.g., a 4-head pump can simultaneously process the single influent and three effluent samples collected for one test condition). This method has been demonstrated in the field, and can significantly reduce the amount of time required for sample filtrations.

Particles captured on each filter are re-suspended in a small volume of DI water. Separation from the filter is assisted 10 minutes of sonication using an ultrasonic cleaner, followed by one minute of agitation on a vortex mixer. Previous experience with an ultrasonic cleaner (Cole-Palmer), model 8890 showed good success, with an output frequency of 42 kHz. The purpose of sonication at this step is to promote the release of DMS from the filters. The filters are then removed from the centrifuge tubes and discarded. DI or tap water can be used as the bath solution for the cleaner tank, and DMS sample containers should be well-sealed and submerged. When an ultrasonic cleaner is continuously used for DMS samples, the temperature of the bath solution should be controlled. Periodically exchanging with cool water or adding ice cubes can help to maintain the temperature inside the cleaner bath at no higher than room temperature.

The aqueous DMS suspensions are then subjected to centrifugation at 3000 rpm for a minimum of 10 minutes. Sample supernatants are discarded by pipetting the liquid from the tube (not by pouring) and not disturbing the sediments at the bottom. The remaining sample concentrate (roughly 1.5 mL) is analyzed by flow cytometry.
COLLIMATED BEAM TESTING FOR THE DYED MICROSPHERES

A collimated beam test is performed for purposes of defining the UV dose-response behavior of the DMS. The influent water from the testing facility should be used as the background water matrix for the collimated beam tests. If the full-scale reactor testing lasts for more than one day, at least one collimated beam test should be conducted for each day of testing, with two exposures per delivered dose. A minimum of two dose-response tests are recommended for the overall validation, one each at the high and low validation UVT.

The concentration of DMS for the dose-response test is typically around $10^8$/L, as compared to the concentration of $10^5$/L used in the field tests; the concentration selected for the collimated beam exposures should be such that it has minimal effect on the UVT transmittance of the DMS suspension while providing high enough concentrations of DMS in the dose-response samples for cytometric analysis. This is the case when the concentrations are at or less than $10^8$ particles per liter.

The collimated beam test for DMS effectively follows the general protocol and apparatus configuration described by the UVDGM, Appendix C. It involves placing liquid samples with the dyed microspheres in an open cylindrical container (e.g., a Petri dish) and exposing the sample to collimated UV radiation for a predetermined period of time. The UV dose is calculated using the measured intensity of the incident UV radiation, UV absorbance of the aqueous sample, and exposure time. Reflection at the air:water interface and beam divergence are also accounted for in calculating the UV dose delivered to each sample, following UVDGM guidance.

In the case of DMS dose-response tests, the response of interest is the change in FI as a function of applied dose. Each dose-exposure generates a fluorescence intensity distribution. The FI distributions for the range of UV doses used in these experiments should vary in a regular, predictable manner. As with biodosimetry, interpolation is practiced between collimated dose results when applied to interpretation of the field sample results.

Non-linear regression is used to define a function to fit to the dose-response FI distributions. A Weibull distribution has been found to fit the data from DMS dose-response experiments well, and the parameters of the Weibull function have been found to vary in a regular manner across the range of applied doses. Therefore, non-linear regression of the dose-response data based on a Weibull distribution is recommended for this interpolation approach. However, it is conceivable that other distribution functions may also be applied for this purpose. The non-linear function used to fit to the DMS dose-response data should be selected so as to allow for interpolation of FI behavior for UV doses between those applied in the dose-response experiment. Interpolation accuracy is particularly important in the lower tails of the FI distributions.

Collimated Beam Apparatus Design and Operation

The collimated beam apparatus used for this test should enclose a low-pressure mercury lamp with monochromatic output at 254 nm. The inner surfaces of the device should be minimally reflective of UV radiation. To prevent ozone formation, lamps that emit 185-nm radiation should
Guidelines for Conducting Experimental Dyed-Microspheres Tests | 39

not be used. The UV radiation enters the suspension with a near zero-degree angle of incidence and is relatively uniform across the surface area. Figure 4-1 provides an example of a typical collimated beam apparatus. The spatial variability of beam intensity should be measured by scanning across the face of the beam at a fixed distance from the bottom of the collimator.

The output from the lamp, as measured with a radiometer should vary by no more than 5% over the exposure time. A stable lamp output can be obtained by driving the lamp with a constant power source and maintaining the lamp at a constant operating temperature. If the line voltage is not sufficiently stable, a voltage regulator may be used to obtain a stable power supply. A stable temperature can be obtained by maintaining steady airflow across the lamp. A collimating device should be designed such that air is forced across the lamp to provide thermal stabilization. The airflow pattern should also be maintained such that air currents are directed away from the object that is being irradiated, thereby reducing the potential for contamination in the experiment.

Figure 4-1. Collimated Beam Apparatus

The UV lamp should be located far enough above the surface of the microsphere suspension that uniform irradiance is obtained across the sample’s surface and UV radiation enters the suspension with a near zero-degree angle of incidence. In order to vary the UV intensity incident on the suspension, the distance between the suspension and the lamp can be adjusted.

The uniformity of the intensity field across the sample’s surface should be assessed by measuring the “Petri Factor,” defined as the ratio of the average irradiance across the suspension
surface to the irradiance measured at the center (Bolton and Linden 2003). The average irradiance is determined by averaging radiometer measurements taken at each point in a 5-mm spaced grid across an area defined by the suspension’s surface. The collimated beam apparatus should have a Petri Factor greater than 0.9.

The lamp and the optical path from the lamp to the suspension should be enclosed to protect the user from exposure to UV radiation. The collimating device should serve this function. The inside surface of the collimating device should have a low UV reflectance and incorporate apertures to improve UV light collimation. A shutter mechanism is sometimes used to control the exposure of the suspension to UV light. The exposure times should be measured with an uncertainty of 5% or less. Exposure times less than 20 seconds are not recommended.

The aqueous suspension should be irradiated in an open cylindrical container (e.g., Petri dish). The diameter of the container should be smaller than the diameter of the UV beam incident on the container. Sample depth should be 0.5 to 2 cm. The material of the container should not adsorb the challenge microorganism or DMS enough to impact its measured UV dose-response.

Sample volumes irradiated in the container should be sufficient for measuring the DMS fluorescence after irradiation. The aqueous suspension should be mixed using a stir bar and a magnetic stirrer at a rate that does not induce vortices. The volume and diameter of the stir bar should be small relative to the volume and depth of the sample.

The irradiance at the center of the suspension’s surface before and after exposure to UV radiation should be measured using a UV radiometer calibrated at 254 nm. During measurement, the elevation of the radiometer’s detector surface (calibration plane) should match that of the suspension’s surface and be perpendicular to the incident UV. The calibration plane of the radiometer should be specified in the radiometer’s calibration certificate.

**Accuracy of Monitoring Equipment Used for the Collimated Beam Tests**

Similar to the recommended procedures for full-scale reactor testing, spectrophotometer measurements of \( A_{254} \) should be verified using NIST-traceable potassium dichromate UV absorbance standards and holmium oxide UV wavelength standards. The measurement uncertainty of the spectrophotometer should be 10% or less.

The radiometers used in the collimated beam tests should come from the manufacturer with a certified uncertainty of 8 percent or less at a 95% confidence level at the intervals suggested by the manufacturer. At minimum, the accuracy of the radiometer used to measure the UV intensity should be verified at least at the beginning and the end of each collimated beam test session using a second radiometer. The two radiometers should read within 5 percent of each other. If the two radiometers do not read within 5% of each other, a third radiometer should be used to identify which radiometer is out of specification. The two radiometers with readings within 5% of each other should be used. If none of the radiometer readings match, at least two of them are likely out of calibration.
If the above criteria are met, the average radiometer measurement can be used in calculations. Alternatively, the radiometer that provides the lowest reading could be used. If these criteria are not met, the radiometers should be recalibrated. The radiometers should also be checked to be sure that the irradiance measurement does not differ by more than 5 percent before and after UV exposure.

In accordance with the UVDGM, the uncertainties of the terms in the UV dose calculation should meet the following measurement uncertainty criteria, and be included in the validation report.

- Depth of suspension \((d) \leq 10\%\)
- Average incident irradiance \((E_s) \leq 8\%\)
- Petri Factor \((P_f) \leq 5\%\)
- \(L/(d + L) \leq 1\%\)
- Time \((t) \leq 5\%\)
- \[
\frac{1 - 10^{-A_254d}}{A_254d} \leq 5\%
\]

Collimated Beam Exposures for Dyed Microspheres

UV doses should be selected to cover the range of expected values for the entire UV dose distribution, using a minimum of 10 exposure levels plus a control [zero (0) UV dose]. For each dose, two replicate UV exposures are recommended to improve the quality of dose-response behavior characterization. The collimated beam test is conducted in the validation test water matrix, either by taking a “seeded” influent sample or spiking the adjusted source water with DMS. In either case the water is spiked to a DMS concentration of approximately \(1 \times 10^8\) DMS/L. Results of typical DMS fluorescence intensity (FI) distributions from a UV dose-response experiment are illustrated in Figure 4-2.

Depending on the width of the reactor’s dose distribution curve (a characteristic of the UV reactor’s integrated hydraulic and intensity distributions), the upper and lower boundaries of doses that are delivered by the system to individual DMS particles can be much higher or lower than the mean equivalent dose. Within practical limits these upper and lower bounds are between 1 mJ/cm\(^2\) and 600 mJ/cm\(^2\). Therefore, in order to span the entire range in the reactor’s dose distribution, the range of doses selected for the dyed microspheres dose response test should cover up to at least three times the expected maximum RED that will be validated for the system. For example, if the maximum expected RED is 100 mJ/cm\(^2\) during a single flow test day, the DMS dose-response test for that day should have a dose range up to at approximately 300 mJ/cm\(^2\).
The following procedure is recommended for collimated-beam testing of a water sample containing dyed microspheres:

1. Measure the $A_{254}$ of the sample using a properly calibrated spectrophotometer. If the water sample collected from the field testing is to be spiked with a higher concentration of DMS, the $A_{254}$ of the sample shall be measured after the DMS concentration adjustment;
2. Place a known aliquot from the water sample into a Petri dish and add a stir bar. Measure the water depth in the Petri dish;
3. Measure the UV intensity delivered by the collimated beam at the surface plane of the sample using a calibrated radiometer;
4. Calculate the required exposure time to deliver the target UV dose following Equation 4-1 (Equation C.6 in the UVDGM Appendix C, Section C.6.3);
5. Block the radiation from the collimating device using a shutter or equivalent;
6. Center the Petri dish with the water sample under the collimated beam;
7. Unblock the radiation from the collimating device and start the timer;
8. When the target exposure time has elapsed, block the radiation from the collimating device;
9. Remove the Petri dish and sample from the collimating apparatus;
10. Re-measure the UV intensity and calculate the average of this measurement and the measurement taken in Step 3. The value should be within 5 percent of the value measured in Step 3;
11. Using Equation 4-1, calculate the UV dose applied to the sample based on the observed experimental conditions (this should be similar to the targeted dose);
12. Repeat steps 1 through 11 for each replicate and targeted UV dose value.

The exposed aqueous solution should be protected from exposure to ambient light. Pour the contents into a centrifuge tube; the exposed DMS suspension is centrifuged to separate and concentrate the DMS to a small volume (usually between 1.5 to 2.0 mL) for cytometric analysis. The concentrate should be stored in a foil-wrapped, capped tube. If the sample is not analyzed immediately, store in the dark at 4°C.

Collimated Beam UV Dose Calculation

The UV dose delivered to a sample under a collimated beam is calculated as:

\[
D_{CB} = E_s P_f (1 - R) \frac{L}{(d + L)} \left(1 - 10^{-A_{254}d} \right)t
\]

\[
\text{Equation 4-1}
\]

where:

- \(D_{CB}\) = UV dose delivered to sample by collimated beam (mJ/cm²)
- \(E_s\) = Average incident UV intensity (before and after irradiation) (mW/cm²)
- \(P_f\) = Petri Factor (unitless)
- \(R\) = Reflectance at the air-water interface at 254 nm (unitless)
- \(L\) = Distance from lamp centerline to suspension surface (cm)
- \(d\) = Depth of the suspension (cm)
- \(A_{254}\) = UV absorbance at 254 nm (unitless)
- \(t\) = Exposure time (s)

The required exposure time for a certain delivered dose may be calculated by rearranging Equation 4-1. The term \(L/(d + L)\) accounts for the divergence of the UV radiation from the collimated beam as it passes through the suspension. The reflectance at the air-water interface \(R\) can be estimated using Fresnel’s Law. At \(\lambda = 254\) nm, the refractive indices for air and water are 1.000 and 1.372, respectively. Based on these values, an \(R\) equal to 0.025 is calculated for the air:water interface for UV radiation at \(\lambda = 254\) nm that is imposed normal to the air:water interface.

ANALYSIS OF DYED MICROSPHERES SAMPLES BY FLOW CYTOMETRY

The samples generated from the field and collimated beam tests are analyzed by flow cytometry. According to the peak excitation and emission wavelengths of cytidine, analysis of the cytidine-based DMS requires an excitation UV laser source at 330 nm and an emission filter centered near 385 nm. However, such laser sources and emission filters are unusual among regular flow cytometers. Experience has shown that flow cytometers with UV laser sources that emit near 350 nm and an emission filter centered near 405 nm can meet analytical needs as well. There are many laboratories in the US that are equipped with flow cytometers that satisfy these characteristics, and are capable of conducting these DMS-specific analyses. Currently, successful sample analyses have been conducted at the Cytometry Labs at Purdue University and the State University of New York at Albany. For available cytometry labs in the United States, a listing of known laboratory websites can be found at following web address:
EPA cytometry labs are also potential sources for analysis services, and information about commercial labs can be obtained by contacting the primary cytometer manufacturers, such as Beckman Coulter, Inc. and BD Biosciences.

**Principles of Flow Cytometry**

Flow cytometry is a technology that is used for counting, examining, and sorting microscopic particles. Simultaneous analysis of multiple optical characteristics of individual particles can be conducted as particles flow through a detection flow cell. This technology has applications in many fields, including molecular biology, pathology, immunology, plant biology and marine biology. Along with other sources, the following webpage sponsored by the Cytometry Lab at Purdue University provides links for introductory information about flow cytometry:

http://www.cyto.purdue.edu/flowcyt/educate/pptslide.htm

**Flow Cytometer Specifications and Settings**

As a general rule, the settings for a flow cytometer should be maintained as stable as possible during sample analysis. The performance of the flow cytometer (e.g., strength of the excitation laser) may vary with time; minor adjustments can be made to set and calibrate the instrument to perform consistently. It is not possible to define a uniform protocol for cytometer settings, since these settings are strongly dependent on the status of the cytometer and the local lab environment. The cytometer laser strength, local power supply, laboratory temperature, as well as the optical filters used for excitation and emission are factors that will influence the selection of cytometer settings.

The operation of a flow cytometer, especially for the analysis of DMS, requires cytometric expertise, experience and training. The analysis of DMS samples by flow cytometry also demands knowledge of physical and chemical properties of DMS, the purpose of using DMS, and typical behavior of DMS in samples collected from UV validations. Generally speaking, flow cytometer settings shall be selected within the following constraints:

- The flow cytometer shall be periodically calibrated using standard cytometry microspheres so that the size and granularity of a pure DMS group can be detected with minimal variation;
- The fluorescence intensity (FI) of DMS should be clearly higher than the primary group of ambient particles. The FI of the DMS in control samples (after pre-irradiation but without further exposure to UV reactor) should be high enough to be differentiated from ambient particles;
- The FI of the DMS samples that are subjected to the highest UV doses (e.g., dose of 600 mJ/cm² from the collimated beam tests) should be within the cytometer’s linear range;
- The sample introduction rate through the detection flow-cell is adjustable for many flow cytometers. The selection of sample flow rate should be at a level that ensures analysis of
a DMS sample within a reasonable time frame. However, it has been observed in previous studies that the sample introduction rate does not have a detectable impact on the FI signal. It is recommended that a constant sample introduction rate be selected and maintained for all DMS samples collected from the same testing day, including corresponding DR samples;

- It is recommended that all DMS samples collected from the same testing day be analyzed within the same flow cytometry day while the instrument settings are kept stable. This can help to maintain the FI measurement on the same relative scale so that the FI measured among the samples can be compared. Otherwise, internal standards can be used to calibrate the instrument on different days to the same relative FI sensitivity;

- Variations of flow cytometer behavior are occasionally observed when the instrument has been used over a relatively long period. When the variation is due to accumulation of ambient particles inside the flow cell, it is necessary to use DI water or an appropriate cytometer solution to rinse the entire sample introduction line. If the problem is due to the variation in laser strength or the pressure of the sample delivery medium (e.g., sheath fluid), minor adjustment can be made on the instrument settings by using an internal standard.

Flow Cytometry Data Analysis

**Gating the Dyed Microspheres**

Flow cytometry can provide information on size, granularity, and fluorescence intensity (FI) of particles in an aqueous suspension, such as DMS. The information collected from cytometric analysis of the DMS samples then needs to be transformed to histogram data files which are presented as DMS abundance for each FI channel. The resulting histograms are used for further computation to estimate the dose distributions. However, the samples usually contain impurities from DMS stock and ambient particles presented in the water source used for validation. These particles usually present significant interference to the target DMS population; therefore, the information about these background particles needs to be eliminated before the histogram files are generated. Gating, which is commonly used in flow cytometry data analysis, can be applied to address this issue.

Gating, by definition, is a post-processing method to differentiate a population space based on optical characteristics of target particles. When gating is applied, only particles inside or outside such gates are of interest and the complement is eliminated from the final data exportation. Gates can be defined based on different characteristics, and particles meeting the criteria of multiple gates can be distinguished by the intersection of these gates. DMS, which possess a well-defined combination of size, granularity, and FI, can therefore be differentiated against background particles through a multiple-gating strategy.

**DMS Size and Granularity**

Although DMS specifications may state a nominal size (e.g., 6.1 μm in diameter), the microspheres provided by the manufacturer usually have a distribution in size and in granularity. Conjugation of the biotinylated dye will not significantly affect microsphere size or granularity. When analyzed by flow cytometry, the size and the granularity characteristics of DMS are defined
by the forward scatter and side (90°) scatter signals, respectively. Both signals can be collected in linear or log scale, but linear scale is usually preferred. An example of a cytometry data file is shown in Figure 4-3 as a cytogram in the form of side scatter linear (vertical axis, symbol SSC-A) versus forward scatter linear (horizontal axis, FSC-A). Note that each “dot” represents a number of particles, varying according to the color scale. Thus, each dot has an associated population density. This example is based on a DMS sample from a dose-response test, where impurities were present mostly in the form of debris and multiplets from the DMS stock; other sources of ambient particles were very limited in this sample. From the cytogram, the primary population of DMS can be easily identified along with some debris and duplets. Different flow cytometer software may display cytograms in slightly different ways, and the notation for forward scatter and side scatter can be different as well.
Figure 4-3. Forward scatter and side scatter signals of a DMS sample detected by flow cytometry (The cytogram was generated using the freeware, WinMDI provided by the Cytometry Lab at Purdue University, Indiana)

Fluorescence Intensity (FI)

Fluorescence intensity of DMS is measured as a relative unit. As such, FI is strongly dependent on the cytometer instrument settings, such as the laser strength, the optical filters used, and the voltage settings. Both linear and log scale can be used for this signal, but linear scale is usually preferred for subsequent data analysis. Figure 4-4 shows an example of FI signal (vertical axis, denoted as Beads-HQ-A) versus the forward scatter signal (horizontal axis, FSC-A). This data set was generated by analysis of the same DMS sample as shown in Figure 4-3, where impurities were present mostly in the forms of debris and multiplets from the DMS stock.
Flow Cytometer Data and Data Reduction (Applying Gating)

As discussed earlier, the objective of gating is to differentiate DMS from ambient particles in the sample data generated by the flow cytometer. Removing these interfering particles from the data set will strengthen the subsequent deconvolution analysis and mitigate uncertainties associated with the results. For LA applications, gating is usually based on the size and granularity of DMS, which can be pre-defined by analyzing a pure DMS stock suspension, and the fact that DMS have much stronger initial fluorescence intensities than the ambient particles in the sample. Special flow cytometry software is required to conduct DMS data reduction, usually either provided with the purchase of a flow cytometer, or ordered from the major brand cytometer manufacturers. A freeware, called WinMDI 2.8, provides sufficient features and has been used for LA applications. This freeware and its tutorial can be downloaded from the following link sponsored by Purdue University Cytometry Lab:

http://www.cyto.purdue.edu/flowcyt/software.htm
The following section provides guidance on the application of gating for DMS data reduction based on WinMDI 2.8. Even though this discussion is based on WinMDI, the concepts are the same when using other cytometry software.

1. **Apply threshold.** The threshold is an electronic feature provided by cytometry software. Applying a threshold does not affect the data file itself, but filters low (population) density dots on the screen, thereby allowing clearer definition of the boundaries of the primary DMS group. It has been demonstrated in previous studies that using a threshold of 10 is reliable and efficient (in this case the threshold level of 10 means retention of those “dots” that represent to 10 or more particles per dot). Figure 4-5 illustrates the effect of applying a threshold of 10 to the cytogram shown in Figure 4-3; the result of this process is that the DMS group is clearly displayed for subsequent gating procedures. All DMS cytometric results are subjected to threshold screening before gating;

![Figure 4-5. Application of threshold of 10 to the cytometric data set shown in Figure 4-3](image)

2. **Define the gate based on the size and granularity of DMS.** When background particles in a DMS sample represent a small fraction of the total particle population, as shown in Figures 4-3 to 4-5, it is relatively easy to identify the DMS group of interest. However, for DMS samples collected from UV reactor validation tests, ambient particles in the background test water can overwhelm some DMS optical signals, thereby making it difficult to identify the DMS group based on these signals. Under these circumstances, advantage is taken of the fact that the size and granularity signals of DMS remain constant (for a fixed set of cytometer settings); therefore, it is possible to gate DMS on the basis of size and granularity for purposes of differentiation of DMS against background particles.
Figure 4-6 illustrates the definition of a gate R1, which is along the edge of the DMS group, and the application of R1 is shown in Figure 4-7. This R1 gate is, effectively, a standard used to isolate the experimental DMS present in a field sample.

Figure 4-6. Definition of a gate based on the size and granularity of DMS using a sample with limited background particles. This sample is usually prepared from the direct dilution of a DMS stock, which is the same DMS source for the reactor validation test.
Figure 4-7. Application of the gate R1, defined in Figure 4-6, to a DMS sample collected from a UV validation challenge test. Left upper panel is before applying gate R1, which shows no clear separation between DMS and background particles. The right upper panel overlays R1 on the raw data set. Then with the application of R1 (eliminating particles outside of gate), the lower panel illustrates a gated particle population with the same size and granularity as DMS.

(3) Define the gate based on the high FI of DMS. After the application of the gate R1, a particle population is defined, as shown in the lower panel of Figure 4-7. This population has the same size and granularity as those of DMS, but it may be contaminated by non-DMS particles. When converting Figure 4-7 to the format of FI versus forward scatter (FSC), as shown in Figure 4-8, a small population of low fluorescence is identifiable as being distinct from the DMS group, which is known with
higher fluorescence. Based on fluorescence characteristics of DMS, another gate can be defined around the edge of the DMS group, as indicated by R2 in Figure 4-8.

Figure 4-8. Definition of gate R2 based on the fact that the DMS possess higher fluorescence than background particles with the similar size and granularity

(4) Intersect the two gates R1 and R2. With the definition of gates R1 and R2, the intersection of the two gates distinguishes a DMS population from the total population shown in the upper left panel of Figure 4-7, resulting in the final gated population illustrated in Figure 4-9, which is assumed to be comprised of DMS only. This gated cytogram can be translated to a histogram, as shown in Figure 4-9(b) as DMS abundance at each FI channel. This histogram can be saved as tab delimited text file and eventually be used for deconvolution computation to estimate a dose distribution. This FI distribution is the final data product from the field tests and subsequent flow cytometry analyses.
Figure 4-9. Upper panel shows a DMS group defined by the intersection of the two gates R1 and R2, and the lower panel is a histogram representing the DMS group’s particle abundance at different FI levels.
CHAPTER 5
VALIDATION: DATA REDUCTION AND ANALYSIS

OVERVIEW

The data sets that result from a reactor validation by LA will include flow cytometry data for samples from UV dose-response experiments, samples collected during reactor testing, and corresponding blanks and controls. The data sets will also include measurements of relevant operational parameters, including flow rate, transmittance, lamp power, etc. The data from the dyed microspheres analyses, which take the form of cytograms for each sample, require subsequent data reduction and deconvolution to yield measurements of the UV dose distribution delivered by the reactor for each operating condition included in the test matrix. Prior to the application of these data reduction methods and statistical analyses, the cytometric data will have been passed through a threshold limit numerical filter, and subjected to gating (Section 4.4). The resulting FI distributions represent the product of the flow cytometry analyses of samples generated from the lab and field experiments.

The FI data are normalized and processed by numerical analyses to yield the dose-distribution observed at each test condition. In turn, these dose distributions are combined via a segregated-flow model, with measured UV dose-response data for one or more target microorganisms to yield estimates of target organism inactivation behavior for the reactor over the range of operating conditions defined by the test matrix. The log-inactivation results can then be analyzed to develop LI-prediction algorithms, similar to the RED equations discussed in the UVDGM. These predictions of reactor behavior are accompanied by estimates of corresponding error, based on the application of appropriate statistical methods, hereto in a manner similar to that of the UVGDM.

This section presents the relevant data reduction and statistical methods in the form of an example. Before the example is presented, however, it is necessary to review the mathematical basis of the deconvolution algorithm, as well as the basic concepts associated with the segregated-flow model. The information presented relative to deconvolution is intended to illustrate the logic behind the method, as well as the nature of the data sets involved in this operation. Basic knowledge of the logic of the segregated-flow model is necessary to understand its application for prediction of microbial inactivation that will be assigned to a reactor for a given operating condition, as well as the statistical methods that will be used to define the validation factor associated with these predictions and the assignment of disinfection credit.

DECONVOLUTION AND MICROBIAL INACTIVATION PREDICTIONS: BASIC PRINCIPLES

The overall goal of the LA validation protocol is to measure the UV dose distribution delivered by a reactor, so that accurate predictions of microbial inactivation may be developed. This process involves the following sequence of numerical operations:

- Numerical interpolation of UV dose-response data
- Application of the deconvolution algorithm
Numerical integration of dose distribution and dose-response data through the segregated-flow model to yield a prediction of microbial inactivation.

The following text provides summaries of these steps and the fundamental scientific and/or mathematical principles on which they are based.

**Numerical Interpolation of UV Dose-Response Data**

UV dose-response experiments are performed by subjecting aqueous suspensions of DMS to well-defined doses of UV radiation in a shallow, well-mixed, batch reactor under a collimated beam. This method of exposure ensures delivery of a uniform (single-valued) UV dose to all DMS in a sample. By repeating this method of exposure over a range of UV doses, it is possible to define the changes in FI distribution that will result from a given UV dose.

For practical reasons, it is not possible to perform UV exposures under a collimated beam for all possible UV doses that could be delivered by a full-scale reactor (i.e., all doses that could comprise a UV dose distribution for a reactor under a given operating condition). Therefore, it is necessary to use an interpolation scheme for the DMS FI distribution as a function of UV dose.

Empirical observations have indicated that the FI distributions for DMS that are based on \( S \rightarrow P \) phototransformation change in a regular, predictable manner. Moreover, it has been observed that the FI distributions from UV dose-response experiments can be fit well by a four-parameter Weibull function, and the parameters of this distribution have been observed to vary in a regular, predictable manner as functions of UV dose. However, there is no (known) theoretical basis to support the use of the four-parameter Weibull distribution for this curve-fitting exercise. As such, it is possible that other functions could also be used for this fitting process, provided that the model parameters vary in a regular, predictable manner, so that interpolation does not suffer because of the fitting function used.

The interpolation function (e.g., four-parameter Weibull) is fit to the FI distributions from the UV dose-response experiment by non-linear regression. The parameters of the interpolation function are determined as a function of applied UV dose. In turn, the trends in these parameters (as a function of UV dose) are fit to appropriate functions so that values of interpolation function parameters may be predicted for any UV dose within the range of doses from the collimated-beam experiment. It is then possible to predict the FI distribution for the DMS at any dose across this same dose range.

To simplify subsequent analyses, UV dose-response behavior of the microspheres is represented only at integer values of UV dose for this dose range. At each (integer-valued) dose within this range, the response of the DMS is described by a normalized FI histogram (i.e., fraction of DMS that yield each integer-valued FI response across the range of FI channels that are available from the cytometer, typically 1024 channels). For example, if the UV dose-response behavior of the DMS is to be represented for doses ranging from 0-200 mJ/cm², then at each integer value of UV dose across this range, the relative abundance of DMS will need to be defined for each of the FI channels of the cytometer. If the cytometer is capable of measuring FI at 1024 channels, this
implies that the UV dose-response behavior of the DMS will be represented by a 1024 x 201 matrix. The elements of the matrix will be generated by the interpolation function described above.

**Deconvolution Algorithm**

It is assumed that \( n \) dose-response distribution curves are predicted by application of non-linear regression to the FI distributions at each dose from the collimated-beam tests (see discussion above). For each dose-response distribution curve, \( m \) different fluorescence intensities are analyzed. For a typical flow cytometer, \( m \) is usually equal to 1024.

Define: \( i = 1, 2, \ldots, m \) = index for counting fluorescence intensity increments; \( j = 1, 2, \ldots, n \) = index for counting dose increments.

For any dose-response distribution curve with dose \( D_j \), the total number of microspheres counted for analysis is defined as:

\[
Sum_j = \sum_{i=1}^{m} c_{i,j}
\]

Equation 5-1

where: \( c_{i,j} \) = number of microspheres at dose \( D_i \) that release fluorescence intensity \( F_i \).

Therefore, fraction of particles receiving dose \( D_j \) that emit fluorescence intensity \( F_i \), \( \gamma_{i,j} \) can be defined as follows:

\[
\gamma_{i,j} = \frac{c_{i,j}}{Sum_j}
\]

Equation 5-2

The matrix \([\gamma]\) is the normalized representation of DMS UV dose-response behavior and is subject to the following constraints:

\[
\sum_{i=1}^{m} \gamma_{i,j} = 1
\]

Equation 5-3

\[
\sum_{i,j} \gamma_{i,j} = n
\]

Equation 5-4

The first constraint implies that the sum of all values of \( \gamma_{i,j} \) must be one at any dose value, \( D_j \). Because there are \( n \) dose increments included in \([\gamma]\), this requires that the sum of all elements in \([\gamma]\) must equal \( n \), as defined by the second constraint. Alternatively, the first constraint implies that all DMS that are subjected to a given dose (\( D_j \)) are accounted for, and more generally, all DMS that are included in the global analysis are accounted for in these calculations.

When a flow-through experiment is performed, a DMS abundance–fluorescence curve is also obtained (see example in Figure 4-9). If the total number of DMS from a flow-through
experiment analyzed by flow cytometer is defined as A, and the number of particles that emit 
fluorescence intensity $F_{I_i}$ is defined as $B_i$, $\beta_i$ can be defined as below:

$$\beta_i = \frac{B_i}{A} = \text{fraction of particles collected from flow-through experiments}$$

that are detected with fluorescence intensity $F_{I_i}$. 

The vector $[\beta]$ (i.e., m by 1 matrix) is subject to the following constraint:

$$\sum_{i=1}^{m} \beta_i \equiv 1$$

This constraint implies that the 100% of particles measured in a sample are accounted for 
in the numerical analysis.

The purpose of flow-through experiments is to measure the UV dose distribution delivered 
by a reactor for a given operating condition, based on changes in DMS FI distributions. If $A_j$ is 
defined as the number of particles that receive dose $D_j$, $\alpha_j$ can be defined as below:

$$\alpha_j = \frac{A_j}{A} = \text{fraction of particles that receive dose } D_i$$

The vector $[\alpha]$ (i.e., n by 1 matrix) represents the dose distribution and is subject to the 
following constraint:

$$\sum_{j=1}^{n} \alpha_j \equiv 1$$

Therefore, $A_j$ or $\alpha_j$ is the value that needs to be quantified to complete the dose distribution 
profile.

As discussed in the dose-response section, microspheres will release a broad range of FI 
values, even when subjected to a uniform UV dose. In flow-through experiments, $B_i$ represents 
the number of particles that emit fluorescence intensity $F_{I_i}$. Therefore, the population of particles 
that contribute to $B_i$ will include particles that emit fluorescence intensity $F_{I_i}$; however, this 
population will be comprised of particles that have received many different UV doses when they 
passed through the UV reactor. By definition, $B_i$ has following relation with $+\alpha_j$ and $\gamma_{i,j}$:

$$B_i \equiv \alpha_i A \gamma_{i,1} + \cdots + \alpha_n A \gamma_{i,n} = \sum_{j=1}^{n} [\alpha_j A \gamma_{i,j}]$$

Equation 5-9
When divided by \( A \) on both sides, \( \beta_i \) can be normalized to:

\[
\beta_i = \sum_{j=1}^{n} [\alpha_j \gamma_{i,j}]
\]

Equation 5-10

In Equation 5-10, \( \beta_i \) and \( \gamma_{i,j} \) are known, and the values of \( \alpha_j \) are unknown. Multiple linear regression is applied to the data to determine the \( \alpha_j \) values. Equation 5-10 can also be expressed in matrix notation as:

\[
[\beta] = [\gamma] \times [\alpha]
\]

Equation 5-11

where,

\[
[\alpha] = \begin{bmatrix} \alpha_1 \\ \alpha_2 \\ \vdots \\ \alpha_n \end{bmatrix} = \text{dose distribution (n depends on dose range)}
\]

Equation 5-12

\[
[\beta] = \begin{bmatrix} \beta_1 \\ \beta_2 \\ \vdots \\ \beta_{1024} \end{bmatrix} = \text{effluent sample FI distribution, as a 1024\times1 matrix}
\]

Equation 5-13

\[
[\gamma] = \begin{bmatrix} \gamma_{1,1} & \gamma_{1,2} & \cdots & \gamma_{1,n} \\ \gamma_{2,1} & \gamma_{2,2} & \cdots & \gamma_{2,n} \\ \vdots & \vdots & \cdots & \vdots \\ \gamma_{1024,0} & \gamma_{1024,1} & \cdots & \gamma_{1024,n} \end{bmatrix} = \text{dose response matrix, 1024\times n}
\]

Equation 5-14

Deconvolution Program

Based on the matrix relationships described in the previous section, MATLAB was used to design a program for DMS data analysis, especially the DR response Weibull function fitting and the deconvolution. This program allows users to interactively conduct the entire DMS data analysis from the point of obtaining FI distributions after cytomtery gating. The program works on principles developed early in the research phase of LA. The deconvolution program uses the dose response matrix \([\gamma]\) to find the best linear combination of dose response FI distributions that will describe the vector \([\beta]\). As a result, Matrix \([\alpha]\) is deconvolved by the program, with the constraints discussed previously, as well as the constraints described below. This deconvolution algorithm searches for the UV dose distribution that best fits the observed FI distributions from
the UV dose-response and flow-through experiments. The fitting process requires definition of an initial “guess” of the UV dose distribution to seed the process. This program was developed based on MATLAB 7.6. An electronic copy this program can be provided upon request.

**Constraints on the deconvolution algorithm**

The solution of the deconvolution algorithm is subjected to two constraints:

\[ 0 \leq [\alpha] \leq 1 \]
\[ \sum_{i=1}^{n} \alpha_i = 1 \]  \hspace{1cm} \text{Equation 5-15}

These two constraints ensure that the solution displays the characteristics of a probability density function (pdf). Values in a pdf can only have values ranging from zero to one, and the sum of the probabilities for an entire distribution (i.e., “area under the curve”) must always equal one. In terms of dyed-microsphere populations, the dose distribution vector, \([\alpha]\), describes the fraction of DMS within a population that have received each discrete dose within the dose range defined for a distribution. The fractions must be between zero and one, and the total sum of these fractions must equal identically one, indicating that 100% of the population is subjected to a UV dose that lies between zero and the upper limit being considered for the dose distribution.

**Numerical starting seed for the deconvolution algorithm**

A starting seed vector is used to initiate the deconvolution algorithm. The algorithm searches for a solution globally, without assuming a pre-defined distribution shape or type (i.e., assuming that the dose distribution is Gaussian or log-normal). The ideal seed vector will satisfy the constraints of the general solution, facilitate rapid convergence on the final answer, and minimize the chance that the algorithm will terminate at a local minimum (i.e., incorrect distribution).

A seed vector that has been observed to satisfy these constraints is a vector containing uniform random numbers ranging from zero to one that have been normalized against the total sum. This seed is coded in MATLAB using a built-in function to generate n uniform random numbers.

\[ x_0 = \text{rand}(n,1); \]
\[ x_0 = \frac{x_0}{\sum_{i=1}^{n} x_0} \]  \hspace{1cm} \text{Equation 5-16}

where,

\[ x_0 \] = elements of the initial seed vector

\[ \text{rand}(n,1) \] = creates an n-by-1 matrix of random numbers
\[ n = \text{number of elements in vector } \alpha \]
\[ i = \text{index for elements in vector } \alpha. \]

This starting solution is generated automatically when the MATLAB program for the dyed-microsphere data analysis is executed from the MATLAB command window.

As described above, random ("seed") values are assigned to \([\alpha]\) to start the deconvolution, and the final result is the vector \([\alpha]\) that minimizes the sum of the squared distances between the predicted \([\beta]\) and experimental \([\beta]\) vectors.

**Dose-Response Matrix Optimization**

Achieving the optimum performance of the deconvolution algorithm is highly dependent on the spacing of dose values, \(D_j\), that are used to interpolate the columns in the matrix \([\gamma]\). If the dose increment used is relatively fine, the \([\gamma]\) matrix will have high multiple collinearity. In this case, there is a possibility that the solution for the dose distribution, \([\alpha]\) will lose uniqueness.

To remedy this issue, an algorithm was created that will allow for the optimum selection of the spacing of the dose values for the matrix \([\gamma]\). This algorithm is built-in to the MATLAB program and requires no manipulation by the user. The algorithm begins by choosing three log-spaced values for UV dose from the minimum dose value used in the dose-response experiment to the maximum dose value used in the dose-response experiment. Once the three dose values are generated the deconvolution is solved for by generating all of the elements in \([\gamma]\); this is done by evaluating the functions for each of the four Weibull distribution parameters as a function of UV dose. In turn, the system of equations represented by equation 5-11 is solved for \([\alpha]\), the dose distribution.

This algorithm successively adds one additional dose value to the set of UV dose values that are generated for the purpose of constructing the dose-response matrix \([\gamma]\). At each iteration, the sum-of-squares of the residuals is calculated. This process will terminate when the minimum of the sum-of-squares residuals is achieved, and the resulting vector \([\alpha]\) will be reported as the deconvolved dose distribution, an example of this minimization of the sum-of-squares of the residual at each addition of a FI distribution to the \([\gamma]\) matrix is shown in Figure 5-1.
Figure 5-1. Relative sum-of-squares residuals as function of the number of columns in the dose response matrix \([γ]\). A demonstration of the effect of the optimizing algorithm for the dose response matrix \([γ]\).

Estimation of Microbial (Log) Inactivation

Once a dose distribution has been determined for a reactor (corresponding to a given set of operating conditions), it is possible to use this information to predict reactor performance, as defined by the ability to inactivate one or more target microorganisms. This calculation is conducted using a mathematical analog of the segregated-flow model, which assumes that a reactor is operating under steady-state conditions, and reactive entities within the reactor do not exchange material during their time in the reactor. For a UV disinfection system, the reactive entities are the individual microorganisms; therefore, the assumption of zero exchange applies rigorously.

The governing equation for the segregated-flow model is:

$$\left( \frac{N}{N_0} \right)_{\text{reactor}} = \int_{0}^{\infty} \left( \frac{N}{N_0} \right)_{\text{batch}} E(D) \cdot dD$$  \hspace{1cm} \text{Equation 5-17}

Where the left-hand side of the equation represents the fraction of target organisms that retain viability after traversing the reactor. The first term on the right-hand side of the equation represents the UV dose-response behavior of the target organism; the product \(E(D)\cdot dD\) represents the fraction of particles (microorganisms) that receive a dose between \(D\) and \(D+dD\). This equation is mathematically and theoretically analogous to the segregated-flow model, as applied for prediction of reactor performance in chemical reactor systems, based on (batch) reaction kinetics and a residence time distribution function.
The data needed for this approach is generally not available in continuous form. Therefore, an approximate solution can be developed by summation as follows:

\[
\left( \frac{N}{N_0} \right)_{\text{reactor}} \approx \sum_i \left( \frac{N}{N_0} \right)_{\text{batch}} E(D_i) \cdot \Delta D_i
\]

Equation 5-18

This summation is performed over the entire dose range delivered by the reactor.

As a simple illustration of the segregated-flow model, consider a reactor that delivers the UV dose distribution represented in Figure 5-2. For this reactor, 25% of the particles passing through the reactor receive a UV dose of 10 mJ/cm²; 50% of the particles receive a UV dose of 20 mJ/cm²; and the remaining 25% of the population receives a UV dose of 30 mJ/cm².

![Figure 5-2. Hypothetical UV dose distribution delivered by a reactor](image)

This contrived dose distribution represents the function \( E(D_i) \cdot \Delta D_i \) for the reactor. For simplicity, we will assume that the target microbe has UV dose-response behavior that is defined by simple first-order kinetics, with an inactivation rate constant of 0.1 cm²/mJ:

\[
\left( \frac{N}{N_0} \right)_{\text{batch}} = e^{-kD}
\]

Equation 5-19
where,

\[ \begin{align*}
N &= \text{concentration of target microbes that retain viability after receiving a given UV dose} \\
N_0 &= \text{concentration of target microbes that retain viability prior to UV irradiation} \\
k &= \text{inactivation rate constant (0.1 cm}^2/\text{mJ, in this case)} \\
D &= \text{UV dose (mJ/cm}^2) \\
\end{align*} \]

For this scenario, application of the segregated-flow model indicates that the reactor will yield the following inactivation response for the target microbe:

\[
\left( \frac{N}{N_0} \right)_{\text{reactor}} = 0.25 \cdot (0.1) + 0.5 \cdot (0.01) + 0.25 \cdot (0.001) = 0.303
\]

Equation 5-20

\[
\log_{10} \left( \frac{N}{N_0} \right) = -1.52
\]

Real reactors will have UV dose distributions that are more complex than the contrived distribution illustrated in Figure 5-2. Also, many microbes of interest will have UV dose-response behavior that is best described by mathematical functions that are more complex than the simple first-order model used in this example. However, the application of the segregated-flow model will follow the same logic as illustrated in this example.

Figure 5-3 is included as an illustration of the validity of the dose distribution estimates developed by this method. The data included in Figure 5-3 were collected from a validation matrix experiment conducted on an LPHO reactor system. The reactor was tested at 33 different operating conditions. For each operating condition, LA was applied. For most of the operating conditions, MS2 biodosimetry was performed; biodosimetry based on T1 as a challenge organism was applied for a smaller fraction of the operating conditions. For some operating conditions, MS2 and T1 biodosimetry were both performed, but in separate experiments.
Figure 5-3. Comparison of predicted challenge organism inactivation (using DMS actinometry, y axis) with measured inactivation responses for the same organism in validation experiments conducted on an LPHO reactor

For each operating condition, the UV dose distribution was estimated by LA. In turn, the dose distribution estimates for each operating condition were integrated with the measured UV dose-response behavior for the relevant challenge organism(s) to yield an estimate of inactivation for the challenge organism; these estimates were then log-transformed for presentation in the form of log inactivation (LI). Figure 5-3 presents a summary of the predictions of challenge organism inactivation as a function of measured challenge organism inactivation. The estimates of LI based on dose distribution estimates developed from LA were quantitatively consistent with the measured inactivation responses of the challenge organisms, and within the error that is inherent in the analysis of microbial responses to UV exposure. It is important to note also that this comparison is based on two challenge organisms (MS2 and T1) that display markedly different UV dose-response behavior. As such, this supports the validity of the dose distribution estimates that were developed by LA.

CASE STUDY FOR APPLICATION OF LA

As a means of illustrating the mechanics of deconvolution and application of the segregated-flow model, an example is presented below. It involves complete analysis of data selected from an actual reactor study, including predictions of target pathogen inactivation as a function of the unit’s operating conditions.
Case Study Background:

An LPHO UV reactor was tested at several sets of operating conditions, as defined by combinations of UVT, lamp power, and flow rate. Each operating condition was tested by biodosimetry (using MS2 coliphage as the challenge organism) and LA. Table 5-1 provides a summary of the operating conditions used in this example.

<table>
<thead>
<tr>
<th>UVT(_{254}) (%/cm)</th>
<th>Lamp Power (% of max)</th>
<th>Flow Rate (gpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>97</td>
<td>100</td>
<td>3960</td>
</tr>
<tr>
<td>97</td>
<td>100</td>
<td>3960</td>
</tr>
<tr>
<td>97</td>
<td>100</td>
<td>2200</td>
</tr>
<tr>
<td>97</td>
<td>100</td>
<td>1760</td>
</tr>
</tbody>
</table>

The data analysis methods required for numerical deconvolution involve manipulation of large matrices and arrays, based on the logic described above. The order of these calculations, the programs used to accomplish them, as well as the purpose of each step are described below:

- SigmaPlot – Transpose text files of cytometric data from row-oriented data to column-oriented data, since FI histograms typically have 1024 bins and MS excel only has 250 columns per worksheet, FI histogram data must be transposed to column oriented data for calculations.
- MS Excel – Perform normalization, which transforms FI histograms into FI density distributions. This step can help users to evaluate the repeatability among replicate samples from the same operating condition, and then the replicate sample FI histograms are combined to one histogram for such flow condition to reduce noise for better deconvolution results.
- MATLAB – The DMS dose-response data are fitted with Weibull function and a linear-spline function is used for the interpolation matrix, \([\gamma]\). Then, flow-through reactor (FTR) FI distributions, \([\beta]\), are imported into the MATLAB-based executable program and the deconvolution algorithm is executed. Multiple field test FI files from the same testing day can be carried out at the same time.
- MS Excel – The dose distribution vector, \([\alpha]\), from the deconvolution algorithm is copied from MATLAB and pasted to MS Excel for further analysis, such as the application of the segregated-flow model to predict (log) microbial inactivation for a challenge or target organism. However, this can be accomplished within the MATLAB-based executable program as well when coefficients of the corresponding DR equation from the microorganisms-of-interest are provided.

**Step 1. Normalization**

For each sample analyzed by flow cytometry, the resulting data sets are in the form of histograms. The histograms are text files that define the abundance of particles that have expressed FI at each of the 1024 FI channels of the cytometer. Figure 5-4 illustrates an FI histogram from a sample that was analyzed by a flow cytometer.
Figure 5-4. Example FI histogram from flow cytometry software. Notice the units of abundance on the y axis.

An FI histogram is converted into a FI (density) distribution by normalizing the FI histogram against the total sum of abundances (total sum of counts) for the sample. This step is necessary to ensure that the distribution of FI in a sample of microspheres is not dependent on the number of DMS collected in the distribution, and because the distribution of FI values for that sample of microspheres must be characteristic of a density function, meaning that area under the curve must equal 1 (i.e., total sum of probabilities is equal to one). The calculation for the relative abundances for each dose bin is straightforward (Equation 5-21); the normalized form of the data set from Figure 5-4 is illustrated in Figure 5-5 (notice the change in scale for the vertical axis).

\[
U_{i,j} = [u_{1,j} \ u_{2,j} \ ... \ u_{1024,j}]
\]

\[
\gamma_{i,j} = \frac{U_{i,j}}{\sum_{1}^{1024} U_{i,j}}
\]

Equation 5-21

where,

- \(U_{ij}\) = array defining absolute abundance values for FI i subjected to dose j
- \(\gamma_{i,j}\) = array containing relative abundance values for FI i subjected to dose j
Step 2: Fitting the Dose-Response FI Distributions

Interpolation of dose-response data is required to yield predictions of FI density functions at doses that are intermediate to those applied in the dose-response experiment. Experience has shown that a four-parameter Weibull distribution function fits the FI distributions from collimated-beam dose-response experiments well (see Figure 5-6). The equation that describes the four-parameter Weibull distribution is:

\[
y = a \left( \frac{c-1}{c} \right)^{\frac{x-x_0}{b} + \left( \frac{c-1}{c} \right)^{\frac{1}{c}}} \exp \left( - \left( \frac{x-x_0}{b} + \left( \frac{c-1}{c} \right)^{\frac{1}{c}} \right) \right)^c + \frac{c-1}{c}
\]

Equation 5-22

In this equation, the parameter \( x_0 \) may be viewed as a “location” parameter, roughly analogous to the mean of the distribution, whereas parameters \( a, b, \) and \( c \) define the shape of the distribution around \( x_0 \).

As indicated previously, there is no (known) theoretical link between the four-parameter Weibull distribution and the UV dose-response behavior of DMS. However, the four-parameter Weibull distribution is mathematically flexible enough to fit to the distribution shapes that are characteristic of DMS dose-response behavior, and the parameters of this function vary in a regular
manner across the range of UV doses that are used in validation experiments. Therefore, the four-parameter Weibull distribution works well for this interpolation process.

The algorithm for fitting the Weibull distribution to FI distributions involves the minimization of the sum-of-squares residuals between the observed FI distribution (FI distribution from dose response experiment) and the predicted FI distribution based on the Weibull distribution equation.

Figure 5-6. Example of four-parameter Weibull distribution fitted to a FI distribution. The black line represents the normalized FI distribution for a sample that was subjected to a UV dose of 60 mJ/cm² under a collimated beam, while the red line represents a non-linear fit of the four-parameter Weibull distribution to the data.

**Step 3: Fitting Distribution Parameters as a Function of UV Dose**

In generating the dose-response matrix, $[γ]$, it is necessary to describe the FI response of dyed microspheres that were subjected to UV doses not covered in the dose-response experiment. This requires interpolation of the distribution parameters as a function of UV dose. After all FI distributions are fitted with a distribution function, the parameters can be plotted as a function of UV dose, and in turn linear splines can be fitted to the parameters.

Figure 5-7 illustrates the fitting of piece-wise linear splines to the four-parameter Weibull distribution from an actual DMS UV dose-response experiment. The model parameters for the four-parameter Weibull distributions tend to follow regular patterns. As such, it is possible to fit spline interpolation functions to these trends so that representative values of distribution parameters may be estimated by interpolation. The piece-wise linear spline functions are of the form:
Given \( n \) dose values, construct \( n-1 \) linear functions \( f(x_i) \), where \( x_i \) is the interval between dose values \( i \) and \( i+1 \)

\[
s_i(x_i) = f(x_i) \quad i = 1, 2, ..., n - 1
\]

Equation 5-23

where,

\[
f(x) = v_0 + v_1 \cdot x \quad \text{linear spline}
\]

Equation 5-24

Although equations 5-23 and 5-24 approximate the observed values of the parameters from the Weibull distribution well, the selection of fitting equations for the distribution parameters is left to the judgment of the person performing the numerical analysis.

Figure 5-7. Four-parameter Weibull distribution parameters fitted with linear splines as a function of UV dose (see equations 5-23 and 5-24)

**Step 4: Generation of Dose-Response Matrix**

The generation of the dose-response matrix requires the use of two sets of equations. The first set of equations are the piece-wise linear spline functions fitted to the distribution function
(e.g., Weibull) parameters as a function of UV dose, and the second set are the equations used to
generate the elements in the dose-response matrix \([\gamma]\), namely the Weibull function is needed for
the generation of interpolated FI distributions (equation 5-22).

The first step in generating the dose-response matrix involves querying the equations for
the fitted curves for the distribution function parameters as a function of UV dose (see Tables 5-2). This procedure is outlined as follows:

- Generate an array of log-spaced UV dose values ranging from zero to the largest UV dose
  applied in the dose response experiment

\[
D = [d_1 \ d_2 \ ... \ d_n]
\]

\[d = \text{values of UV dose}\]

\[n = \text{total number of UV doses in array } D\]

- Evaluate the parameter equations for each dose bin, for example if \(x_0\) is to be predicted,
  perform this calculation on each value of UV dose in array \(D\) for all parameters.

\[
\begin{bmatrix}
  x_{0,1} \\
  x_{0,2} \\
  \vdots \\
  x_{0,n}
\end{bmatrix}
= 
\begin{bmatrix}
  s_1(x_i) \\
  s_2(x_i) \\
  \vdots \\
  s_n(x_i)
\end{bmatrix}
\]

\text{Equation 5-25}

Apply the parameters obtained from this step to the equation for the Weibull distributions
for each FI value from 1 to 1024.

A summary of the output of this operation is illustrated in Figure 5-8 for three sample UV
doses within a distribution. Note that the example shown in Figure 5-8 was calculated through an
Excel spreadsheet for the purpose of demonstration, but the actual dose-response matrix, not
shown here, is prepared by the MATLAB-based program. After the dose-response matrix is
developed, the deconvolution algorithm can be executed.
Figure 5-8. Example output of FI distribution predictions for doses of 0, 100, and 400 mJ/cm² from a DMS dose-response experiment

**Step 5: Preliminary Analysis and Quality Control on Flow-through Test Sample Results**

Three replicate effluent samples are usually collected from each operating condition tested with DMS. Each of the replicate generates a FI distribution histogram, and these data are normalized against the total DMS counts within the same sample. The relative DMS counts, or DMS fraction, from these three replicates are plotted against the fluorescence intensity, as shown on the left in Figure 5-9 to evaluate their similarities, such that no significant bias resides among the replicates. The histogram data (before normalization) from the three replicates at each FI value are then mathematically summed and assigned as the new value for that FI. This combination process is done to reduce the noise of cytometric measurements. Figure 5-9 shows that noise around the histogram is significantly reduced (see the plot on the right in Figure 5-9). This has been demonstrated in previous studies to be beneficial to the deconvolution process. The
combined histogram for such operating condition is then converted back to a new text file, which will be loaded by the MATLAB-based program for deconvolution. Note that this text file needs to be tabbed and row oriented, which are the formats to be read by the deconvolution program.

![Graph 1](image1)

**Figure 5-9. Combination of FI Distributions from Flow-through Replicate Samples for Noise Reduction**

**Step 6: Run Deconvolution Algorithm**

Computing the dose-distribution of a reactor using LA is performed by determining the “best fit” linear combination of each FI distribution for doses in the dose-response matrix subject to the constraints defined in Section 5.2.3.1. This process can be illustrated by creating a mixed sample of dyed microspheres containing known dose values. This example is an illustration of a laboratory procedure known as a convolution experiment (Blatchley et al., 2006).

When performing a collimated-beam dose-response experiment, a population of dyed microspheres is subjected to known, predetermined dose values. Consider an example in which sub-populations of dyed microspheres are exposed to 8 distinct UV doses: 0, 10, 20, 30, 60, 100, 150, and 300 mJ/cm². At the end of each dose exposure, equal volumes of the DMS suspension are placed in a container, a mixed sample. This process is illustrated schematically in Figure 5-10.
The dose distribution of this mixed sample can be directly determined without deconvolution because by design, equal volumes of each dose exposure were used to develop the mixed sample. The dose distribution of this sample would simply be a uniform distribution with probabilities of 1/(number of doses), in this case 1/8 (where 8 is the number of doses measured in the convolution experiment) (see Figure 5-11).

Because the dose distribution is known, and the FI distribution of each sample (corresponding to a fixed UV dose) is also known, it is possible to predict the FI distribution of the mixed sample as the weighted average of the FI distributions of the individual samples that were used to create the convolution sample. An example of the agreement between the measured FI distribution of a mixed sample and the estimate of this distribution based on a weighted average (convolution) is provided in Figure 5-12.
Figure 5-11. Dose distribution of mixed sample in convolution experiment


Figure 5-12. Measurements of FI from convolution experiment. FI distributions based on measurements of sample developed from equal-volume mixture of subsamples that had been exposed to known doses under collimated beam (dose mixture) were compared with calculated FI distribution based on convolution of data from individual doses (convolution)
Now, consider the case of an effluent sample of dyed microspheres collected from a UV reactor. This effluent sample is similar to the mixed sample constructed in the convolution experiment, in that it contains a mixture of UV doses delivered by the UV reactor to the dyed microspheres; however, in this case, the dose distribution of the sample is unknown. The goal of the deconvolution algorithm is to determine the distribution fraction of these unknown doses from information that is known. However, included in the information that is known about this sample is the UV dose-response behavior of the same microspheres that comprise the sample. Figure 5-13 provides a summary of the measured behavior of the microspheres from a single LA experiment.

![Figure 5-13. Known FI distribution information for an effluent sample from LPHO UV reactor (left: dose response matrix, right: FI distribution for effluent DMS sample from a continuous-flow reactor)](image)

As described previously, the dose-response data (Figure 5-13, left panel) will be used to define a dose-response matrix by interpolation. With this information and the FI distribution for an effluent sample, the deconvolution algorithm is applied to find the “best” fit combination of doses from the FI distributions from the dose-response interpolation matrix to describe the FI distribution displayed in the right panel of Figure 5-13.

The deconvolution algorithm is executed using a function in MATLAB. This algorithm determines the minimum of a function with two constraints on the solution vector (see previous discussion for constraints on the solution). The algorithm is an iterative scheme where an initial numerical seed is required to start the iterations. The initial numerical seed is required to be a vector comprised of normalized uniform random numbers from values 0 to 1 (see Figure 5-14) and normalized against its total sum of all elements in the seed vector. The number of elements in this vector is equal to the number of dose bins. This vector is generated within MATLAB code that was previously described.
Figure 5-14. Example of initial numerical seed to start deconvolution algorithm

The algorithm is initiated with the seed vector, and iteratively converges on a solution, subject to the criterion of minimization of the sum of residual squared errors between the calculated FI distribution and the measured FI distribution for the sample:

$$\min_{\alpha} \| \gamma \alpha - \beta \|_2^2$$  \hspace{1cm} \text{Equation 5-26}

where,

$$\| \cdot \|_2^2 = \text{square of the Euclidian length of a vector (i.e. the l}_2\text{ vector norm)}$$

$$= \left[ \sum_{i=1}^{n}(\gamma \alpha - \beta_i)^2 \right]^{1/2}$$

$$\gamma = \text{dose response matrix}$$

$$\alpha = \text{vector containing distribution fractions at each dose}$$

$$\beta = \text{vector for effluent sample FI distribution relative abundance}$$

$$\ i = \text{the ith element of the vectors}$$

$$\ n = \text{total number of bins in } \beta = 1024.$$

The algorithm continues to iterate until the value of Equation 5-24 is minimized. At the solution, the algorithm stores the values for the dose-distribution vector, $\alpha$, into its corresponding location within the data structure and can be exported for further analysis as needed. An example of a solution for the deconvolution algorithm is illustrated in Figure 5-15; this solution is an actual dose distribution measured using LA for an LPHO reactor. The solution is presented (top panel in
Figure 5-15) in the form of a probability density function (or PDF) and a cumulative distribution function (or CDF).

Recall that this algorithm finds the “best” combination of FI distributions from the dose response matrix, $\gamma$, to describe the FI distribution for the effluent UV reactor sample, $\beta$. This fact can be verified by convolving the solution from the algorithm with the dose response matrix. An illustration of the result of this calculation is presented in (Figure 5-15 bottom panel).

![Image of Figure 5-15](image)

**Figure 5-15. Solution for deconvolution algorithm, an estimated dose distribution for an LPHO reactor (top panel: deconvolved dose distribution (PDF and CDF); bottom panel: FI distribution from LPHO UV reactor (dotted blue) fitted with the convolution (solid red) of the dose response matrix $[\gamma]$ with the deconvolved dose distribution vector $[\alpha]$).**

**Step 7: Prediction of Microbial Inactivation**

If the UV dose distribution delivered by a reactor is known, it is possible to predict process performance of the reactor, as defined by microbial inactivation, for any organism for which UV dose-response behavior is known. Mathematically, this prediction is accomplished by application of the segregated-flow model; an illustrative example of the application of the segregated-flow model was presented at the beginning of this chapter. In the following section, an example of the application of this model is presented for a real UV dose distribution, and an actual microbial dose-response relationship.

Challenge organism inactivation prediction can be accomplished after microbial dose-response data have been fitted with an appropriate UV dose-response equation. Many microbial
dose-response relationships have been presented in the literature. The selection of an appropriate
dose-response equation is a matter of judgment.

For biodosimetric applications, microbial dose-response relationships are often presented
in a form that facilitates RED calculation, wherein “Dose” is calculated as a function of log\(_10\) inactivation (log I). As an example, a common form of dose-response relationship for coliphage MS2 as a challenge organism is the following:

\[
Dose = A \cdot \log I^2 + B \cdot \log I
\]

Equation 5-27

where,

\[
Dose = \text{UV dose } [\text{ mJ/cm}^2]
\]

\[
A = \text{Regression parameter A}
\]

\[
B = \text{Regression parameter B}
\]

\[
\log I = \log \text{ inactivation, } \log_{10}(N_0/N) = -\log_{10}(N/N_0)
\]

For prediction of microbial inactivation by the segregated flow model, the inverse form of
this relationship is required. Specifically, it is necessary to have a function that describes log\(_{10}\) as
a function of UV dose. For Equation 5-27 above, this form of the dose-response relationship can
be found by application of the quadratic equation:

\[
I = 10 \frac{B - \sqrt{B^2 + 4A \cdot \text{Dose}}}{2A}
\]

Equation 5-28

where,

\[
I = \text{inactivation, } (N/N_0)_{\text{batch}}
\]

\[
A = \text{Regression parameter A from Equation 5-27}
\]

\[
B = \text{Regression parameter B from Equation 5-27}
\]

\[
\text{Dose} = \text{dose bins used to generate dose response matrix}
\]

The values for the variable Dose are the values of the dose bins used to predict FI
distributions in the dose response matrix, an example of the values of dose include, D = [0 1 3 5 9 10 ... 299]. Once inactivation is predicted from the dose bins, the discrete form of the segregated-
flow model can be applied to predict a reactor’s microbial inactivation of MS2. The data presented
in Figure 5-3 illustrate the accuracy of this method for MS2 and another common biodosimetry
challenge organism, coliphage T1.

**Step 8: Prediction of Target Organism Inactivation**

The methods to predict target organism inactivation are the same as described for
prediction of MS2 inactivation. The dose-response behavior of the challenge organism must be
quantified in terms of a dose-response equation. The dose-response behavior of *Cryptosporidium*
**PROTOCOL FOR VALIDATION OF UV DISINFECTION REACTORS USING LAGRANGIAN ACTINOMETRY**

*C. parvum*, *Giardia lamblia*, and viruses are defined by regulation; these dose-response relationships are listed in Table 5-2.

**Table 5-2. UV dose requirements for inactivation of C. parvum, G. lamblia and Adenovirus**

(from *UV Disinfection Guidance Manual*, USEPA 2006)

<table>
<thead>
<tr>
<th>Target Pathogens</th>
<th>Log Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>1.6</td>
</tr>
<tr>
<td>Giardia</td>
<td>1.5</td>
</tr>
<tr>
<td>Virus</td>
<td>39</td>
</tr>
</tbody>
</table>

DEVELOPMENT OF LOG INACTIVATION FOR SETPOINT AND CALCULATED APPROACHES

The discussion and calculations presented in Sections 5.2 and 5.3 yield the final product of the LA experimental validation: The log-inactivation of a specific microbe or pathogen at a known combination of operating variables. Depending on the reactor control strategy, the number of test point that are sampled yields an equivalent number of log-inactivation predictions. At this point, the protocol again corresponds to the UVDGM in developing a setpoint performance target, or establishing a UV reactor control algorithm based on the validation results. In this case, however, log-inactivation results are used, instead of the RED that is the focus of the UVDGM.

DETERMINING THE MAXIMUM LOG INACTIVATION FOR THE UV INTENSITY SETPOINT APPROACH

Reference is made to Section 3.4.1 with respect to testing a reactor that operates with a minimum intensity setpoint. If the UV sensor is in the ideal condition, the two test points noted in Section 3.4.1 should yield equivalent LI results. If not, selecting the lowest LI from these two test conditions accounts for UV reactor designs where the sensor is not in the ideal location.

Replicate LI values (typically 3-5 values) should be averaged to produce one LI for each test condition. From these average values, the lowest LI should be selected and used in the validated LI calculation. If variable-setpoint operations will be used at the water treatment plant (i.e., different UV intensity setpoints for different flow rate ranges), the maximum LI value should be identified for each flow rate range.

**Developing a Log Inactivation Equation for the Calculated LI Control Approach**

If the reactor uses a dose or LI algorithm control approach, the validation testing results are used to develop a LI-monitoring equation. Test design for this approach is discussed in Section 3.4.2. The variables in the LI equation are one or more of the key operating variables associated with the UV reactor. Typically flow, UVT, sensor output, input power, number of lamps, etc. are used to develop this equation.
UVDGM Section 5.8.3 discusses the use of multivariate linear regression to fit an equation to the validation test data. This approach is also recommended for the LA protocol, developing LI as a function of the key reactor variables. Since replicate samples are combined to reduce the noise around the FI distribution for each operating condition tested, one LI result is determined at each given test point. Variables should include those that can be monitored by the reactor or water plant on a continuous basis as inputs to the LI algorithm. Goodness of fit should be tested using standard statistical methods, and the lack of any significant bias to a given variable should be verified (see Section 5.8.3 in UVDGM).

**DERIVING THE VALIDATION FACTOR (VF)**

This section presents the LA analog to the UVDGM method of determining uncertainty (variance) for validation of UV reactors. The general approach is essentially equivalent to that of the UVDGM with respect to the uncertainty components that are addressed. As such, the following discussions closely follow those found in Section 5.9 of the UVDGM.

Several uncertainties and biases are involved in using experimental testing to define a validated LI and operating conditions. The LI is divided by VF to quantitatively account for key areas of uncertainty. The UVDGM states this operation by the equation:

\[
VF = B_{RED} \times \left(1 + \frac{U_{Val}}{100}\right)
\]

Where:

- \(VF\) = Validation Factor
- \(B_{RED}\) = RED bias factor
- \(U_{Val}\) = Uncertainty of validation, expressed as a percentage

Note that a polychromatic bias (\(B_{POLY}\)) is also used in the UVDGM expression for the validation factor, VF, when using medium pressure (polychromatic) lamps. It allows elimination of the term if the reactor is equipped with germicidal sensors, whereby less than 10% of the radiation accepted by the sensor is greater than 300 nm, or the sensor is positioned less than 10 cm from the nearest lamp when the water to be treated has a UVT less than 80%. (Refer to Section 5.9, pg 5-36, and Appendix D of the UVDGM for an explanation of the \(B_{POLY}\)). Modern reactors are invariably equipped with acceptable sensors.

In biodosimetry, the RED bias (\(B_{RED}\)) is a correction factor that accounts for the difference between the UV sensitivity of the target pathogen and the UV sensitivity of the challenge microorganism. It is included by the UVDGM to reflect the potential bias that may occur because the dose-distribution of the reactor is unknown. The \(B_{RED}\) is computed and provided in lookup tables UVDGM Appendix G, based on default dose-distributions reflective of a relatively inefficient UV reactor (see Appendix D of the UVDGM for a full explanation of the RED bias). Because the LA method allows for the direct measurement of dose-distribution, and this dose-distribution is then used to compute the log-inactivation, the use of the \(B_{RED}\) is not needed. In
effect, it becomes 1.0, and the VF equation is reduced to a function of the experimental uncertainty, $U_{Val}$:

$$VF = \left(1 + \frac{U_{Val}}{100}\right)$$

Some sources of uncertainty are not included in the VF. Instead, as with the UVDGM guidance on biodosimetry, certain UV reactor monitoring components must meet performance requirements (flow meters, radiometers, spectrophotometer, mixing, power, etc.), and test results must be within QA/QC criteria (see Section 3.5). The next section (5.5.1) addresses experimental uncertainties that are included in the VF.

**Uncertainty of Validation ($U_{Val}$)**

The Uncertainty of Validation ($U_{Val}$), also referred to as the experimental uncertainty, has between 1 and 3 input variables, based on how well the validation testing adhered to QA/QC limits. Refer to Figures 5.4 and 5.5 in the UVDGM for decision trees that illustrate the components included in $U_{Val}$. Basically, the $U_{Val}$ equations are as follows, depending on the reactor’s control strategy:

**UV Intensity Setpoint Approach:**

$$U_{Val} = \left(U_{SP}^2 + U_{S}^2 + U_{DR}^2\right)^{\frac{1}{2}}$$

**Calculated LI Approach:**

$$U_{Val} = \left(U_{IN}^2 + U_{S}^2 + U_{DR}^2\right)^{\frac{1}{2}}$$

Where:

- $U_{Val}$ = Uncertainty of validation
- $U_{SP}$ = Uncertainty of setpoint
- $U_{IN}$ = Uncertainty of interpolation for the calculated L approach
- $U_{S}$ = Uncertainty of sensor value, expressed as a fraction
- $U_{DR}$ = Uncertainty of the dose-response FI distribution Weibull fits

**Sensor Uncertainty ($U_{S}$)**

The accuracy of the sensor measurements is briefly addressed in Section 3.3.4 of this protocol, which defers to the UVDGM, Section 5.5.4. Specific criteria are established when comparing duty sensors to reference sensors. In effect, the references (three are typically used) must be with 10% of their average, and each duty sensor must be within 10% of the average reference sensor reading. If this criterion is met during validation, then $U_{S}$ can be ignored. If it is greater than 10%, than the actual variance is included in the $U_{Val}$ equation.
Dose-Response Uncertainty ($U_{DR}$)

In the UVDGM, all the uncertainties associated with a collimated beam, biodosimetric dose-response experiment and the subsequent dose response data analysis are integrated into one combined uncertainty factor, $U_{DR}$. The UVDGM sets uncertainty bounds for the experimental data; if the results fall within these criteria, the $U_{DR}$ can be ignored. In LA, the collimated beam experiments are very similar to the biodosimetric methods, except that a FI distribution is determined, which in turn is fitted with Weibull function (See Section 5.3.2.3). The key attributable uncertainties include the test operations, the cytometry analyses, the cytometry gating and data reduction, and the regression fitting of the FI distributions. Quantifying the uncertainties of the LA dose-response process is not practical; instead, the process is incorporated into the overall deconvolution scheme and included in the uncertainty of the LI or RED predictions. $U_{DR}$ is not considered in the LA UVal.

Intensity Setpoint Uncertainty ($U_{SP}$)

The uncertainty in the setpoint value is based on a prediction interval at a 95-percent confidence level. Similar to the treatment recommended in the UVDGM, it is calculated as follows:

1. Calculate the average and standard deviation of the LI values generated for each test condition.

2. Calculate the uncertainty of the setpoint LI using:

$$U_{SP} = \frac{t \times SD_{LI}}{LI} \times 100$$

Where:

- $LI$ = Average LI value determined for each test condition
- $SD_{LI}$ = Standard deviation of the LI values measured for each test condition
- $t$ = t-statistic for a 95-th confidence level, based on the number of replicates

3. Select the highest $U_{SP}$ from all test conditions for calculating the VF.

Interpolation Uncertainty ($U_{IN}$)

The interpolation uncertainty for the log inactivation (LI) algorithm integrates the uncertainties associated with field testing and the associated calculations of LI. Each effluent sample collected from LA testing is subjected to cytometry analysis, deconvolution to determine the measured dose distribution, and then integration with the dose-response behavior function of a target pathogen for its estimated log inactivation. These log inactivation values, together with the corresponding operation conditions are used to develop a LI-calculation algorithm, which can be built into the reactor control panel for LI prediction in field operations. The uncertainty of interpolation ($U_{IN}$) is calculated as the lower bound of the 95-percent confidence band for the LI-prediction equation. This confidence band reflects the noise in the dose-distribution-predicted LI
about that fit. The uncertainty of interpolation, $U_{IN}$, is evaluated in accordance with the following equation as an analog to the method defined in the UVDGM:

$$U_{IN} = \left( \frac{t_{stat} \times SD}{LI_{Calc}} \right) \times 100\%$$

Where,

- $U_{IN}$ = the uncertainty of interpolation, expressed as a percentage;
- $t_{stat}$ = t-statistic of the data population at 95% confidence level;
- SD = standard deviation of the errors between algorithm-calculated log inactivation and the log inactivation estimated from DMS measured dose distributions;
- $LI_{Calc}$ = the algorithm-calculated LI for any given operation point

DETERMINING THE VALIDATED LOG INACTIVATION

The last step in the validation protocol, as presented in Section 5.10 of the UVDGM, is to adjust the LI results by the VF to determine the validated LI for the UV reactor, using the following equation:

$$\text{Validated LI} = \frac{LI}{VF}$$

Where:

- LI = the maximum LI for the UV Intensity Setpoint Approach; or the LI as calculated using the LI-monitoring equation for the Calculated LI approach.
- VF = the Validation Factor, as described and calculated in Section 5.5.

Because the method and assumptions for this step rely on the LI monitoring strategy of the UV reactor, they are discussed separately below.

Determining the Validated LI and Operating Conditions for the UV Intensity Setpoint Approach

For the UV intensity setpoint approach, the validated LI equation above produces one validated LI for a given intensity setpoint corresponding to the minimum LI. When the UV reactor is operating at a UV intensity level above the setpoint, the true LI accomplished is always equal to or greater than the validated LI.

Validated operating conditions are as follows:

- The UV intensity measured by UV sensors must be greater than the UV intensity setpoint.
- The flow rate must be equal to or less than the flow rate tested.
- The lamp status for each lamp (i.e., on/off setting) must be equivalent to the settings used during testing.
Determining the Validated LI and Operating Conditions for the Calculated LI Approach

For the calculated LI approach, the validated LI varies based on operational parameters. Typically, measured values of UVT, UV intensity, and flow rate are entered into the LI-monitoring equation to calculate LI for the targeted pathogen. LI is divided by the VF to produce the validated LI. One VF can be used. An equation for VF can also be used if $U_{IN}$ is expressed as a function of LI.

Validated operating conditions for the calculated LI approach are as follows:

- The operating UVT must be equal to or greater than the minimum UVT evaluated during validation testing.
- The operating flow rate must not exceed the flow rate evaluated during validation testing.


