Spectrophotometry Theory

PURPOSE: This document provides basic training on the function of an absorption spectrophotometer and the principles that apply to the testing of a spectrophotometer.

- Function of a spectrophotometer.
- Relevance of the Operational Qualification tests.
- Nature and handling of solid state filters.

An absorbance spectrophotometer is used to identify and quantify a molecule of interest in a test solution.

Identification of a molecule of interest in a solution is possible because many molecules in solution absorb light at wavelengths characteristic of the molecule. Wavelengths that are not absorbed by a solution are transmitted through the solution to a light detector.

The intensity of light absorbed by a solution is proportional to the concentration of the molecules in that solution. The relationship between light absorbed by a molecule of interest in solution and the concentration of that molecule is linear (Beer's law). The light that is not transmitted through the solution to the light detector is the light that was absorbed by the solution.

$$\text{Intensity}_{\text{Original}} - \text{Intensity}_{\text{Transmitted}} = \text{Intensity}_{\text{Absorbed}}$$

The main elements of a spectrophotometer are:

- **Light Source**: emits white light (tungsten, halogen, xenon, etc).
- **Monochromator**: a diffraction grating or prism-like device that splits the monochromatic light into its component wavelengths (spectrum).
- **Exit Slit**: a physical opening that allows the exit of only the selected wavelength; the exit slit excludes all but the wavelength selected by the spectrophotometer's operator.
- **Cuvette Holder**: physical mounting securely holds the test cuvette in the path of the selected wavelength. The test cuvettes are containers made of specialized plastic, glass, or transparent quartz.
- **Light Detector**: measures light transmitted through the test cuvette; it measures the light that travels through the test cuvette.
- **Data Viewer**: a mechanism to present humanly readable absorption or transmission data (printout, visual display, etc.)

The absorbance spectrophotometer beams light to a mechanical device that splits the light into its spectral components, the monochromator. The monochromator rotates and thusly aims the selected wavelength to the exit slit. Only the wavelength that passes through the exit slit is transmitted through the test cuvette. The light transmitted through the test cuvette is measured by a light detector. The intensity of the light absorbed by the test solution is the difference between the intensity of the light entering the test solution and that of the light exiting the test solution.

$$(I_0 - I_T = A)$$

A UV/VIS spectrophotometer's light source emits a spectrum of different wavelengths, measured in nm. The spectrum ranges from the UV wavelengths through the visible (VIS) range and into the near infrared range (NIR).

- UV (200 – 400 nm wavelengths)
- Visible (400 – 700 nm)
- Near Infrared (700 – 1100 nm)

These wavelengths are used in the analysis of biological molecules.

- 230 Organic impurities
- 260 Nucleic acids (aromatic structures, oligos, ssDNA, RNA)
- 280 Proteins (aromatic side chains of amino acids)
- 260/280 Nucleic acid concentration and purity (protein or phenol contamination)
- 260/320 DNA/RNA concentration
- 320 Reference wavelength for nucleic acids
- 340 Background correction for scattering of light by particulates, damaged cuvettes.
- 595 – 750 Direct UV and colorimetric assays for protein concentration

Below is a list of standard spectrophotometer qualification and calibration testing:

- **Photometric Accuracy** is verified because a spectrophotometer's ability to accurately measure absorbance is critical to each of its measurements. The spectrophotometer's measurement of the absorbance of the test cuvette must match that documented on the test filter's calibration certificate. Photometric Accuracy is the same as Absorbance Accuracy.
- **Wavelength Accuracy** is verified because each peak of absorption is in reference to a specific wavelength. The ideal test cuvette for the measurement of wavelength accuracy has sharp, clearly defined transmission peaks. The test is a scan of a range of wavelengths. The measured peaks of absorbance must be located at the wavelengths documented on the calibration certificate.
- **Stray Light** is any wavelength, other than the one selected by the operator, that reaches the light detector. The mechanical device that splits the white light into its component wavelengths is the prism-like monochromator. When it splits light into its spectral components, some light is scattered; no monochromator is 100% efficient. The light beam transmitted through the exit slit probably includes some scattered wavelengths, "stray light".

  The light detector detects light, all light that reaches it. It cannot differentiate between the light of the selected wavelength and that of stray light. Because the light detector sums the two sources of light, the detected light transmitted through the sample is higher than the true value. More transmittance is calculated as less absorbance, and less absorbance is reported as a lower than true concentration of the light absorbing molecule.

  Stray light's negative effect on absorption measurement is greater in the UV range (190 – 300 nm). Lucigen uses the spectrophotometer to measure the concentration and activity of biological activity in the UV range. A spectrophotometer's ability to accurately measure the concentration of a high absorbance test solution is a key specification of its quality.
- **Linearity** verification provides evidence that the spectrophotometer functions in compliance with Beer's Law, absorbance vs concentration is linear. The test is a series of measurements of differing optical densities at a single wavelength.

- **Spectral Resolution/Bandwidth** is a measurement of the ability of a spectrophotometer to differentiate between two wavelengths that are adjacent to each other in the spectrum. Two peaks are considered to be "resolved" if the absorption between the two peaks is less than 80% of the maximum peak.

  Spectral resolution increases as the exit slit width is decreased, but there are tradeoffs to consider when increased spectral resolution is sought:
  - As the exit slit width is decreased, the intensity of the light transmitted through the test solution is also decreased.
  - When the light transmitted through the test solution is decreased, the signal to electronic noise ratio is decreased; the true signal is less distinct from the background noise. In an extreme case, as the intensity of the noise signal approaches the intensity of the test signal, the spectrophotometer cannot discriminate between the two. All electronic systems create electronic noise when analog data is converted to digital data; noise is inherent in an analog to digital signal conversion.
  - As the signal to noise ratio decreases, the spectrophotometer's repeatability and reliability also decreases.

  The measurement of spectral resolution was required for spectrophotometer validation by European Pharmacopoeia for a few years before the FDA required it for the US Pharmacopoeia. Lucigen is not a pharmaceutical company; Lucigen determined that this requirement is not applicable to RUO (research use only) manufacturers.

SOLID-STATE FILTERS

These tests were developed for use with the WAV-1, WAV-7, and FUV-0.5D FireFlySci solid state standards (also referred to as "filters"). Their calibration certifications are attached to the QCBD equipment record LEN 00878.

WAV-7 is a Didymium Glass filter. WAV-1 is a Holmium-Oxide Filter.

- WAV-1 for wavelength accuracy (240 -640 nm).
- WAV-7 for wavelength accuracy (330 – 875 nm).
- WAV-7 for absorbance accuracy (270 – 340 nm).
- FUV-0.5D absorbance accuracy (200 – 700 nm).
- FUV-0.5D radiant stray light anywhere on the continuous spectrum from 200 – 700 nm.

The amount of light absorbed by a test solution is directly proportional to the:

- Concentration of the absorbing molecule in the test solution.
- Distance between the two inner surfaces of the cuvette. The distance the light travels through the test solution is the pathlength. The filters have a standard pathlength of 10 mm.

To maintain the lifetime calibration of the FireFlySci solid state filters, strictly adhere to these handling requirements.

- **Always wear powder-free gloves** when handling solid-state calibration standards. Fingerprints are the main root cause for incorrect readings.
- ***Never* touch the optical surfaces** even when wearing gloves; always pick up the standards by their sides.
- **Do not use micro-fiber cloths or lens tissues**. Manufacturer's tests show that these cause smudging and scratching of the optical glass. Use compressed air to clean a filter's surface per the manufacturer's web site. FireFlySci.com