Validating UV/Visible Spectrophotometers

UV/Visible spectrophotometers are widely used by many laboratories – including those found in academia and research as well as industrial quality assurance. The technique is mainly used quantitatively (although some qualitative analysis can also be performed).

For any type of critical determination, whether it be clinical, pharmaceutical or industrial QC, environmental analysis or research, it is essential that the instrument is performing according to specification. In some of these applications, it is important that the instrument performance is monitored regularly and that there is documentary evidence that this is the case.

This will apply to any measurements to do with human or animal health (clinical or pharmaceutical/nutraceutical), food or labs offering an accredited measurement service (e.g. in accordance with ISO 17025). Validation is also a key requirement of Good Laboratory Practice (GLP).

Testing frequency

One common question from laboratories relates to the frequency of testing. There are no regulations, as such, regarding this but for any human health or nutrition related analysis, a testing frequency of at least monthly should be considered. If the instrument is in a high sample throughput environment, then some additional weekly testing (e.g. absorbance and wavelength check) should be performed.

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Testing wavelength accuracy

Wavelength accuracy is normally assessed by using either a sample containing a series of very sharp peaks such as a solution of holmium perchlorate or a holmium oxide and/or didymium doped glass filter or by measuring the emission from a lamp. If the instrument is equipped with a deuterium (D2) lamp as the UV source, this can be used. An external mercury (Hg) lamp can also be used. This is less convenient than using the previously mentioned methods but methodology exists (e.g. in the Ph. Eur. tests) for its use as an alternative to a glass or liquid standard. The advantage of emission lines is that they are inviolate (i.e. the emission wavelengths don’t change over time).

Using the deuterium lamp

If the instrument uses a deuterium lamp, it is possible to measure the very sharp emission line at 656.1 nm by switching off the visible lamp and measuring the energy (single beam mode). There is also a weaker emission line at 486.0 nm that can be used. This is not as rigorous as using other wavelength standards (as fewer lines are available) but it does serve as a very convenient and accurate way to assess that the instrument’s optics are aligned correctly. Some PerkinElmer instruments (such as the LAMBDA™ 25/35/45) use the 656.1 nm emission line of D2 upon switch-on to calibrate the monochromator. Others (such as the LAMBDA 800/900 and LAMBDA X50 series) can perform this calibration on demand (and store the values in the instrument software). UV WinLab™ v5 and v6 have methods to check this and produce a report. These methods use the rear beam for the procedure (just in case a sample has been left in the front position by mistake). The 486.0 nm emission line (but not the 656.1 nm line) is one of the possible test points in the Ph. Eur. 5.2 (2005).

Glass and liquid filters

The most commonly used glass (or quartz) filter is holmium oxide. This provides a quick and easy way to check wavelength accuracy. This standard gives a series of lines in the visible region. It does not provide strong absorption peaks into the UV region and so a solution of holmium oxide in perchloric acid (holmium perchlorate) is recommended by some pharmacopoeias but for general non-pharmacopoeia instrument testing, holmium oxide glass is widely used. Another material that is used is “didymium” – a mixture of praseodymium and neodymium. This is recommended by the U.S. Pharmacopoeia (USP 24) as well as others such as the JP (Japanese Pharmacopoeia). As with holmium oxide, it is also available in solution form. Other wavelength standard solutions commercially available include samarium perchlorate, which gives a range of wavelengths in the UV and visible regions between 230 and 560 nm and “rare earth sulphate” which gives far UV wavelengths between 201 nm and 253 nm.

<table>
<thead>
<tr>
<th></th>
<th>Holmium oxide quartz</th>
<th>Holmium perchlorate solution</th>
<th>Didymium glass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelengths</td>
<td>279.2 nm</td>
<td>241.15 nm</td>
<td>440.4 nm</td>
</tr>
<tr>
<td></td>
<td>453.7 nm</td>
<td>361.5 nm</td>
<td>513.4 nm</td>
</tr>
<tr>
<td></td>
<td>637.7 nm</td>
<td>287.15 nm</td>
<td>879.3 nm</td>
</tr>
<tr>
<td></td>
<td>360.9 nm</td>
<td>536.3 nm</td>
<td>481.0 nm</td>
</tr>
<tr>
<td></td>
<td>536.5 nm</td>
<td></td>
<td>684.5 nm</td>
</tr>
</tbody>
</table>

Figure 2. Wavelengths for mercury emission lamp.

Figure 3. Wavelengths for holmium oxide glass, holmium perchlorate solution and didymium glass.

Using a mercury emission source

The use of a mercury (Hg) emission lamp is another way to check wavelengths. This lamp offers more lines compared to deuterium – particularly in the UV region. The practicalities of using such a lamp are a little more difficult. The mercury lamp needs to be placed in the instrument’s lamp compartment. The principal mercury emission lines are shown in Figure 2.
Photometric (absorbance) accuracy testing

As with wavelength accuracy testing, either solutions or glass/quartz filters can be used. For general applications, neutral density grey glass filters (such as the NIST® 930D set) are a convenient way to test the photometric accuracy of instruments. These offer a number of wavelengths in the visible region. For testing in the UV either “metal on quartz” glass filters or solutions must be used. Metal on quartz filters have a reflective coating applied to a quartz plate. This can cause issues due to the strong back reflection sent back through the instrument’s optics so they may not be suitable for all types of instruments. The coating needs to be treated with care as any damage will alter the optical properties.

The most commonly used solution for checking absorbance accuracy is potassium dichromate. The original 1988 Ph. Eur. method tests absorbance at four wavelengths – 235, 257, 313 and 350 nm using between 57.0 and 63.0 mg of potassium dichromate in 0.005 M sulphuric acid diluted to 1000 mL. Since 2005 a second solution has been added to provide an additional test point at 430 nm. This uses the same amount of potassium dichromate but is made up to 100 mL (i.e. it is ten times more concentrated than the original solution). In both cases the A 1%/1 cm value is recorded and checked against the target range.

Nicotinic acid (0.1 M hydrochloric acid) in a range between 6 and 24 mg/L can be used to assess photometric accuracy in the far UV region (at 213 nm and 261 nm). This is not an official procedure but reference materials are commercially available for this test.

Care has to be taken when preparing the solutions as potassium dichromate is hygroscopic and so it is important that this is dried thoroughly otherwise the weight will be incorrect and this could result in an unnecessary failure.

Double aperture correction for photometric accuracy

No instrument has a perfectly linear detector response and so the observed photometric accuracy will have a greater deviance from the certified values as a result. It is possible to correct for this systematic error by using a technique called double aperture correction. This procedure is only available for instruments at the upper performance range. To correct for this, it is necessary to purchase a double aperture accessory. This accessory consists of a series of shutters – either manual or motorized – which are able to measure the top and bottom half of the beam. The mechanism must be highly precise such that there is no overlap between the two measurements. It is then possible to calculate a correction factor that can be used for subsequent measurements. Instrument manufacturers will quote photometric accuracy both with and without double aperture correction as part of their specifications. It will also be used by some national standards laboratories as part of their determinations but it is very rare to find it used elsewhere due to its complexity and offering a precision that is unnecessary for most measurements.

Stray light testing

Stray light can be defined as the amount of unwanted wavelengths that are present in the sample beam. In a conventional “dispersive” instrument, the monochromator is before the sample and the sample receives one wavelength at a time. After the sample, there is only the detector. This means that any stray light that gets past the monochromator will be seen by the detector. Stray light has a strong impact on the instrument’s ability to record accurately at higher absorbances and is a key specification when assessing the quality of a spectrophotometer. For a single monochromator instrument (as found in most chemical and biochemical laboratories) the best achievable stray light value (using ASTM® methodology) will be around 0.01%T. This equates to one in every 10,000 photons being due to stray light. This may sound like a small amount but it has to be remembered that absorbance is a logarithmic scale and so 3AU (equivalent to 0.1%T) equates to only one in a thousand photons actually passing through the sample and reaching the detector. If the stray radiation is mainly “far” stray light (i.e. wavelengths some distance from the actual analytical wavelength) then it is unlikely to be absorbed by the sample and thus will cause the linearity to become worse at higher absorbances as the absorbance becomes swamped by the [constant] stray light. If linear performance greater than 3A is required, systems with more than one monochromator must be considered – either premonochromator (using a smaller auxiliary
monochromator) or a full double monochromator (two equivalent monochromators). It should, however, be taken into consideration that for most liquid samples, they can be diluted or measured in short pathlength (e.g. 5 mm) cuvettes and so measurement at such a high absorbance value shouldn’t be necessary – multiple monochromator systems are usually only necessary for materials and optics analysis where it is not possible to adjust the sample.

All tests should be carried out using a 1 cm (10 mm) quartz cuvette.

Stray light testing for pharmacopoeia

The European Pharmacopoeia (Ph. Eur.) performs the test in absorbance (unlike ASTM® where transmittance is used as the ordinate mode) and the recorded value of a 12 g/L aqueous potassium chloride solution at 198 nm should be 2A or greater. This test was revised in 2005 as the test wavelength was moved from 200 nm (which had been used since 1988) to 198 nm. The original 200 nm wavelength was influenced somewhat by the sample temperature and whether or not the solution had been degassed (sealed cuvettes were less prone to this) and so moving the test point down to 198 nm made the test more robust.

Stray light tests should be carried out on the basic instrument and in a configuration specified by the manufacturer for that particular test. If any accessories are fitted (e.g. sippers, integrating spheres, cell changers, etc.) these can have a detrimental effect on the stray light value due to additional light leakage, etc. Clear plastic tubing entering the sample chamber from outside can act as a fiber optic and transfer light from outside to the inside of the instrument. It is recommended to either use black tubing or to shroud the tubing (an outer black tubing sheath works well) for about 5 cm prior to entering the sample area in order to minimize this.

Stray light testing to ASTM® methodology

ASTM® stray light testing involves the use of two solutions – 10 g/L sodium iodide (NaI) for 220 nm and 50 g/L sodium nitrite (NaNO₂) for 340 nm and 370 nm. These solutions have a very sharp cut-off in the UV region. This means that if light is detected at a wavelength where the sample is supposed to be completely blocking, this must be due to stray light passing through the transparent part of the sample spectrum. As a further confirmation, it is possible to block the beam (the metal frame of our glass filters inserted “sideways on” into the instrument makes an ideal beam blocker) and compare the measurement of the stray light solution to the blocked beam – the difference between the two giving the stray light value. A similar test can be performed at 650 nm using a 0.1 g/L methylene blue solution or measuring around the cut-off of pure acetone between 250 and 320 nm.

Figure 7. Effect of instrumental stray light on absorbance linearity.

Figure 8. ASTM® stray light test at 220 nm using a 10 g/L sodium iodide (NaI) solution showing stray light to be less than 0.01% T.

Figure 9. Stray light testing according to Ph. Eur. using 12 g/L KCl solution showing the difference in stray light in a premonochromator instrument (LAMBDA 45) compared to a single monochromator instrument (LAMBDA 25/35). It also shows the improvement in the result when using 198 nm as the test point instead of 200 nm.
Some pharmaceutical companies use their own stray light test solutions such as a saturated solution of lithium carbonate (at 227 nm) or sodium chloride (at 205 nm) as part of their in-house procedures but these are not officially recognized by the major pharmacopoeias.

**Resolution testing**

The European Pharmacopoeia introduced a resolution test as part of the original 1988 methodology. This is based on measuring a solution of 0.02% w/v solution of toluene in hexane, measuring the ratio of the peak (at 269 nm) and the trough (at 266 nm) and measuring the ratio. This ratio should be 1.5 or greater unless prescribed in the monograph. This means that, although it is highly desirable for an instrument to pass this test, it is not essential but most manufacturers regard this to be a part of making an instrument “pharmaceutical compliant” and some carry out the measurement as part of their final test procedure. In practice, an instrument with a bandpass (slit width) of around 1.5 nm should pass this test. For a fixed bandpass instrument, the measurement should give fairly constant results as there is no mechanical movement involved. If the instrument has variable bandpass – particularly if the slits can be varied continuously using a stepper motor “jaw” mechanism, test results may vary more and so the test should be carried out more frequently.

![Figure 10. Resolution test using 0.02% w/v toluene in hexane (in this example ratio is approx 1.9).](image)

In 2005 the European Pharmacopoeia introduced a second method involving a solution of toluene in methanol and based on second derivative (D2) spectroscopy. At the time of writing, this test is not widely used.

**Additional testing**

It may be useful to perform additional tests on the instrument using methodology not contained in pharmacopoeias but using a subset of the manufacturers’ own in-house service procedures. These can give an indication of things such as lamp deterioration. Some examples are:

**Absorbance stability (drift)** – measuring how an absorbance changes (usually in absorbance per hour) under stipulated conditions.

**Baseline flatness** – the instrument is autozeroed with nothing in the sample compartment across its working range and this is then measured. Some manufacturers specify flatness over a restricted range (e.g. 200-800 nm) which may not be the full working range of the instrument. This is normally the case with photomultiplier based instruments where the photomultiplier suffers a fairly major loss of sensitivity above 800 nm.

**Noise** – this is measured at a particular wavelength under prescribed conditions. It may be measured at zero absorbance or at a specified absorbance value (e.g. 1A).

**Reproducibility** (both in terms of wavelength and photometric accuracy).

**Testing reflectance**

Some laboratories wish to test the accuracy in reflectance. These tend to be the more highly specified instruments and they must be equipped with a suitable reflectance attachment. This is usually an integrating sphere for diffuse and total reflectance and either a relative or absolute attachment for specular reflectance.

For diffuse reflectance, it is possible to purchase diffuse reflectance standards. These are either grey standards where differing amounts of carbon black are added to the white material or color standards where the white material is dyed. For perfectly diffuse materials, Spectralon® is probably the best material. This is a proprietary white sintered PTFE material which is resistant to yellowing and can be cleaned easily. When measuring these standards, it is best to use as large a beam as possible. The standards aren’t completely homogenous and so, for very small beams, it is possible to pick up individual “smuts” of carbon which will give an inconsistent result. By measuring the %RSD (Relative Standard Deviation) of these standards, the expected tolerance can be assessed.
For measuring specular reflectance, the most commonly used material is a front surface coated aluminum mirror as this reflects across the UV/Vis/NIR range. These mirrors are less reliable in the UV range as any surface scratches will contribute to higher scatter in the UV region due to the physics of scatter phenomena. One other issue with these mirrors is that they are often required to be traceable as part of a laboratory gaining accreditation (e.g. ISO 17025). Traceable mirrors often have a poor specification – such as ±1% reflectivity and this may not be good enough for some applications (such as the measurement of laser mirrors where accuracy of 0.1%R is highly desirable). The reason for this wide tolerance is that national standards labs compare their standards with other laboratories and so, the more laboratories are involved, the higher the statistical spread of data which increases the specified uncertainty.

When testing reflectance, there can be some debate about the "correct" result for a particular sample. Most standards are fairly ideal in nature so, for example, Spectralon® is almost perfectly diffuse and an aluminum mirror is almost perfectly specular. Actual samples may lie somewhere in between – displaying a mixture of both types of reflectance. In order to address this issue, some industries (e.g. paint manufacturers) sometimes use calibrated ceramic tiles as they have a higher specular component.

When measuring reflectance (unless using an absolute accessory), it is necessary to zero the instrument and accessory using a standard whose reflectance is known. It is then necessary to perform a mathematical correction to remove the contribution of this standard and convert the relative measurement into an absolute one. If this is not done (i.e. the relative value is used) for a set of absolute standards, the results will be invalid.

Testing in the Near Infrared (NIR) region

Some spectrophotometers also have near infrared capability and can measure in excess of 900 nm and so it is necessary to assess performance in this region. There are some test procedures that are applicable in this region. These include:

- Wavelength accuracy of the NIR grating using the second order deuterium emission line at 1312.2 nm or 972 nm (i.e. two times 656.1 nm and 486 nm, respectively).
- Wavelength accuracy using a holmium oxide which gives a peak at 1938 nm.
- Wavelength accuracy using a McCrone standard. This is a composite standard which give additional lines in the NIR from 481 nm to 2326.8 nm (available from the U.K. NPL).
- Stray light using water at 1420 nm chloroform at 2365 nm.
- Photometric accuracy using grey glass standards. Some grey glass standards have test points in the NIR region in addition to the UV/Visible range.
- Reflectance – both Spectralon® and aluminum mirrors can be used to check reflectance in the NIR region. Spectralon® cuts off above 2500 nm.

Validating PerkinElmer UV/Vis and UV/Vis/NIR Spectrophotometers Using UV WinLab software

UV WinLab software (v4 and higher) contains features to aid with the calibration of spectrophotometers.

For medium performance instruments (LAMBDA 20, 25/35, 40/40P and 45), there is the Instrument Performance Verification (IPV) module. This module contains over twenty tests that the user can perform to check performance. Some tests require a standard or an external mercury lamp whereas others do not.
Calibration utility for the high performance LAMBDA instruments

For the high performance instruments, there is a collection of Service Methods. These are similar in nature to the IPV methods but there are more of them (reflecting the additional complexity of these instruments) and they are regular analytical methods arranged into a collection rather than being part of a separate software module. There are also additional service methods for some major accessories such as the integrating spheres and the Universal Reflectance Accessory (URA).

<table>
<thead>
<tr>
<th>Test</th>
<th>Standards Required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength accuracy – third party lamp</td>
<td>Third party lamp (e.g. mercury)</td>
</tr>
<tr>
<td>Wavelength accuracy glass</td>
<td>Calibrated standards set (B0507805). Traceable to German PTB</td>
</tr>
<tr>
<td>Wavelength accuracy solution</td>
<td>Pharmacopoeia standards set (B2500100) using holmium perchlorate (Ph. Eur. test)</td>
</tr>
<tr>
<td>Wavelength accuracy – D₁</td>
<td>No standard required – uses instrument’s deuterium lamp (486 nm and 656.1 nm)</td>
</tr>
<tr>
<td>Wavelength repeatability – D₂</td>
<td>No standard required – uses instrument’s deuterium lamp (486 nm and 656.1 nm)</td>
</tr>
<tr>
<td>Resolution – benzene vapor</td>
<td>Sealed Benzene cell (toluene acceptable substitute)</td>
</tr>
<tr>
<td>Resolution – toluene</td>
<td>Pharmacopoeia standards set (B2500100) using toluene in methanol (Ph. Eur. test)</td>
</tr>
<tr>
<td>Photometric accuracy glass</td>
<td>Calibrated standards set (B0507805). Traceable to German PTB</td>
</tr>
<tr>
<td>Photometric accuracy – K₂Cr₂O₇</td>
<td>Pharmacopoeia standards set (B2500100) using K₂Cr₂O₇ solution (Ph. Eur. test)</td>
</tr>
<tr>
<td>Photometric accuracy – KNO₃ solution</td>
<td>KNO₃ solution</td>
</tr>
<tr>
<td>Photometric repeatability glass</td>
<td>Calibrated standards set (B0507805). Traceable to German PTB</td>
</tr>
<tr>
<td>Baseline stability (flatness)</td>
<td>No standard required</td>
</tr>
<tr>
<td>Noise (three tests)</td>
<td>No standard required</td>
</tr>
<tr>
<td>Absorbance zero stability (drift)</td>
<td>No standard required</td>
</tr>
<tr>
<td>Stray light NaI (ASTM®)</td>
<td>Stray light standards (B2500099)</td>
</tr>
<tr>
<td>Stray light NaNO₂ (ASTM®)</td>
<td>Stray light standards (B2500099)</td>
</tr>
<tr>
<td>Resolution (toluene in methanol – Ph. Eur. 2005)</td>
<td>Toluene in methanol solution</td>
</tr>
<tr>
<td>Photometric accuracy – K₂Cr₂O₇ at 430 nm (Ph. Eur. 2005)</td>
<td>Solution of K₂Cr₂O₇</td>
</tr>
</tbody>
</table>

Calibration utility for the high performance LAMBDA instruments

The high performance LAMBDA instruments also have an onboard facility to adjust the instrument’s slit and wavelength settings. This calibration utility can be accessed by right-clicking on the instrument icon (in the instruments node of UV WinLab). Unlike other tests described here, it will measure and correct for any wavelength shift detected – it can be regarded as a kind of “autotune” routine. It also calibrates the continuously variable slit mechanism as well as checking the 0%T offset. The wavelength calibration is based on the 656.1 nm deuterium emission line (the 486.0 nm line can be also used optionally). The NIR monochromator can also be checked using the second order 1312.6 nm line. Please note that the photomultiplier is used for all tests (even the NIR wavelength accuracy – due to the fact that it is a second order effect and so the detector is still actually detecting at 656.1 nm). Where possible, it is best to calibrate the instrument using the standard detector rather than with an accessory. This is because very narrow slits are used for the wavelength calibration and so sensitivity is important. For dedicated “S” and “R” versions of the LAMBDA 650 and 750, the calibration routine in v6.0.4 (released in October 2011) has been enhanced in order to allow successful calibration when the sphere or URA are installed in place of the standard detector.

Figure 12. Service methods for the high performance instruments.
Validating solvents

Water is a commonly used solvent in UV/Visible absorption spectroscopy. It is a near perfect material offering transparency over the UV/Visible range (but having stronger absorption bands in the NIR region) and ideal for the wide range of water-soluble analytes.

Some materials (e.g. organic species) will not dissolve in water and others (mainly biological compounds) may require other substances to be present in order to stabilize the material (e.g. buffer solutions).

It is necessary, therefore, to check that the analysis is being conducted at a wavelength where the solvent is sufficiently transparent for the assay to be stable and reproducible. This can be done by assessing the cut-off point of the solvent. The most commonly used definition is the point at which the solvent has a transmittance of 10% (1 Absorbance) in a 1 cm cuvette. This can be ascertained quite easily by scanning the solvent or buffer on its own in a 1 cm quartz cuvette (using air as the autozero) and observing the point at which the spectrum reaches 1 absorbance.

Beyond the cut-off point, the solvent becomes completely opaque and the instrument will try as best it can to continue measuring (particularly if solvent is in both sample and reference beams) but what is observed is not useful analytically and routine methods should be constructed in such a way that they don’t collect data where the solvent is beyond its cut-off point as it will only cause confusion in trying to interpret meaningless results. Measuring the sample in energy (single beam) mode will give a clearer picture of what the instrument’s detector is observing.

Setting tolerances

When devising a validation test, it is necessary to set realistic tolerances for the test, otherwise it will fail unnecessarily. The tolerance should comprise of three parts which should be added together. These are:

- The uncertainty of measurement given on the standard certificate (or published emission wavelength if using a source emission line).
- The manufacturer’s specification for the instrument that relates to that particular standard (e.g. a wavelength accuracy test will be influenced by the instrument’s wavelength accuracy specification as well as the tolerance of the standard).
- Any influence to the measurement coming from the operator or other parts of the system.

Validating assays

So far, the instrument validation itself has been discussed. An instrument may be working perfectly but may still not be fit for purpose of a particular assay. This may be because the instrument has unsuitable specifications for that particular determination. As a trivial example, a perfectly functional instrument covering the range 190 to 900 nm would be unsuitable for measuring absorbance at 1000 nm.

Cuvette validation

Testing of the type of cuvette chosen for a particular assay should also be included in any validation of the assay. This should be in terms of design (macro, semi-micro, micro, ultra-micro, whether the walls are blackened, etc.), construction material (quartz, fused silica, NIR silica, glass, etc.) and the manufacturing tolerance of the nominal pathlength (usually to German norm DIN 58963-1/2).
Mean Value

\[ \bar{x} = \frac{1}{n} \sum_{i=1}^{n} x_i \]

The arithmetic mean value of a set of data points.

Standard Deviation

\[ s_x = \sqrt{\frac{1}{n-1} \sum_{i=1}^{n} (x_i - \bar{x})^2} \]

Relative Standard Deviation (%RSD)

\[ RSD(\%) = \frac{s_x \times 100}{\bar{x}} \]

Residual Error (of linearity data or a calibration curve)

\[ s_y = \sqrt{\frac{1}{df} \sum_{i=1}^{n} (y_i^* - y_i)^2} \]

Variance

\[ s_x^2 = \frac{1}{(n-1)} \sum_{i=1}^{n} (x_i - \bar{x})^2 \]

Where:
- \( n \) Number of data points.
- \( x_i \) The analytical value of the data point (e.g. concentration).
- \( y_i \) The ordinate value of the data point (e.g. absorbance).
- \( y_i^* \) Corresponding point on the calibration curve.
- \( df \) Number of degrees of freedom (linear curve without intercept = 1, linear curve plus intercept term = 2, quadratic curve without intercept = 2, quadratic curve with intercept = 3).

### Error assessment

#### Random errors

Random errors are caused by unavoidable deviations of the analytical results obtained with \( n \) determinations of the same sample from the related value (mean value of unknown samples, known value with reference samples). A measure for the random error is the repeatability, estimated from measurement values of completely independently repeated measurements. Each repeated measurement must be carried out as if it were a separate unknown sample.

#### Systematic errors, bias

Systematic errors (bias) are the deviations of the mean value obtained from \( n \) repeated measurements (expectation) from the related value (true or conventional true value). If there are systematic errors, this difference is bigger than the range of confidence of the mean value. The respective range of confidence is calculated from the standard deviation according to the given statistical accuracy. The systematic deviations are also characterized by the term **trueness**.

### Using statistics to evaluate the data

Statistical analysis techniques provide a useful “toolkit” in the assessment of validation data – both in terms of the instrument calibration and also with respect to a particular assay.

It is also necessary to determine the robustness of a calibration or assay in terms of its accuracy and precision. The diagram below gives a good visual representation of these two terms.

Assessing precision is useful when dealing with autosamplers – either “sippers” or carousel type devices. These devices rely on pumping the sample through a flowcell and are, therefore, prone to carryover. Although it is possible to devise operating parameters that will avoid this (e.g. minimum pumping times), they will be affected by things such as sample viscosity, temperature, etc.

### Statistical assessment of the measurement result

The following statistical formulas are useful in assessing precision and accuracy:

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Cut-off (nm) (&gt;1 AU in 1 cm cuvette)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>190</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>200</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>200</td>
</tr>
<tr>
<td>Methanol</td>
<td>205</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>210</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>233</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>256</td>
</tr>
<tr>
<td>Toluene</td>
<td>254</td>
</tr>
<tr>
<td>Acetone</td>
<td>330</td>
</tr>
</tbody>
</table>

Solvent manufacturers offer different purity grades of solvents and the UV cut-off point will normally be stated on the bottle. Some approximate cut-off points of common solvents are shown in the table below.
Standards Available from PerkinElmer

The following standards materials are available from PerkinElmer:

<table>
<thead>
<tr>
<th>Part Number</th>
<th>Description</th>
<th>Traceability</th>
</tr>
</thead>
<tbody>
<tr>
<td>B0507805</td>
<td>Photometric and Wavelength Accuracy Reference Material Set</td>
<td>German PTB</td>
</tr>
<tr>
<td></td>
<td>Set of secondary reference materials traceable to NIST® to verify the ordinate and abscissa (wavelength) accuracy of UV/Vis and UV/Vis/NIR spectrometers. The set comprises four glass filters mounted in precision aluminum spring mounts. They are designed for use with the standard 10 mm cuvette holders of most UV/Vis and UV/Vis/NIR spectrometers. The set is provided in a robust aluminum container. There are three neutral density glass filters for checking the ordinate accuracy and a holmium oxide glass filter for checking the wavelength accuracy of a spectrometer. Each filter has a unique identification number. The certified ordinate and abscissa values and tolerances of each filter are quoted in the accompanying Certificate of Calibration. Measured wavelengths for the three neutral density filters are 440.0, 546.1, 635.0, 1700, and 2300 nm. The glass filters are chosen to provide nominal absorbance values of 0.3 A (50%), 0.5 A (30%) and 1 A (10%).</td>
<td></td>
</tr>
<tr>
<td>N1010545</td>
<td>Holmium Oxide Glass Filter</td>
<td>U.S. National Institute of Standards and Technology (NIST®)</td>
</tr>
<tr>
<td></td>
<td>Holmium oxide glass filter for checking the wavelength accuracy of a UV/Vis or UV/Vis/NIR spectrometer. The filter is mounted in precision aluminum spring mount and is provided in a robust aluminum container, with a Certificate of Calibration.</td>
<td></td>
</tr>
<tr>
<td>B2500099</td>
<td>Stray Light Solutions Reference Material Set</td>
<td>Instrument calibrated using NIST® 930D standards</td>
</tr>
<tr>
<td></td>
<td>Set comprising four solutions in sealed cuvettes – one reference solution and three test solutions. The cuvettes are designed for use with the standard 10 mm cuvette holders of most UV/Vis and UV/Vis/NIR spectrometers. The test solutions comprising sodium iodide, sodium nitrite and potassium chloride, which act as high-pass filters with very precise cut-off wavelengths. Potassium Chloride (KCL) for measuring stray at 200 nm, Sodium Iodide (NaI) for measuring stray light at 220 nm, and Sodium Nitrite (NaN02) for measuring stray light at 340 and 370 nm. The set is provided in a robust aluminum container, with a Certificate of Calibration.</td>
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</tbody>
</table>

Shewhart Charts

Shewhart Charts (or Means Control Charts) are a useful tool to trend data – either within a particular analytical batch (if there are a statistically significant number of samples) or over a time period. They are useful for trending both analytical data and the results from calibrations so that any change in instrument performance can be monitored and the result of any change in the measurement system (such as a lamp change) can be assessed. UV WinLab uses a database as the primary repository for data and so it is possible to query this database using a combination of search criteria such as user, date range, instrument, keywords in either the method or task (data) name in order to select data that fits the search criteria.

In the example shown, the deviation from the deuterium emission line at 656.1 nm is trended to show the mean deviation is approximately 0.016 nm (specification is 0.08 nm for this particular instrument – a LAMBDA 1050 UV/Vis/NIR spectrophotometer). Statistical limits (based on 2 and 3 standard deviations – $2\sigma$ and $3\sigma$) are also shown on the diagram.

![Figure 16. Shewhart chart showing deviation from deuterium emission line at 656.1 nm.](image)
## Standards Available from PerkinElmer, continued

<table>
<thead>
<tr>
<th>Part Number</th>
<th>Description</th>
<th>Traceability</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2500100</td>
<td><strong>Pharmacopoeia Reference Material Set</strong>&lt;br&gt;Set comprising six solutions in sealed cuvettes – two reference solutions and four test solutions to verify calibration in accordance with pharmacopoeia monographs:&lt;br&gt;- Ordinate (photometric) accuracy; potassium dichromate. Measured wavelengths are 235, 257, 313 and 350 nm&lt;br&gt;- Abscissa (wavelength) accuracy; holmium perchlorate&lt;br&gt;- Level of stray radiation; potassium chloride for measuring stray radiation at 200 nm&lt;br&gt;- Resolution; toluene in hexane&lt;br&gt;The cuvettes are designed for use with the standard 10 mm cuvette holders of most UV/Vis and UV/Vis/NIR spectrometers. The set is provided in two robust aluminum containers with Certificates of Calibration.</td>
<td>Instrument calibrated using NIST® 930D standards</td>
</tr>
<tr>
<td>PELA9057 1.25” dia.)</td>
<td><strong>Calibrated Spectralon® Diffuse Reflectance Standard</strong>&lt;br&gt;250 - 2500 nm, reported every 50 nm. CD-ROM provided with data every 1 nm.</td>
<td>U.S. National Institute of Standards and Technology (NIST®)</td>
</tr>
<tr>
<td>PELA9058 2” dia.)</td>
<td><strong>Spectralon® UV/Vis/NIR Diffuse Reflectance Reference Materials</strong>&lt;br&gt;Spectralon® diffuse reflectance reference material sets consist of a diffuse white reference material and a selection of three diffuse gray reference materials. Each reference material in the set is supplied with complete diffuse reflectance data from 250 nm to 2500 nm, and is mounted in an anodized aluminum frame. The set is packed in an airtight storage case.&lt;br&gt;- Durable, chemically inert&lt;br&gt;- Reflectance values – 99%, 75%, 50% and 2%&lt;br&gt;- Washable</td>
<td>U.S. National Institute of Standards and Technology (NIST®)</td>
</tr>
<tr>
<td>PELA9010 1.25” dia.)</td>
<td><strong>Spectralon® UV/Vis/NIR Diffuse Reflectance Reference Materials</strong>&lt;br&gt;Spectralon® diffuse reflectance reference material sets consist of a diffuse white reference material and a selection of seven diffuse gray reference materials. Each reference material in the set is supplied with complete diffuse reflectance data from 250 nm to 2500 nm, and is mounted in an anodized aluminum frame. The set is packed in an airtight storage case.&lt;br&gt;- Durable, chemically inert&lt;br&gt;- Reflectance values – 99%, 80%, 60%, 40%, 20%, 10%, 5% and 2%&lt;br&gt;- Washable</td>
<td>U.S. National Institute of Standards and Technology (NIST®)</td>
</tr>
<tr>
<td>PELA9018 1.25” dia.)</td>
<td><strong>Set of Four Color Standards (Red, Green, Blue, and Yellow)</strong></td>
<td>U.S. National Institute of Standards and Technology (NIST®)</td>
</tr>
<tr>
<td>PELA9019 2” dia.)</td>
<td><strong>Set of Four Color and Four Grey 2” Standards (Red, Green, Blue, Yellow, Plus Four Greys) (99%, 50%, 20%, 2%)</strong></td>
<td>U.S. National Institute of Standards and Technology (NIST®)</td>
</tr>
<tr>
<td>L6310204</td>
<td><strong>UV/Vis/NIR Second Surface Reference Mirror</strong>&lt;br&gt;Metal-dielectric multi-layer second surface reference mirror design optimized for stability and maximum reflectance in the UV/Vis/NIR range from 200 nm – 2,600 nm for checking the accuracy of absolute and to correct the spectra of non-absolute specular reflectance accessories.&lt;br&gt;- Easy to clean, protective 2 mm silica front plate&lt;br&gt;- Smooth reflectance curve without water related absorption bands in the NIR range&lt;br&gt;- Size 50 mm x 100 mm&lt;br&gt;- Calibration at 8 degrees incidence in the wavelength range 200 nm - 2,500 nm, calibration uncertainty typical &lt; 0.25%&lt;br&gt;Delivered in a lightweight protector case, accompanied by the calibration report and a CD-ROM with the calibration data in different Excel® formats.</td>
<td>Manufactured by OMT Solutions, Eindhoven, Netherlands This is not traceable to a national standard as it offers greater accuracy than any traceable material commonly available standard material.</td>
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</tbody>
</table>
Standards Available from PerkinElmer, continued

<table>
<thead>
<tr>
<th>Part Number</th>
<th>Description</th>
<th>Traceability</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1010504</td>
<td>Disposable Reference Mirror for Reflectance Spectroscopy</td>
<td>U.S. National Institute of Standards and Technology (NIST®)</td>
</tr>
</tbody>
</table>

This mirror is necessary to check the accuracy of absolute and to correct the spectra of non-absolute specular reflectance accessories. The mirror is a secondary reference material and is calibrated according to GLP regulations, wavelength range 250 to 2,500 nm.

Recalibration of standards

Most standards can be recalibrated. In some cases (e.g. aluminum mirror), the standard itself is an inexpensive material and also gets scratched over time and so it is not practical to have it recalibrated. It is, therefore, referred to as "disposable".

Some liquid standards can present issues with couriers/airlines when being sent back for recalibration due to the materials (albeit only a trace) that they contain.

Further reading


