

Qualification standard and method utilizing Raman activity of H₂O

BACKGROUND OF THE INVENTION

[0001] The disclosed invention relates to a method and a system for applying a primary reference standard for adjusting and/or calibrating high-sensitivity optical spectrometry instruments

5 **[0002]** The invention belongs to the technical fields of Protein Detection.

[0003] Optical spectroscopy equipment typically requires regular calibration and qualification during its production and throughout its service life. Calibration standards are commonly utilized for these purposes and can be nominally separated in this technological field into two categories:

10 **[0004]** First Y-axis, or intensity, calibrations with a calibration of optical peak intensities. And second X-axis, or wavenumber/wavelength, calibrations – calibration of peak locations

[0005] Intensity calibrations can be performed by measuring a broad spectrum reference material, e.g. SRM 2241, and fitting the resultant curve to a defined
15 polynomial curve of the spectrum; or by measurement of a defined standard with multiple peaks with measurement and fitting of relative peak ratios.

[0006] An example is the NIST Standard Reference Material (SRM) 2241, which comprises of optical glass with broadband luminescence spectrum. After illumination with a 785 nm line the resultant emission has a spectral shape that
20 can be defined by a polynomial fit.

[0007] Wavenumber calibrations on the other hand can be performed by measurement of a defined standard or solution with multiple peaks, and fitting of measured versus known peaks enable development of a correction table.

[0008] An example for this option is a 1:1 mixture of Toluene:Acetonitrile, a solution with known Raman peak locations.

[0009] These examples are typical for multiple types of spectrometer calibration and qualification, including Raman spectrometers. Figure 2 below provides visual
5 context for x and y-axis calibrations.

[0010] Calibration processes for fluorescence imaging microscopes can include measurement of independent optical detector paths for correction mapping of their alignment.

[0011] An example for that is the polystyrene fluorescent dye beads, which are
10 fluorescently stained on outer and inner portion of bead to enable focus through measurement and correction of detector and pixel locations for each detector path.

[0012] Other calibration processes for flow cytometry can include alignment verification, measurement of counting accuracy, and sorting efficiency. Examples
15 include an alignment approach where polystyrene microspheres are fluorescent stained and a counting approach where fluorescent microspheres are sold as calibrated suspension to be run through flow cytometer.

[0013] This leads to the task of the optical qualification and verification of the next generation instruments for Single Molecule Counting (SMC). The Raman
20 standards addressed above most closely relate to this application, and these standards can offer numerous advantages, like Broad spectrum standards with a well known intensity versus wavelength ratios, defined standards with narrow transitions and peaks which enable peak ratio calculation, numerous and well-defined peak locations which enable x-axis calibration and a consistent Raman
25 cross-sections and no traditional photobleaching, as opposed to fluorescently labeled standards.

[0014] The Raman standards are well-suited to their intended applications, but also have some severe disadvantages, for example the Raman standards are usually expensive (SRM 2241 >\$3500), the analysis require complex post-

processing and the standards are sensitive to preparation and require special sample fixturing during use.

[0015] Therefore such standards are not optimal for some high-sensitivity instruments such as SMC instrumentation which utilizes fixed fluorescent band
5 detection instead of a grating-based detection path.

[0016] This provides the task to develop an improved way of applying Raman standards for optical qualification and verification and if possible to provide a more inexpensive Raman standard itself.

10 BRIEF SUMMARY OF THE INVENTION

[0017] This task can be solved by a Method for applying a primary reference standard for adjusting and/or calibrating high-sensitivity optical spectrometry instruments via a computer, comprising the following steps of Using a high-purity H₂O solution as a primary standard stored in a container; Exciting the standard
15 using at least one excitation laser causing a Raman scattering; Collecting and measuring the Raman scattering, either in form of Stokes, Anti-Stokes, or both, from the standard using an instrument's detector; Applying a correction methodology for correction of variations in the Raman scattering caused by the container to increase accuracy of measurement; Measuring and integrating the
20 intensity over a fixed detection bandwidth of the spectrum of the Raman scattering to calculate the instrument optical efficiency; and adjustment and/or calibration of the high-sensitivity optical spectrometry instrument based on the calculated instrument optical efficiency. The use of a pure H₂O solution as the primary standard provides robustness and minimizes measurement errors and
25 handling issues associated with the prior art. The broad Raman spectra of the H₂O solution can be measured, and the signal is measured of the fixed detection band. The method's improved accuracy and robustness make it an alternative for measurement and potential calibration and/or adjustment of high-sensitivity optical spectrometry instruments.

[0018] Advantageous and therefore preferred further developments of this invention emerge from the associated sub claims and from the description and the associated drawings.

5 **[0019]** One of those preferred further developments of the disclosed method comprise that high-sensitivity optical spectrometry instruments are used for the detection of protein concentrations in a prepared assay solution. This highlights the method's application in protein concentration detection, emphasizing its suitability, sensitivity, and quantitative analysis capabilities in the context of prepared assay solutions.

10 **[0020]** Another one of those preferred further developments of the disclosed method comprise that the protein detection is done by a single molecule detection approach. The single molecule detection approach enhances the detection method's sensitivity, improves quantification capabilities, enables the detection of rare events, expands the dynamic range, and provides valuable
15 insights into protein behavior. This make single molecule detection a powerful tool for protein detection within the described method framework.

[0021] Another one of those preferred further developments of the disclosed method comprise that a fixed optical band pass is used to collect the Raman scattering from the H₂O solution. Utilizing a fixed optical band pass to collect and
20 filter the Raman scattering in the method results in an improved signal-to-noise ratio, increased specificity, reduced interference, improved instrument performance, and customizability. This collectively contributes to more accurate, specific, and reliable measurements in the high-sensitivity optical spectrometry instrument.

25 **[0022]** Another one of those preferred further developments of the disclosed method comprise that the measurement is corrected for variation in background from the container as standard holder. With the optical radiation from the container as the standard holder offers advantages such as simplified setup, cost-effectiveness, inherent alignment, reduced measurement errors, enhanced
30 stability, and compatibility with various container types. These advantages

collectively contribute to improved accuracy, reliability, and usability of the method in optical spectrometry instruments.

[0023] Another one of those preferred further developments of the disclosed method comprise use of a well plate container with bottom clear substrate for
5 reading of qualification standard in one or multiple wells of the well plate. Well plates are widely used in laboratory settings and offer a standardized format for sample handling. By using such a plate as the container, the method becomes compatible with existing laboratory workflows and equipment. In summary, utilizing a qualification well plate with a clear substrate as the container offers
10 advantages such as versatility, multi-sample capacity, and compatibility with automation. These advantages collectively enhance the method's efficiency, reliability, compatibility, and usability in laboratory settings.

[0024] Another one of those preferred further developments of the disclosed method comprise that the standard is pipetted into the well plate and surveyed by
15 scanning through the bottom clear substrate of the plate. This offers advantages such as precision in standard preparation, contamination prevention, ease of sample handling, enhanced scanning capabilities, flexibility in measurement techniques, and simplified experimental setup.

[0025] Another one of those preferred further developments of the disclosed
20 method comprise that the instrument optical efficiency is determined in form of parameters of alignment of detection and excitation paths, quantification of drift or optical loss, changes in excitation optical power and changes in detector efficiency. These parameters enable comprehensive assessment including: performance evaluation and benchmarking, measurement of stability and
25 reliability, excitation power optimization, and evaluation of detector efficiency. These advantages collectively enhance the ability to measure and ensure the instrument's performance, accuracy, and reliability in optical measurements, ensuring optimal efficiency and quality control.

[0026] Another one of those preferred further developments of the disclosed
30 system comprise that the fixed detection bandwidth in the spectrum of the Raman

scattering is between 670nm and 704nm. That offers advantages including targeted measurement, reduction of background noise, optimal sensitivity, minimization of spectral interference, compatibility with specific analytes, consistency in measurements, and simplified data analysis. These advantages collectively contribute to more accurate, selective, and reliable Raman scattering analysis within the specified wavelength range.

[0027] Another one of those preferred further developments of the disclosed system comprise that the correction methodology is applied by the computer, e.g. in form of internal hardware or external computing, by using a correction factor equation in form of $\text{MeasurementCorr} = \text{AvgH2O} - \text{Corr} \times \text{AvgEmpty}$, with MeasurementCorr being the corrected, background removed Raman integrated intensity, AvgH2O being the average integrated intensity from the measured Raman scattering of the standard filled container, AvgEmpty being the average integrated intensity from the empty container and CORR being a correction factor. Applying the correction methodology using the given correction factor equation offers advantages including robust background removal, increased accuracy of the Raman measurement, specificity in measurement, , reproducibility, and traceability. These advantages collectively contribute to improved measurement data quality and reliability in the analysis of Raman scattering measurements.

[0028] Another one of those preferred further developments of the disclosed system comprise that the correction factor is constant for a singular instrument design, but varies for different instrument designs, excitation bands and/or detection bands. By having a constant correction factor for a singular instrument design but allowing variations for different instruments, excitation bands, and detection bands this offers advantages such as instrument-specific correction, customization for different configurations, improved accuracy for specific bands, cross-instrument comparability, adaptability to instrument upgrades, validation and calibration processes, and improved data reproducibility. These advantages collectively contribute to accurate and reliable measurements, customized

correction methodologies, and enhanced comparability and reproducibility of data across different instrument configurations.

[0029] A further component of the claimed invention is a system for for applying a primary reference standard to adjust and/or calibrate high-sensitivity optical spectrometry instruments via a computer, configured to perform the following steps of: Using a high-purity H₂O solution as a primary standard stored in a container; Exciting the standard using at least one excitation laser; Collecting and measuring the Raman scattering, either in form of Stokes, Anti-Stokes, or both, from the standard using a detector instrument; Applying a correction methodology for correction of variations in the Raman scattering caused by the container to increase accuracy of measurement; Measuring and integrating the intensity over a fixed detection bandwidth of the spectrum of the Raman scattering to calculate the instrument optical efficiency; and Adjusting and/or calibrating of the high-sensitivity optical spectrometry instrument based on the calculated instrument optical efficiency

[0030] Another component of the disclosed invention is a Computer program comprising instructions which cause the involved computers to carry out the following method steps of Using a high-purity H₂O solution as a primary standard stored in a container; Exciting the standard using at least one excitation laser; Collecting and measuring the Raman scattering, either in form of Stokes, Anti-Stokes, or both, from the standard using the instrument's detector; Applying a correction methodology for correction of variations in the Raman scattering caused by the container to increase accuracy of measurement; Measuring and integrating the intensity over a fixed detection bandwidth of the spectrum of the Raman scattering to calculate the instrument optical efficiency; and possible adjustment and/or calibraiton of the high-sensitivity optical spectrometry instrument based on the calculated instrument optical efficiency. The program parts responsible for the single method steps are running on the respective computer parts. How the program itself is partitioned depends on the computer hardware being involved. It is possible to use a main software running on one of the mentioned computers or a separate computer which controls local client

programs. Other options include equal instances of the software who communicate with each other and so on.

[0031] Only requirement for this computer program to perform the whole method as described is, that the used program and its respective hardware components
5 are able to perform the method completely and automatically. Such a program can then be stored on a Computer-readable storage medium and/or data carrier signal which cause the involved computers to carry out the method steps of Using a high-purity H₂O solution as a primary standard stored in a container;
10 Exciting the standard using at least one excitation laser; Collecting and measuring the Raman scattering, either in form of Stokes, Anti-Stokes, or both, from the standard using a detector instrument; Applying a correction methodology for correction of variations in the Raman scattering caused by the container to increase accuracy of measurement; Measuring and integrating the intensity over a fixed detection bandwidth of the spectrum of the Raman scattering to calculate
15 the instrument optical efficiency; and Adjusting and/or calibrating of the high-sensitivity optical spectrometry instrument based on the calculated instrument optical efficiency. The storage medium can be stored on any suitable digital memory like an usb drive, a harddisk, a flashdrive and so on. From that memory it can also be provided via remota communication means using respective data
20 carrier signals, like ethernet, wired or wireless, or any other suitable network transmission means, for transmitting the software to its target hardware.

BRIEF DESCRIPTION OF SEVERAL VIEWS OF THE DRAWINGS

[0032] Figure 1: showing an example of the optical layout of an high-
25 sensitivity optical spectrometry instrument

[0033] Figure 2: showing an example qualification performance using a polystyrene spectrum.

[0034] Figure 3: showing a graphic showing approximate emission spectra that results form excitation of the pure H₂O reference used for this invention.

- [0035] Figure 4: showing the approximate Raman spectrum and a possible detection window for a channel
- [0036] Figure 5: showing the plot of real data displaying high correlation between SMC excitation and detection path failures and integrated and corrected Raman measurand from H₂O standard scanned in a well plate
- [0037] Figure 6: showing a well plate template for use with a SMC next generation instrument
- [0038] Figure 7: showing an illustration of importance of experimentally derived background correction and correction factor
- 10 [0039] Figure 8: showing a workflow diagram with the process to scan qualification plate for SMC next generation performance evaluation

DETAILED DESCRIPTION OF THE INVENTION

- [0040] The invention will be explained in more detail by presenting one preferred exemplary embodiment.
- [0041] The invented method can be applied for different high-sensitivity optical instruments, in particular SMC. An example of the optical path of a confocal detection instrument is shown in Figure 1.
- [0042] The inventions preferred embodiment is described in the follows and shows the use and methodology of H₂O as a primary reference standard for high-sensitivity optical spectrometry instrumentation. It comprises the following features:
- [0043] A high-purity H₂O solution is used as the primary standard, by way of measuring Raman scatter. This H₂O standard is inexpensive and widely available. The standard is pipetted into a sample holder such as a well plate, so that the standard can be measured using typical instrument operation. This is a significant advantage for instruments that measure liquid samples as the standard can utilize the same overall measurement hardware and processes.

- [0044]** The standard is then excited using the instrument excitation laser(s).
- [0045]** Raman scattering – either Stokes, Anti-Stokes, or both – is measured and collected using the instrument detector(s). The instrument collects the scatter by of of optical band pass filtering in the detection path(s).
- 5 **[0046]** A correction methodology for correction of variations in the process, e.g. non-H₂O related, is applied, especially optical radiation from standard holder, i.e. well plate substrate autofluorescence, to increase accuracy of measurement.
- [0047]** - Measurement of intensity is gathered over a fixed detection bandwidth of detector(s) and integrated for measurement of instrument optical efficiency.
- 10 **[0048]** - Measured intensity directly relates to instrument optical efficiency – and thus presents a robust measurement of instrument condition and performance: (1) alignment of detection and excitation paths; quantification of drift or optical loss; (2) changes in excitation optical power; and (3) changes in detector efficiency.
- 15 **[0049]** Figure 3 highlights now the Raman spectra of H₂O (left), and an image of the well plate utilized to scan this standard in the SMC process. The SMC next generation instrument samples approximately 600 to 1400 cm⁻¹ in the detection path. The H₂O standard is pipetted into well plate, and sample scanned from bottom in epi-fluorescence format.
- 20 **[0050]** Figure 4 helps to describe the methodology of the invented system calibration. A fixed band of the H₂O Raman spectrum is sampled, and the integrated scatter intensity is collected, integrated, and used as a measure of the instrument system optical collection efficiency.
- [0051]** It has to be noted that no specific intensity versus wavelength data is
- 25 required, only the integrated optical scatter intensity over the detection window. This integrated intensity can be reported as energy over a fixed time bin (e.g. Joules) or measured power – such as Watts or even photons/second. A relatively flat portion of the Raman scattering can be used and may be advisable for less sensitivity to filter band manufacturing variances.

[0052] A simplified formula (Equation 1) for the measured integrated intensity of the Raman scatter can be described as follows:

[0053]
$$I_{Raman} \propto I_{excitation} \times \sigma_{Raman} \times EFF_{collection}$$

[0054] Where I_{Raman} is the measured Raman integrated intensity; $I_{excitation}$ is the
5 excitation power of the system, in particular the laser power, σ_{Raman} is the Raman cross-section of H₂O, assumed fixed and invariant under consistent conditions; $EFF_{collection}$ is the collection efficiency of the system, which results from the Optical collection efficiency, the % transmission, numerical aperture of objective, filter band, and the Detector quantum efficiency, and the efficiency of optical
10 conversion to electrical signal.

[0055] Very important to this process is that the Raman cross-section of H₂O can be considered invariant and constant if the sample is kept at approximately consistent conditions (i.e. room temperature). This is pre-requisite for a consistent and trace-able measurement standard.

15 [0056] The measured Raman integrated intensity can be used to evaluate the performance of critical factors that describe common failure modes and therefore require period qualification. These are: First the excitation path, comprising of the intensity of the laser and the alignment or obstruction of the laser to the confocal volume; Second the detection path, comprising of the alignment or obstruction of
20 detection path and confocal volume and the detector for the quantum efficiency.

[0057] The resulting measurand has high linearity to excitation and detection path changes, i.e. drift, misalignment, or general failures. Figure 5 is comprised of real experimental data from the SMC next generation instrument and shows high linearity with near 1:1 sensitivity between the measurand and changes in the
25 excitation and detection path. Any path failures shown here were induced in a challenge study of the concept.

[0058] The application of any liquid standard requires a kind of container to hold the standard. This may offer advantages as many instruments in the biological application space work with liquid samples, and the H₂O solution can be
30 prepared and dispensed in the same sample holder as is normally used.

- [0059]** For implementation of the H₂O standard to the SMC instrument a so-called qualification well plate is used. The standard is thereby pipetted into this well plate and surveyed by scanning through the bottom clear substrate of the plate. While this application is convenient as it mirrors the preparation and scanning of an SMC assay, it does have a challenge: The liquid is scanned through the bottom clear substrate of the well plate which is unfortunately not invariant (consistent / constant) and can significantly reduce accuracy of the measurement due to the following problems:
- [0060]** a) The substrate has autofluorescence, resulting in a noticeable and changing background.
- [0061]** b) The background will add to the Raman measurement and be indistinguishable.
- [0062]** c) The autofluorescence is not typically consistent across a well plate, or between different well plates and well plate lots.
- [0063]** d) The autofluorescence can change over time, e.g. via photobleaching.
- [0064]** To fix this problem, two solutions can be applied to the preferred example:
- [0065]** 1. Simultaneous measurement of 'empty' well plate wells (substrate only) with the following steps:
- [0066]** a. Empty wells are measured near the H₂O sample wells to ensure similarity between material measurements and correction.
- [0067]** b. Multiple empty wells and H₂O sample wells are measured for statistical averaging.
- [0068]** c. Empty wells could be measured prior to filling with H₂O. However, this adds inconvenience and additional process steps..
- [0069]** 2. For the second solution, which is especially preferred for the working example a novel subtraction formula is used to correct for large changes in background with the following features.

[0070] a. This subtraction formula uses a correction factor.

[0071] b. The correction factor is determined empirically and compensates for differences between the empty well measurement and H₂O well measurement. Simply put, H₂O wells will have a slightly lower impact from plate substrate

5 background based on a variety of factors including Fresnel reflection differences (COP-to-Air versus COP-to-H₂O interface).

[0072] c. The correction factor can be considered constant for a singular instrument design, but can be optimized for different instrument designs, excitation bands, and/or detection bands.

10 [0073] d. The correction factor increases accuracy of the subtraction and is especially necessary when there are large changes in substrate background.

[0074] An image of a typical implementation of the qualification plate and correction factor approach for use with the SMC next generation instrument is shown in Figure 6. 12 wells are empty for background correction, and 12 wells
15 are filled with H₂O.

[0075] The correction factor equation (Equation 2) used for correction of plate substrate background is shown below.

$$[0076] \text{Measurement}_{\text{corr}} = \text{Avg}_{\text{H}_2\text{O}} - \text{CORR} \times \text{Avg}_{\text{Empty}}$$

[0077] Where Measurement_{corr} is the primary measurand of the qualification
20 process, with the corrected, background removed Raman integrated intensity; Avg_{H₂O} is the average integrated intensity from the H₂O sample wells, as a measure of the H₂O Raman signature; Avg_{Empty} is the average integrated intensity from the empty wells, as a measure of the plate substrate background and CORR is the correction factor with 0.85 being used for the SMC next
25 generation instrument.

[0078] Figure 7 illustrates the importance of the background correction and the modified correction factor discussed in Equation 2. When using the 0.85X correction factor (top), plate substrate changes via different plates, photobleaching of substrate fluorescence, and other factors is nearly eliminated

showing a flat Raman integral measurement over 4 months. When not using this 0.85X factor and performing a simple subtraction (middle), the slope trend increases dramatically by 33X. When no correction/subtraction is used (bottom), the trend increases still, showing the very large effect of background fluorescence variability on the measured result.

[0079] This type of sensitivity and correction may not be required for all spectroscopy instrumentation but is important for sensitive measurement systems such as the SMC system as shown in figure 1.

[0080] In the following a specific preferred mode of operation is shown for the preferred working example of the invention. It describes a general use of the invention to qualify the SMC next generation instrument from figure 1 in the field. A qualification plate is prepared by pipetting 12 wells of H2O solution into a well plate as shown in Figure 6. H2O and empty wells are scanned, and a background correction algorithm is used (see Equation 2) to calculate the background corrected, integrated Raman measurement. This measurand is then compared to acceptance limits to determine if the instrument is passing or failing optical performance.

[0081] A workflow of this process is shown in the Figure 8.

[0082] Typical Pass/Fail limits for the SMC next generation red laser scan are shown below. Note that for tighter testing specifications, such as when conducting an end-of-line test, multiple detectors may be surveyed, e.g. measurement and specification limits of Stokes and Anti-Stokes Raman intensity.

Red Laser	Red Detector (Avg TP/s)
Target – Corrected TP/s	Mean Corrected TP/s from build of the instrument
Pass/Fail - Corrected TP/s	+/- 12% of the target

[0083] This approach approach has numerous advantages of which the most important are as follows:

[0084] 1. Streamlined and inexpensive solution

[0085] The use of H₂O in solution as a Raman standard is a streamlined solution for high sensitivity optical spectrometry instrumentation. H₂O is a biocompatible liquid and can be prepared in a format similar to standard

5 application use for many instrument formats, such as by pipetting into a well plate. Highly pure H₂O is readily available and inexpensive as it is used for many applications including cell biology, buffer preparation, and chromatography.

[0086] For comparison – consider the given preferred working example using as a further example high-purity H₂O, EMD #W4502, to use of NIST SRM #2241 (a
10 common Raman intensity standard). In the SMC application, less than 1 mL of H₂O solution is required for each qualification plate costing less than \$0.10 bulk reagent per plate. The solution can be pipetted directly into a well plate for measurement using standard hardware and processes. SRM #2241 would require custom fixturing and measurement processes for many applications
15 including SMC, and typically costs several thousand dollars. Clearly H₂O can be disposed of after use, eliminating worries of contamination and storage whereas SRM #2241 would require careful storage and re-use, likely only as a high-level traceable standard.

[0087] 2. Robust solution with background correction

20 **[0088]** A typical confocal volume size of 10 cubic microns will contain approximately 335 billion H₂O molecules in a primarily aqueous solution. High purity, yet relatively inexpensive H₂O can offer less than part per billion trace impurities – yielding a highly averaged resultant measurement with a high fraction of H₂O to non-H₂O molecules surveyed. Along with H₂O's consistent Raman
25 cross-section (at room temperature), this provides a robust Raman integral intensity measurement and thus a robust and repeatable standard.

[0089] The disclosed background subtraction methodology greatly reduces the impact of the well plate substrate background in the process. The well plate substrate background is particularly significant as it can vary between plate lots

due to substrate chemistry and impurities and also experience photobleaching over time due to re-scanning. See Equation 2 and Figure 7 for reference.

[0090] 3. High linearity to optical efficiency, including excitation/detection path condition

5 **[0091]** As described in Equation 1 and shown graphically in Figure 5, the present invention has high sensitivity and linearity (near 1:1) to optical efficiency through the instrument. Detailed challenge experiments have been conducted showing that excitation and detection path failures in the instrument are easily discriminated in the measurand.

10 **[0092]** 4. Useful as end-of-line manufacturing tool, and service qualification tool

[0093] As previously described, this solution is readily deployed using standard procedures by pipetting H₂O into a well plate or any other standard holder and subsequent instrument scanning. The resulting measurement is both robust, due to the background subtraction as can be seen in Figure 7, and sensitive to major
15 instrument failure modes - see Figure 5. This provides a tool that has low false negatives and high confidence in evaluation of instrument optical efficiency and thus overall system performance.

[0094] The tool is robust, and easily rolled out as an 'end-of-line' test to guarantee instrument operation after it has been assembled and prior to stocking
20 for inventory. It is also valuable as a tool for continual measurement of instrument performance over its lifetime, by field personnel and/or scientist customers.

CLAIMS

1. A Method for applying a primary reference standard for adjusting
and/or calibrating high-sensitivity optical spectrometry instruments
via a computer, the following steps comprising:
- Using a high-purity H₂O solution as a primary standard stored
in a container.
 - Exciting the standard using at least one excitation laser
causing a Raman scattering.
 - Collecting and measuring the Raman scattering, either in form
of Stokes, Anti-Stokes, or both, from the standard using an
instrument's detector.
 - Applying a correction methodology for correction of variations
in the Raman scattering caused by the container to increase
accuracy of measurement.
 - Measuring and integrating the intensity over a fixed detection
bandwidth of the spectrum of the Raman scattering to
calculate the instrument optical efficiency.
 - Adjustment and/or calibration of the high-sensitivity optical
spectrometry instrument based on the calculated instrument
optical efficiency.
2. The Method according to claim 1, wherein
the high-sensitivity optical spectrometry instruments are used for the
detection of protein concentrations in a prepared assay solution.
3. The Method according to claim 2, wherein
the protein detection is done by a single molecule detection
approach.

4. The Method according to claim 1, wherein
a fixed optical band pass is used to collect the Raman scattering
from the H₂O solution.
5. The Method according to claim 1, wherein
the measurement is corrected for variation in background from the
container as standard holder.
6. The Method according to claim 1, wherein
a well plate with a bottom clear substrate is used as container.
7. The Method according to claim 6, wherein
the standard is pipetted into the well plate and surveyed by
scanning through the bottom clear substrate of the plate.
8. The Method according to claim 1, wherein
the instrument optical efficiency is determined in form of parameters
of alignment of detection and excitation paths, quantification of drift
or optical loss, changes in excitation optical power and changes in
detector efficiency.
9. The Method according to claim 1, wherein
the fixed detection bandwidth in the spectrum of the Raman
scattering is between 670nm and 704nm.
10. The Method of claim 1, wherein
the correction methodology is applied by the computer by using a
correction factor equation in form of:
$$\text{Measurement}_{\text{Corr}} = \text{Avg}_{\text{H}_2\text{O}} - \text{Corr} \times \text{Avg}_{\text{Empty}},$$

with $\text{Measurement}_{\text{Corr}}$ being the corrected, background removed
Raman integrated intensity, $\text{Avg}_{\text{H}_2\text{O}}$ being the average integrated

intensity from the measured Raman scattering of the standard filled container, Avg_{Empty} being the average integrated intensity from the empty container and CORR being a correction factor.

- 5 11. The Method of claim 10, wherein
the correction factor is constant for a singular instrument design, but
varies for different instrument designs, excitation bands, and/or
detection bands.
- 10 12. A System for applying a primary reference standard to adjust and/or
calibrate high-sensitivity optical spectrometry instruments via a computer,
configured to perform the following steps of:
- Using a high-purity H₂O solution as a primary standard stored in a
container.
 - 15 • Exciting the standard using at least one excitation laser.
 - Collecting and measuring the Raman scattering, either in form of
Stokes, Anti-Stokes, or both, from the standard using the
instrument's detector.
 - Applying a correction methodology for correction of variations in the
20 Raman scattering caused by the container to increase accuracy of
measurement.
 - Measuring and integrating the intensity over a fixed detection
bandwidth of the spectrum of the Raman scattering to calculate the
instrument optical efficiency.
 - 25 • Adjusting and/or calibrating of the high-sensitivity optical
spectrometry instrument based on the calculated instrument optical
efficiency.

13. A Computer program comprising instructions which cause the involved computers to control the respective configured hardware components to carry out the following method steps:

- 5 • Using a high-purity H₂O solution as a primary standard stored in a container.
- Exciting the standard using at least one excitation laser.
- Collecting and measuring the Raman scattering, either in form of Stokes, Anti-Stokes, or both, from the standard using the instrument's detector.
- 10 • Applying a correction methodology for correction of variations in the Raman scattering caused by the container to increase accuracy of measurement.
- Measuring and integrating the intensity over a fixed detection bandwidth of the spectrum of the Raman scattering to calculate the instrument optical efficiency.
- 15 • Potential adjustment and/or calibration of the high-sensitivity optical spectrometry instrument based on the calculated instrument optical efficiency.

14. A Computer-readable storage medium and/or data carrier signal having stored thereon the computer program of claim 13 which cause the involved computers to control the respective configured hardware components to carry out the following method steps:

- 25 • Using a high-purity H₂O solution as a primary standard stored in a container.
- Exciting the standard using at least one excitation laser.
- Collecting and measuring the Raman scattering, either in form of Stokes, Anti-Stokes, or both, from the standard using the instrument's detector.
- 30 • Applying a correction methodology for correction of variations in the Raman scattering caused by the container to increase accuracy of measurement.

- Measuring and integrating the intensity over a fixed detection bandwidth of the spectrum of the Raman scattering to calculate the instrument optical efficiency.
- Potential adjustment and/or calibration of the high-sensitivity optical spectrometry instrument based on the calculated instrument optical efficiency.

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