

## **NEGATIVE TUNE STANDARD FOR MASS SPECTROMETRY**

### **CROSS-REFERENCE TO RELATED APPLICATIONS**

[001] This application claims the benefit of U.S. Provisional Patent Application No. 63/511,843 filed July 3, 2023, the contents of which are incorporated herein by reference in their entirety.

### **FIELD OF THE INVENTION**

[002] The present disclosure relates to methods and compositions for assessing a mass spectrometer's linearity response and mass axis calibration when in negative ionization mode through the use of an oligonucleotide and novel compositions related thereto.

### **BACKGROUND OF THE INVENTION**

[003] Mass spectrometry (MS) is an important analytical technique utilized in many scientific disciplines. It measures the mass-to-charge ratio ( $m/z$ ) of one or more molecules present in a sample following an ionization process. The results of the measurements are usually presented as a spectrum, which plots the mass-to-charge ( $m/z$ ) ratio against the relative abundance of the molecule(s) of interest.

[004] The MS instrument comprises a detector configured for analyzing a sample by measuring the  $m/z$  ratio of ions received by the detector. The mass spectrometry results may be presented as a mass spectrum, a plot of intensity as a function of the mass-to-charge ratio in relation to molecular abundance, or a chromatographic representation of the summation of ions observed (i.e., a total ion chromatogram (TIC)). Mass spectrometry is useful for identifying and quantifying components of complex mixtures.

[005] Analysis by mass spectrometry typically begins with the samples being ionized by any number of means, including but not limited to, Matrix Assisted Laser Desorption Ionization (MALDI) or Electrospray Ionization (ES). In MALDI techniques, analytes are embedded in a solid, IR or UV-absorbent matrix. The matrix embedded analyte is placed in the ion source of a mass spectrometer. The matrix is vaporized by a short laser pulse and the analyte molecule is thereby transported into the gas phase in a non-fragmented state. The analyte is ionized by colliding and reacting with the matrix ions generated at the same time. A voltage is applied which accelerates the ions into a field-free flight tube. Due to their different masses, the ions in the ion source are accelerated to different speeds with the smaller ions reaching the detector earlier than the larger ions. The varying times of flight are converted into the different masses of the ions.

[006] Electrospray ionization allows for the ionization/vaporization of polar molecules. The sample is dissolved in a solvent and then pumped through a thin capillary which is raised to a high potential. Small charged droplets are sprayed from the ES capillary into a bath gas at atmospheric pressure and travel down a pressure and potential gradient towards an orifice in a mass-spectrometer high-vacuum system. As the droplets traverse this path, they break into smaller droplets until either an ion desorbs from the droplet or the solvent is removed.

[007] Ionization can also produce unwanted adducts of the analytes which complicate the quality and resolution of the MS spectrum.

[008] Ionization may create positive ions (cations) or negative ions (anions), which can then be introduced to the MS instrument. Mass spectrometry instruments are often utilized with a cation sample, which is commonly referred to as “positive mode.” Alternatively, mass spectrometry

instruments may be utilized with an anion sample, which is referred to in this disclosure as “negative ionization mode” or “negative mode.”

**[009]** To ensure accuracy and reliability, MS instruments need to be qualified. Qualification is a scheduled process to determine whether the MS instrument elicits reproducible relative abundance results that follow a linear standard as the concentration or volume of an analyte is increased. Qualification is the formal verification of accuracy, linearity, and other system attributes performed through instrument calibration using a pre-defined standard as intended by the vendor. Installation Qualification (IQ) is generally required for new or pre-owned instruments, and it is usually based on factory specifications and follows manufacturer guidelines. Operational Qualification (OQ) verifies and records a MS instrument’s ability to meet the specified operational criteria after an installation, following repetitive use or after major service intervention. Performance Qualification (PQ) is a customer or instrument supplier defined performance protocol that is run on a system configuration that is representative of its regular operational state (ie (U)HPLC/MS). PQ is usually run after any service intervention.

**[0010]** Linearity for an MS instrument refers to whether a linear relationship exists between the amount of an analyte and the resulting MS signal(s) from that analyte. Linearity can be demonstrated by introducing a standard in a plurality of concentrations or volumes and evaluating the MS signals(s) produced by the standard relative to signal(s) known or expected from that standard. A perfectly linear result is not necessarily a requisite for a successful qualification. In some instances, the degree of linearity for successful qualification is defined by the intended use for the MS instrument. Non-linear regression analysis (binomial, exponential, polynomial,

logarithmic, exponential, etc...) of results may also be used if sample concentration to instrument response can be consistently assessed.

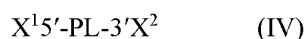
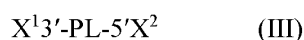
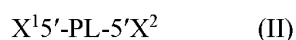
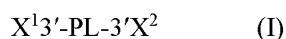
**[0011]** Additionally, MS instruments must regularly have their mass axis calibrated to ensure accuracy and reliability of the  $m/z$  plot or spectrum. Mass axis calibration is a scheduled process to determine whether compounds of a given mass to charge are being appropriately assigned to the corresponding peaks in the MS spectrum. This process consists of analyzing a standard with one or more molecules of a known mass and ionization state on the MS instrument and ensuring that the mass peak or peaks match the  $m/z$  of known or expected  $m/z$  for that standard. If the resulting mass spectrum matches the known standard, no further maintenance is necessary. But if the resulting mass spectrum does not align with the calibration curve or expected  $m/z$  values are not within acceptable tolerances, further maintenance may be needed. Mass axis calibration can occur prior to an analysis, or the mass axis calibration can be dynamically adjusted based on the observed changes in a standard's  $m/z$  that is present throughout an analysis.

**[0012]** Prior approaches to qualifying a MS instrument in negative mode have been adversely impacted by compounds used for the analysis of oligonucleotides including 1,1,1,3,3,3-hexafluoro-2-propanol ("HFIP"), triethylamine ("TEA"), hexylamine, dibutylamine, and dihexylamine. The residual ion pair reagents remaining on the internal optics and flow paths of MS instruments oversaturate the detector making standards currently on the market unusable for assessing accuracy and linearity.

**[0013]** There is a need for improved methods and standards for performing a qualification of the negative mode of MS instruments by assessing the systems response calibration, especially those used to analyze oligonucleotides with ion pair reagents.

## SUMMARY OF THE INVENTION

[0014] The present disclosure provides novel methods and standards for evaluating the performance of an MS instrument or system. A standard used for evaluation comprises one or more oligonucleotides having a structure of Formula I, Formula II, Formula III, or Formula IV



wherein PL is a phosphate linkage PL is a phosphate linkage comprising  $\text{-PO}_4\text{-}$ ,  $\text{-PO}_3\text{-O-PO}_3\text{-}$ ,  $\text{-PO}_3\text{S-}$ ,  $\text{-PO}_3\text{BH}_2\text{-}$  or  $\text{-PO}_3\text{-O-PO}_2\text{-O-PO}_3\text{-}$ ; and  $X^1$  and  $X^2$  are independently a nucleoside, a nucleotide, or an oligonucleotide. In some embodiments, one or more (or all) of the oligonucleotides of the standard ranges from 2 to 100 nucleobases in length. The methods and use of the standard can include evaluating one or more mass spectrometry signals from the MS instrument, wherein the evaluating comprises identifying one or more mass peaks corresponding to the standard, and comparing the identified mass peaks to one or more known or expected mass peaks for the standard. The MS instrument has a negative ionization mode, and wherein the standard is introduced as the sample for a negative mode mass spectrometry analysis. The present methods can also comprise performing qualification of the mass spectrometer instrument's negative mode.

[0015] The present disclosure also provides a novel method for assessing an MS instrument's mass axis calibration via standardization or dynamically adjusting to the difference of experimentally measured  $m/z$ 's vs theoretical  $m/z$ 's during an analysis.

### BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG. 1 is a schematic view of an example of a mass spectrometry (MS) instrument.

[0017] FIG. 2 shows an HPLC chromatogram from an oligonucleotide product synthesized in Example 1.

[0018] FIG. 3 shows a linearity curve obtained from MS analysis of an oligonucleotide over a range of amounts.

[0019] FIG. 4 shows a linearity curve obtained from MS analysis of an oligonucleotide over another range of amounts.

[0020] FIGs. 5 to 7 shows linearity curves obtained from MS analysis of an oligonucleotide having several fragment ions.

### DEFINED TERMINOLOGY

[0021] It is to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present teachings will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those working in the fields to which this disclosure pertain. The defined terms are in addition to the technical and scientific meanings of the defined terms as commonly understood and accepted in the technical field of the present teachings.

[0022] All patents and publications referred to herein are expressly incorporated by reference.

[0023] As used herein, the terms “a,” “an,” and “the” include both singular and plural referents, unless the context clearly dictates otherwise. Thus, for example, “a compound” includes one compound and plural compounds. The terms “first” and “second” are terms to distinguish different

elements, not terms supplying a numerical limit, and a device having first and second element can also include a third, a fourth, a fifth, and so on, unless otherwise indicated.

**[0024]** A “nucleotide” refers to a sub-unit of an oligonucleotide (whether DNA or RNA or analogue thereof) which includes a phosphate group, a sugar group and a heterocyclic base, as well as analogs of such sub-units. Other groups (e.g., protecting groups) can be attached to any component(s) of a nucleotide. A “nucleoside” or “nucleoside moiety” referred to an oligonucleotide subunit including a sugar group and a heterocyclic base, as well as analogs of such sub-units.

**[0025]** The terms “nucleoside” and “nucleotide” are intended to include those moieties that contain not only the known purine and pyrimidine bases, e.g. adenine (A), thymine (T), cytosine (C), guanine (G), or uracil (U), but also other heterocyclic bases that have been modified. The pyrimidine, purine, heterocyclic base and modified heterocyclic bases are termed herein “nucleobases”. Such modifications include methylated purines or pyrimidines, acylated purines or pyrimidines, alkylated heterocycles or other heterocycles. Such modifications include, by way of examples, diaminopurine and its derivatives, inosine and its derivatives, alkylated purines or pyrimidines, acylated purines or pyrimidines, thiolated purines or pyrimidines, and the like, or the addition of a protecting group such as acetyl, difluoroacetyl, trifluoroacetyl, isobutyryl, benzoyl, 9-fluorenylmethoxycarbonyl, phenoxyacetyl, dimethylformamidine, dibutylformamidine, N,N-diphenyl carbamate, or the like. In addition, the terms “nucleoside” and “nucleotide” include those moieties that contain not only conventional ribose and deoxyribose sugars, but other sugars as well. Modified nucleosides or nucleotides also include modifications on the sugar moiety, e.g., wherein one or more of the hydroxyl groups are replaced with halogen atoms or aliphatic groups,

or are functionalized as ethers, amines, or the like. Modified nucleosides or nucleotides also include modifications on the internucleotide linkage or backbone moiety. "Analogues" refer to molecules having structural features that are recognized in the literature as being mimetics, derivatives, having analogous structures, or other like terms, and include, for example, polynucleotides incorporating non-natural (not usually occurring in nature) nucleotides, unnatural nucleotide mimetics such as 2'-modified nucleosides (for example 2'-MOE, 2'-OMe, 2'-F, 2'-cEt, LNA, UNA and the like), peptide oligonucleotides, oligomeric nucleoside phosphonates, phosphorothioates, and any polynucleotide that has added substituent groups, such as protecting groups or linking groups.

**[0026]** An "oligonucleotide" refers to a compound containing a plurality of nucleoside moiety subunits that are linked by internucleotide linkages. As such, the term also refers to a compound containing a plurality of nucleotide moiety subunits or residues. An oligonucleotide might contain ribonucleosides, or deoxyribonucleosides or a mixture thereof. An oligonucleotide may comprise natural and/or non-natural nucleosides, nucleoside analogs and modified nucleosides and may comprise natural (phosphate diester) and/or non-natural internucleotides linkages (e.g. phosphorothioate, phosphonate, boranophosphonate, phosphoramidate, amido linkage such as peptide nucleic acid (PNA)).

**[0027]** In some embodiments, the oligonucleotide can be referred to according to the naming convention of [NUMBER]-mer. Where the [NUMBER] specifies the number of nucleosides linked together by phosphate linkages. For example, a "2-mer" or dimer refers to a compound having two nucleosides linked together by a single phosphate linkage. A "5-mer" refers to five nucleosides linked together by four phosphate linkages.

[0028] The term "connected" means that two or more components are fluidically connected, or physically connected, or both. The term "fluidically connected" means that two components are in fluid communication and includes direct connections between the two components as well as indirect connections where one or more other components are in the flow path between the two components. For example, a first component and a second component are fluidically connected if an outlet from the first component is physically connected to an inlet of the second component, or if a conduit connects the first and second components, or if one or more intervening components, such as a valve, a pump, or other structure, is between the two components as fluid flows from the first component to the second component, or vice versa.

[0029] "Mass peaks" or "mass spectrometry signals" as used herein refer to the peaks shown in the resulting spectrum or chromatographic peak (e.g. Extracted Ion Chromatogram (EIC) or Total Ion Chromatogram (TIC)) when a sample is analyzed by an MS instrument.

[0030] "Introducing" as used herein means injecting, flowing, infusing, charging, or any other suitable way of adding a sample to an MS instrument.

[0031] It is contemplated that methods and materials similar or equivalent to those described herein can also be used in the practice of the present teachings. Some exemplary methods and materials are now described.

#### DETAILED DESCRIPTION

[0032] The present methods and compositions are directed to evaluating performance of an MS instrument or system. In some embodiments, this evaluation may comprise or be part of a

qualification of the MS instrument. In some embodiments, this evaluation may include calibrating the mass axis of the mass spectrometer.

**[0033]** The present methods and standard compositions are for a mass spectrometry (MS) instrument. The MS instrument may comprise a detector configured for analyzing a sample by measuring the mass-to-charge ( $m/z$ ) ratio of ions received by the detector. The result may be presented as a mass spectrum, a plot of intensity as a function of the mass-to-charge ratio. Mass spectrometry is useful for distinguishing components of complex mixtures. The MS instrument may be any device configured for separating, sorting or filtering analyte ions on the basis of their respective masses (e.g., mass-to-charge ratios, or  $m/z$  ratios). Examples of MS instruments include, but are not limited to, multipole electrode structures (e.g., mass filters, ion traps), time-of-flight (TOF) analyzers, single quadrupole (SQ) LC/MSD, a triple quadrupole (TQ or QQQ) LC/MS, a quadrupole time of flight (TOF/Q-TOF), electrostatic analyzers (ESAs), and magnetic sectors.

**[0034]** The MS system 100 may generally include, in order of ion process flow, an ion source 104, a mass analyzer 108 downstream from the ion source 104, and an ion detector 112 positioned to receive ions from the mass analyzer 108. From the perspective of FIG. 1, the MS system 100 defines a flow path for ions and gas molecules successively through the foregoing devices generally in the direction from left to right, as depicted by horizontal arrows. The MS system 100 also includes a vacuum system (not shown) for maintaining various interior regions of the MS system 100 at controlled, sub-atmospheric pressure levels. The vacuum system may include vacuum lines communicating with the various interior regions via vacuum ports or exhaust ports, one or more vacuum-generating pumps, and associated components. The vacuum lines may also remove non-analytical neutral molecules from the ion path of the MS system 100. For simplicity,

additional ion processing devices, ion optics, electronics, and other hardware that may be included in the MS system 100 are not shown. For example, in some embodiments the MS system 100 may include an ion mobility analyzing stage.

**[0035]** The ion source 104 may be any type of continuous-beam or pulsed ion source suitable for producing analyte ions for spectrometry, as appreciated by persons skilled in the art. Depending on the type of ionization implemented, the ion source 104 may operate at vacuum, or at or near atmospheric pressure. Examples of ion sources include, but are not limited to, electron ionization (EI) sources, chemical ionization (CI) sources, photo-ionization (PI) sources, electrospray ionization (ESI) sources, atmospheric pressure chemical ionization (APCI) sources, atmospheric pressure photo-ionization (APPI) sources, field ionization (FI) sources, plasma or corona discharge sources, laser desorption ionization (LDI) sources, and matrix-assisted laser desorption ionization (MALDI) sources. The sample to be analyzed may be introduced to the ion source 104 by any suitable means, including hyphenated techniques in which the sample material is an output 116 from an analytical separation instrument such as, for example, a gas chromatography (GC), sample or standard infusion pump, or liquid chromatography (LC) instrument (as discussed in more detail below). Additionally, the sample may be plumbed or pumped directly to the ion source of a mass spectrometer without need of any other analytical instrumentation.

**[0036]** In some embodiments of the present disclosure, at least one mass analyzer (e.g., the mass analyzer 108) is based on a quadrupole mass filter or linear ion trap, as described further below. The ion detector 112 may be any device configured for collecting and measuring the flux (or current) of mass-discriminated ions outputted from the mass analyzer 108. Examples of ion

detectors include, but are not limited to, multi-channel detectors (e.g., micro-channel plate (MCP) detectors), electron multipliers, photomultipliers, image current detectors, and Faraday cups.

**[0037]** In general operation, a sample is introduced into the ion source 104, and the ion source 104 produces ions from ionizable compounds in the sample and transmits the ions to the mass analyzer 108. The mass analyzer 108 selectively transmits the ions to the ion detector 112 on the basis of mass-to-charge ( $m/z$ ) ratio. The mechanism of mass selection or filtering depends on the type of mass analyzer. The ion detector 112 receives the ions and produces ion measurement signals, from which a mass spectrum of the sample is constructed.

**[0038]** As also illustrated in FIG. 1, in some embodiments the MS system 100 may be configured for implementing tandem MS (MS/MS). For example, the MS system 100 may be configured as a QqQ, qTOF, or QqTOF, TQTOF instrument. Thus, the MS system 100 may include a first mass analyzer 120 upstream of the mass analyzer 108 (the second, or final, mass analyzer in such embodiments), and an ion fragmentation device such as a collision cell 124 between the first mass analyzer 120 and the second mass analyzer 108. The first mass analyzer 120 is configured for selecting precursor ions of a specific  $m/z$  ratio or  $m/z$  ratio range and is typically, but not necessarily, configured as a quadrupole mass filter. The collision cell 124 typically includes a non-mass-resolving, RF-only ion guide enclosed in a cell. The cell is pressurized by an inert gas to a level sufficient for producing fragment ions from the precursor ions by collision-induced dissociation (CID) as appreciated by persons skilled in the art. However, a fragmentation device other than a CID-based device may be utilized such as, for example, a device configured for implementing electron capture dissociation (ECD), electron transfer dissociation (ETD), or infrared multiphoton dissociation (IRMPD). The second mass analyzer 108

then resolves the fragment ions on the basis of mass ( $m/z$  ratio) and transmits the mass-resolved fragment ions to the ion detector 112, which outputs measurement signals from which mass spectra are then produced. In tandem MS embodiments, both the first mass analyzer 120 and the second mass analyzer 108 may be based on a linear quadrupole electrode structure. Alternatively, the second mass analyzer 108 may be another type of mass analyzer such as, for example, a three-dimensional Paul trap, a time-of-flight (TOF) analyzer, an electrostatic trap, a static electric and/or magnetic field sector instrument, or an ion cyclotron resonance (ICR) cell (FT-ICR or FTMS).

**[0039]** As further illustrated in FIG. 1, one or more of the ion processing devices (e.g., first mass analyzer 120, collision cell 124, second mass analyzer 108, or other ion guides not specifically shown) may be preceded or succeeded by ion optics 128, 132, 136, and 140. The ion optics 128, 132, 136, and 140 may include various types of lens elements such as, for example, aperture lenses (ring electrodes centered on-axis, plate electrodes with apertures centered on-axis, split-plate or split-cylinder electrodes with open slots or gaps centered on-axis), parallel plate electrodes, multipole rod electrodes, etc. Any of the ion optics 128, 132, 136, and 140 may be or include a field terminator as described herein.

**[0040]** The MS system 100 may also include a computing device (or system controller) 144. The computing device 144 is schematically depicted as representing one or more modules (or units, or components) configured for controlling, monitoring and/or timing various functional aspects of the MS system 100 described above. One or more modules of the computing device 144 may be, or be embodied in, for example, a desktop computer, laptop computer, portable computer, tablet computer, handheld computer, mobile computing device, personal digital assistant (PDA), smartphone, etc. The computing device 144 may also schematically represent all voltage sources

not specifically shown, as well as timing controllers, clocks, frequency/waveform generators and the like as needed for applying voltages to various components of the MS system 100. The computing device 144 may also be configured for receiving the ion detection signals from the ion detector 112 and performing tasks relating to data acquisition and signal analysis as necessary to generate chromatograms, drift spectra, and mass ( $m/z$  ratio) spectra characterizing the sample under analysis. The computing device 144 may also be configured for providing and controlling a user interface that provides screen displays of spectrometric data and other data with which a user may interact. The computing device 144 may include one or more reading devices on or in which a tangible computer-readable (machine-readable) medium may be loaded that includes instructions for performing all or part of any of the methods disclosed herein. For all such purposes, the computing device 144 may be in signal communication with various components of the MS system 100 via wired or wireless communication links (as partially represented, for example, by a dashed line between the computing device 144 and the ion detector 112). Also for these purposes, the computing device 144 may include one or more types of hardware, firmware and/or software, as well as one or more processors, memories, and databases.

**[0041]** As noted above, the MS system 100 may include at least one quadrupole ion guide assembly that includes a quadrupole ion guide and at least one field terminator. The quadrupole ion guide may be any of the ion processing devices described above that is configured as a linear quadrupole electrode structure, and which is preceded and/or succeeded by a field terminator. As one non-limiting example, a quadrupole ion guide assembly may include the first mass analyzer 120 (e.g., a mass filter) and a preceding field terminator (pre-filter) schematically depicted as ion

optics 128 and/or a succeeding field terminator (post-filter) schematically depicted as ion optics 132.

**[0042]** The MS instrument may comprise, or be part of a system that comprises, more than one mass analyzer, particularly when ion fragmentation is desired. As an example, the mass analyzer may include a mass filter followed by a collision cell or other ion fragmentation device, which in turn is followed by another mass filter or analyzer.

**[0043]** The MS instrument may be part of an analytical system that comprises other instruments, such as chromatography instruments. The MS instruments can be configured for direct injection mass spectrometry, liquid chromatography/mass spectrometry, gas chromatography/mass spectrometry, ion mobility/mass spectrometry, supercritical fluid chromatography/mass spectrometry, or any combination thereof. In some embodiments, the MS analysis is by liquid chromatography/mass spectrometry (LC/MS), or by ultra-high pressure liquid chromatography/mass spectrometry (UHPLC/MS).

**[0044]** In some embodiments, the MS is configured with only a sample pump feeding into the MS system's source or being co-delivered with the effluent of a (U)HPLC.

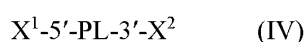
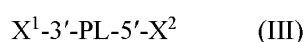
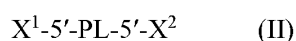
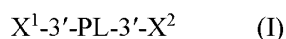
**[0045]** In some embodiments, the present methods and systems also comprise a liquid chromatography instrument fluidically connected to the MS instrument. Liquid chromatography includes, but is not limited to, normal phase liquid chromatography (NPLC), hydrophilic interaction liquid chromatography (HILIC), reversed phase liquid chromatography (RPLC), including ion-pairing reversed phase liquid chromatography (IP-RPLC), high performance liquid chromatography (HPLC), high turbulence liquid chromatography (HTLC) and ultra performance liquid chromatography (UPLC).

[0046] In some embodiments, the liquid chromatography comprises hydrophilic interaction liquid chromatography (HILIC). HILIC can be used to separate small polar compounds on polar stationary phases. HILIC uses hydrophilic stationary phases with reversed-phase type eluents, and elutes analytes in order of increasing polarity. Suitable mobile phases for HILIC include acetonitrile (ACN) and other aprotic solvents miscible with water. Ionic additives, such as ammonium salts, can be used to modulate mobile phase pH and ion strength.

[0047] In some embodiments, the liquid chromatography comprises ion-pairing reversed-phase liquid chromatography (IP-RPLC). IP-RPLC is a technique for separation of organic ions and partly ionized organic analytes. IP-RPLC uses the same types of stationary and mobile phases as RPLC, with the addition of an ion pair reagent to the mobile phase. The ion pair reagent can be, for example, alkylsulfonate, an alkylsulfate or an alkylammonium salt, and it can change the retention time of ionic analytes. In some embodiments, the mobile phase buffer comprises hexafluoroisopropanol (HFIP) or triethylamine (TEA).

[0048] The present methods can include introducing one or more samples to the MS instrument, simultaneously with the standard or separately (before or after) the standard. In some embodiments, a standard is introduced to the MS instrument simultaneously with a sample of interest, and the known mass of the standard is used to dynamically or continuously correct or adjust the MS instrument's  $m/z$  calibration. In some embodiments, the methods comprise mixing a standard and a sample to form a mixture. The mixing can be performed before introducing the mixture to the MS instrument, or the mixing can be performed within the MS instrument by combining the delivery of the separate components in a reservoir, or by combining the flow of each component through the use of a T-junction.

[0049] The present methods employ a standard suitable for introduction to a mass spectrometer and comprising one or more oligonucleotides. Examples of oligonucleotides include compounds according to Formulas I, II, III, or IV:



wherein PL is a phosphate linkage. The phosphate linkage can comprise  $-PO_4-$ ,  $-PO_3S-$ ,  $-PO_3BH_2-$ ,  $-PO_3-O-PO_3-$ , or  $-PO_3-O-PO_2-O-PO_3-$ .  $X^1$  and  $X^2$  can be a nucleoside, a nucleotide, or an oligonucleotide.  $X^1$  and  $X^2$  can be the same (such as deoxythymidine) or different (such as a deoxythymidine and a deoxycytidine). In Formula I,  $X^1-3'-$  indicates that the  $3'O-$  of the  $X^1$  sugar group is attached to the phosphate linkage. Put another way, the  $3'O-$  groups in  $X^1-3'-$  and  $-3'-X^2$  are part of the  $-PO_4-$  group between  $X^1$  and  $X^2$ . Likewise, the  $-3'-X^2$  indicates that the  $3'O-$  of the  $X^2$  sugar group is attached to the phosphate linkage. In Formula II,  $X^1-5'-$  indicates that the  $5'O-$  of the  $X^1$  sugar group is attached to the phosphate linkage, and the  $-5'-X^2$  indicates that the  $5'O-$  of the  $X^2$  sugar group is attached to the phosphate linkage. Similar respective linkages can be found in Formulas III and IV.

[0050] In some embodiments of the present methods, the oligonucleotide of the standard is in solution, or it is solubilized from a solid. Any of the oligonucleotides in the standard can be from 2 to 100 nucleobases in length. In some embodiments, one or more (or all) of the oligonucleotides is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length. In some embodiments, one or more (or all) of the oligonucleotides is

25-30, 30-35, 35-40, 40-45, 45-50, 50-55, 55-60, 60-65, 65-70, 70-75, 75-80, 80-85, 85-90, 90-95, 95-100 nucleotides in length.

**[0051]** In some embodiments, an oligonucleotide in the standard comprises MOE, 2'fluoro, 2'OMe, 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3 -methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5 -propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, 0(6)-methylguanine, 2-thiocytidine, methylated bases, intercalated bases, a nucleotide comprising a modified sugar e.g., Locked Nucleic Acid (LNA), Unlocked Nucleic Acid (UNA), 2',4'-Constrained 2'-O-Ethyl c(Et), 2',4'-Constrained 2'-O-Methoxyethyl (cMOE) 2'-fluororibose (2'-F), 2'-O-methyl (2'-OMe), 2'-O-methoxyethyl (2'-O-MOE), ribose, 2'-deoxyribose, arabinose, and hexose, or any combination thereof.

**[0052]** In some embodiments, one or more oligonucleotides in the standard further comprises an alkyl spacer, such as dodecyl, undecyl, decyl, nonyl, octyl, heptyl, hexyl, pentyl, butyl, propyl, ethyl, and methyl. In some embodiments, an oligonucleotide in the standard comprises a GalNAc or PEG residue. In some embodiments, an oligonucleotide in the standard comprises a 2', 3' and/or 5' synthesis protecting group, such as tert-butyldimethylsilyl (TBDMS), methoxymethyl ether (MOM), tri-iso-propylsilyloxymethyl (TOM), methoxyethoxymethyl ether (MEM), dimethoxytrityl (DMT), p-Methoxybenzyl ether (PMB), methylthiomethyl ether, Pivaloyl, ether, silyl ether, methyl ester, thiomorpholine-4-carboxylate (TC), bis(2-acetoxyethyl-oxy)methyl (ACE), and trityl. In some embodiments, one or more of the oligonucleotides in the standard comprises a nucleobase synthesis protecting group (for example, an acetyl, isobutyl, t-

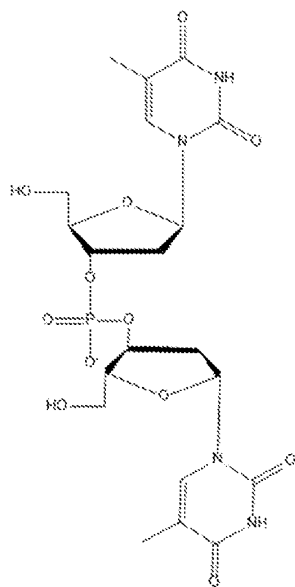
butyl phenoxyacetyl (TAC), dimethylformamidine (dmf), or benzoyl group). In some embodiments, one or more of the oligonucleotides in the standard comprises a phosphate having a cyanoethyl (CNET) protecting group.

**[0053]** As an aspect of the present disclosure, a standard (which may also be called a standard composition) is provided for mass spectrometry analysis, comprising a known amount of at least one oligonucleotide in a solvent that can solubilize the compounds, and any other components that can be present in the composition. In some embodiments, the oligonucleotide can be used to calibrate a mass spec operating in positive or negative ionization mode. In some embodiments, the oligonucleotide can be used in calibrating a mass spectrometer with an atmospheric pressure chemical ionization (APCI) mass spectrometer or a mass spectrometer with an electrospray (ESI) ion source. In some embodiments, an oligonucleotide can be selected to enable calibration across a broad mass range and can provide reference mass peaks in both positive and negative ionization modes. In various aspects, substantially the same components can be used for both APCI and electrospray mass spectrometry. To provide calibration across a broad mass range, the oligonucleotide can comprise a mixture of different discrete oligonucleotides in accordance with some embodiments of the disclosure. In embodiments, the calibration provides for linear or substantially linear mass calibration over the entire mass range being analyzed.

**[0054]** Tuning is the process of adjusting parameters to optimize mass accuracy, resolution, and monitor signal abundance (sensitivity) of tune ions. Calibration is the process of assigning accurate masses based on the known masses of standard compounds.

**[0055]** The compositions, kits, and methods described herein provide various advantages over other compounds and methods that have been used to evaluate MS instrument performance. For example, in accordance with some embodiments of the present disclosure, the oligonucleotides provided herein can provide for better accuracy, sensitivity, and consistency. The oligonucleotides disclosed herein, and the compositions, kits, and methods comprising them provide for improved ionization, mass signal and sensitivity in the negative mode of MS analysis.

**[0056]** In some embodiments, the standard comprises an oligonucleotide having the structure:



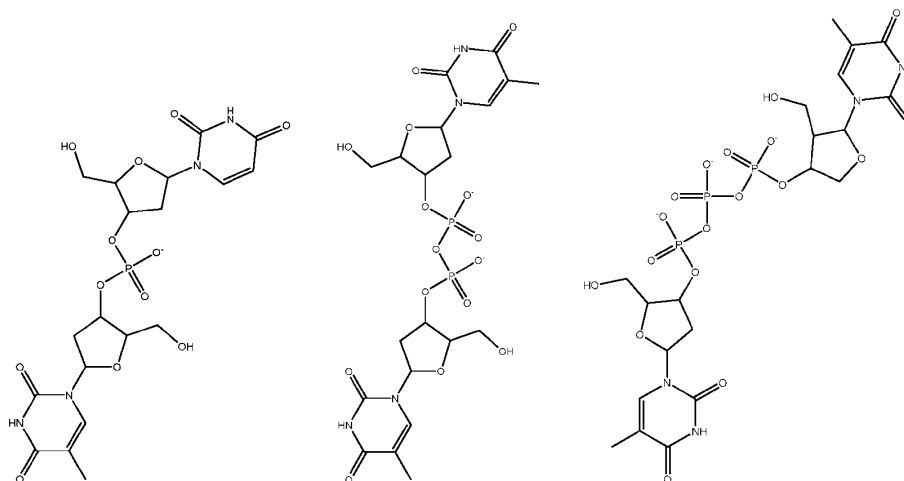
deoxythymidine-3'-PL-3'-deoxythymidine

**[0057]** The present inventors have found that such compounds are especially suited for use as a standard for an MS instruments in negative ionization mode. Without intending to be bound by theory, such compounds are especially suited because the single central phosphate molecule

produces a particularly distinct peak in the resulting spectrum from a mass spectrometry analysis. Moreover, the thymine nucleobases do not require further processing prior to introducing the standard composition to the MS instrument. Similar oligonucleotides that include other nucleobases may be utilized, but some of these may require further processing, such as nucleobase deprotection, prior to introduction due to protecting groups on the other nucleobases.

**[0058]** Examples of oligonucleotides include, but are not limited to deoxythymidine-3'-PL-3'-deoxythymidine, deoxythymidine-5'-PL-5'-deoxythymidine, deoxyuridine-3'-PL-3'-deoxyuridine, deoxyuridine-5'-PL-5'-deoxyuridine, thymine-3'-PL-3'-thymine, thymine-5'-PL-5'-thymine, uridine-3'-PL-3'-uridine, uridine-5'-PL-5'-uridine, deoxythymidine-3'-PL-3'-deoxyuridine, deoxythymidine-5'-PL-5'-deoxyuridine, deoxythymidine-3'-PL-3'-thymidine, deoxythymidine-5'-PL-5'-thymidine, deoxythymidine-3'-PL-3'-deoxycytidine, deoxythymidine-5'-PL-5'-deoxycytidine, deoxycytidine-3'-PL-3'-deoxycytidine, deoxycytidine-5'-PL-5'-deoxycytidine, deoxyadenosine-3'-PL-3'-deoxyadenosine, deoxyadenosine-5'-PL-5'-deoxyadenosine, deoxyguanosine-3'-PL-3'-deoxyguanosine, deoxyguanosine-5'-PL-5'-deoxyguanosine, and MOE, 2'fluoro, 2'OMe, 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, 0(6)-methylguanine, 2-thiocytidine, methylated bases, intercalated bases, a nucleotide comprising a modified sugar (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose), or any combination

thereof. By way of further illustration, other examples of oligonucleotides include, but are not limited to,



**[0059]** In some embodiments of the present methods and compositions, the standard composition is a solution. The solution can be an aqueous or non-aqueous solution. The standard composition can comprise one or more oligonucleotides in a solvent or diluent. In some embodiments the solvent or diluent can comprise aqueous or organic solvents. Examples of solvent or diluent may include but are not limited to water, acetonitrile, methanol, ethanol, propanol, isopropanol, butanol, dichloromethane, dimethylformamide, N-methyl-2-pyrrolidone, acetone, toluene, benzene, chloroform, dimethylformamide, organic acids (e.g., acetic acid), amines (e.g., dimethyl amine), or combinations thereof, such as acetonitrile and water, acetone and water, ethanol and water, methanol and water, dilute formic acid in water, dilute acetic acid in water, or dilute ammonium hydroxide in water, but any suitable solvent or diluent can be used.

**[0060]** The evaluation of the mass spectrometry results comprises identifying one or more “mass peaks” or “mass spectrometry signals” corresponding to the oligonucleotide in the

resulting spectrum EIC, or TIC. The identified (or multiple) m/z peak(s) that correspond to the oligonucleotide standard are utilized in varying quantities through concentration or injection volumes. The linear, binomial, logarithmic, or other statistically relevant metric trend line results are assessed for comparability to a predefined acceptance criteria. The amount of variance in linearity, or other regression model, contributes to an r-squared analysis. Utilizing the method of the present disclosure with the deoxythymidine-3'-PL-5'-deoxythymidine yielded linearity results that met pre-defined acceptance criteria (e.g. an  $R^2$  value of the trend line being greater than 0.900, 0.950, 0.990, 0.995, or 0.999).

**[0061]** In some embodiments, the present method further comprises determining the differences between an expected intensity for one or more of the plurality of oligonucleotides in the oligonucleotide, and the corresponding actual intensity of the one or more of the plurality of oligonucleotides in the mass spectrum, wherein the expected intensity is determined from a series of known different concentration injections or assessments of the one or more of the plurality of oligonucleotides, and adjusting the mass spectrometer based on the differences between the expected intensity and the actual intensity in the mass spectrum as part of the qualification or calibration of the mass spectrometer.

**[0062]** In another aspect, the disclosure provides for methods for calibrating a mass spectrometer. In some embodiments, the method comprises: a) obtaining a mass spectrum of at least one standard composition comprising a plurality of oligonucleotides, or a kit comprising at least one oligonucleotide, according to any of the aspects and embodiments described herein; b) determining the differences between the expected mass peaks for the plurality of oligonucleotides in the standard composition, and the corresponding actual mass peaks in the

mass spectrum; and c) adjusting the mass spectrometer based on the differences between the expected mass peaks and the actual mass peaks.

**[0063]** The present methods can be used for calibrating, standardizing, or qualifying an MS/MS mode of the MS instrument using the mass spectrometry signals from the evaluation of the standards disclosed herein.

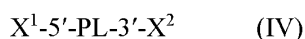
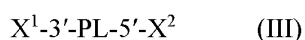
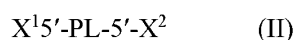
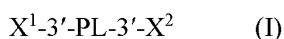
**[0064]** The present methods can further comprise loading the standard into a standard vessel. The standard is introduced to the MS instrument by flowing from the standard vessel. In some embodiments, the standard is continuously flowed to the MS instrument for a selected or predetermined period.

**[0065]** In another aspect, the present methods further comprise connecting the MS instrument to an oligonucleotide synthesis reaction chamber, and receiving a sample from the oligonucleotide synthesis reaction chamber. Oligonucleotide synthesis reaction chambers and methods are discussed in Dellinger et al. US Patent No. 7,572,907; Leproust US Patent No. 8,415,138; Dellinger et al. US Pat. App. Pub. 2020/0181124 A1; and Epps et al. International Patent App. No. PCT/US23/10608.

**[0066]** In some embodiments, the standard is introduced to the MS instrument on a continuous basis during analysis of the sample from the oligonucleotide synthesis reaction chamber. The sample from the oligonucleotide synthesis reaction chamber may comprise an oligonucleotide comprising a synthesis protecting group, such as acetyl, isobutyl, TAC, dmf, benzoyl, TBDMS, MOM, MEM, DMT, PMB, Methylthiomethyl ether, Pivaloyl, ether, silyl ether, methyl ester, and trityl. In some embodiments, the sample from the oligonucleotide synthesis reaction chamber comprises an oligonucleotide comprising a phosphate with a CNET

protecting group. In some embodiments, the sample from the oligonucleotide synthesis reaction chamber comprises an oligonucleotide comprising a GalNAc and/or PEG residue.

**[0067]** The present disclosure also provides standard compositions comprising a plurality of oligonucleotides having a structure of Formulas I to IV:



wherein  $X^1$ ,  $X^2$  and PL have the meanings described above. In some embodiments, the standard comprises two or more, three or more, four or more, or a greater number of oligonucleotides having a structure of Formulas I to IV. In some embodiments, each of the oligonucleotides of the standard has a different length and/or a different molecular weight. For instance, a standard can comprise two oligonucleotides having a structure of Formula I, wherein the oligonucleotides have different lengths and/or different molecular weights. In some embodiments, the standard may be a solution or a solid. In solution can mean either an aqueous or non-aqueous solution.

### **EXAMPLES**

#### Example 1

**[0068]** 3'-O-(5'-thymidylyl)thymidine was prepared by solid phase synthesis using an inverted dT CPG synthesis support. The solid synthesis support has its 3' terminal dimethoxytrityl protecting removed with dichloroacetic acid. A deoxythymidine phosphoramidite was coupled with the support loaded inverted dT by traditional oligonucleotide synthesis by co-delivering the amidite with 5-ethylthiotetrazole (ETT), recirculating the charged ETT and phosphoramidite

solution, and then removing the charged phosphoramidite's terminal DMT protecting group off with DCA. Coupling was followed by performing a diethylamine wash, performing a cleavage/deprotection step using ammonium hydroxide, and evaporating the ammonia off using a rotovap until a pH of  $< 10$  was reached.

**[0069]** The synthesized 2-mer was then tested for purity. The synthesis product was introduced into a 2.1 x 100mm, 1.7  $\mu\text{m}$  Waters Acquity UPLC BEH™ Shield RP18 column. 85% of a first mobile phase (mobile phase A) of 90% 10 mM ammonium acetate at a pH of 8.0, and 10% methanol. 15% of a second mobile phase (mobile phase B) of 50% Ultra-Pure Water and 50% methanol. FIG. 2 shows the chromatogram of the synthesis product. A purity result of 96.8% was obtained.

**[0070]** Samples of the oligonucleotide were prepared at 1 OD/mL (Optical Density per milliliter), 5 OD/mL, and 10 OD/mL concentrations, and 4  $\mu\text{L}$  samples of each were injected into an MS instrument with a flow rate of 0.3 mL/minute for 5 minutes and a column temperature of 40 degrees Celsius.

## Example 2

**[0071]** The linearity testing featured a mobile phase of 20mM ammonium formate in a 75/25 mixture of methanol and water. Samples of the oligonucleotide were prepared at a 0.1 OD/mL concentration. Volumes of 1, 3, 5, 7, 10, 15, and 20  $\mu\text{L}$  were injected in separate analyses into an MS instrument with a flow rate of 0.3 mL/minute and a run time of 5 minutes. The MS results are shown in Table 1.

Table 1

| 0.1 OD/mL   |                   |             |
|-------------|-------------------|-------------|
| uL Injected | Total OD's Loaded | EIC Area    |
| 1           | 0.0001            | 2525349.83  |
| 3           | 0.0003            | 7506551.2   |
| 5           | 0.0005            | 13421682.66 |
| 7           | 0.0007            | 18654149.2  |
| 10          | 0.001             | 27548055.03 |
| 15          | 0.0015            | 42647606.04 |
| 20          | 0.002             | 56982775.48 |

[0072] These results were reviewed and a linearity curve was generated by integrating the extracted ion chromatogram (EIC) of 545.1 m/z and comparing to the amount of the oligonucleotide used in each analysis. FIG. 3 shows the linearity curve calculated via linear regression, and demonstrates that the 0.1 OD/mL sample concentration injected over a 1-20  $\mu$ L volume range yielded an acceptable trend response from the MS instrument.

[0073] This process of linearity testing was then repeated with 1, 3, 5, 7, 10, 15, and 20  $\mu$ L injections of 1.0 OD/mL 2-mer specimen. The MS results are shown in Table 2.

Table 2

| 1 OD/mL     |             |             |
|-------------|-------------|-------------|
| uL Injected | OD's Loaded | EIC Area    |
| 1           | 0.001       | 25941490.48 |
| 3           | 0.003       | 86217618.4  |
| 5           | 0.005       | 137924110.9 |
| 7           | 0.007       | 183144898.5 |

|    |       |             |
|----|-------|-------------|
| 10 | 0.01  | 240071633.4 |
| 15 | 0.015 | 317582581   |
| 20 | 0.02  | 386038984.8 |

[0074] These results were reviewed and a linearity curve was generated by integrating the extracted ion chromatogram of 545.1 m/z and comparing to the concentrations loaded. FIG. 4 shows the linearity curve calculated via binomial regression which demonstrates that the 1.0 OD/mL sample concentration a 1-20  $\mu$ L injected over a 1-20  $\mu$ L volume range yielded an acceptable trend response from the MS instrument.

#### Example 3

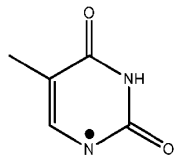
[0075] An oligonucleotide compound comprising five nucleotides (a 5-mer with the 3' end being a 3' coupling) was synthesized by repeating the synthesis steps described in Example 1. This oligonucleotide compound contains three charge states or m/z values, (485.3 (3<sup>rd</sup>), 728.1 (2<sup>nd</sup>), and 1457.0 (1<sup>st</sup>) m/z's), which usually correspond to phosphate linkages in an MS result.

[0076] Samples of this 5-mer were injected into and analyzed by the MS instrument under the conditions described in Example 1. The MS spectrum displayed the two known charge states of the 5-mer, indicating good performance of the MS instrument and providing the opportunity to dynamically adjust its mass axis calibration based on changes in the observed mass spectrum.

#### Example 4

[0077] An oligonucleotide compound comprising of two nucleotides (a 2-mer) was synthesized by repeating the synthesis steps described in Example 1. This oligonucleotide

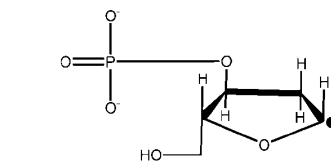
compound can be injected onto a mass spectrometer with a collision cell or other fragmenting capability enabling mass spectrometry / mass spectrometry (MS/MS) or tandem mass spectrometry. The theoretical MS/MS fragments of inverted dT-dT standard are shown below.

Chemical Formula:  $C_5H_5N_2O_2^{\bullet+}$ 

Exact Mass: 125.04

Molecular Weight: 125.11

m/z: 125.04 (100.0%), 126.04 (5.5%)

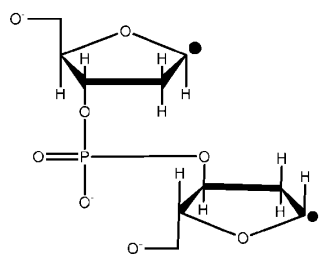
Chemical Formula:  $C_8H_{18}O_8P^{2-}$ 

Exact Mass: 195.01

Molecular Weight: 195.09

m/z: 97.51 (100.0%), 98.01 (5.7%), 98.51 (1.4%)

Elemental Analysis: C, 30.78; H, 4.13; O, 49.21; P, 15.88

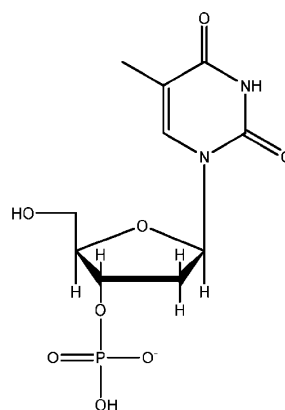
Chemical Formula:  $C_{10}H_{14}O_8P^{2-3-}$ 

Exact Mass: 293.04

Molecular Weight: 293.19

m/z: 97.68 (100.0%), 98.02 (11.3%), 98.35 (2.2%)

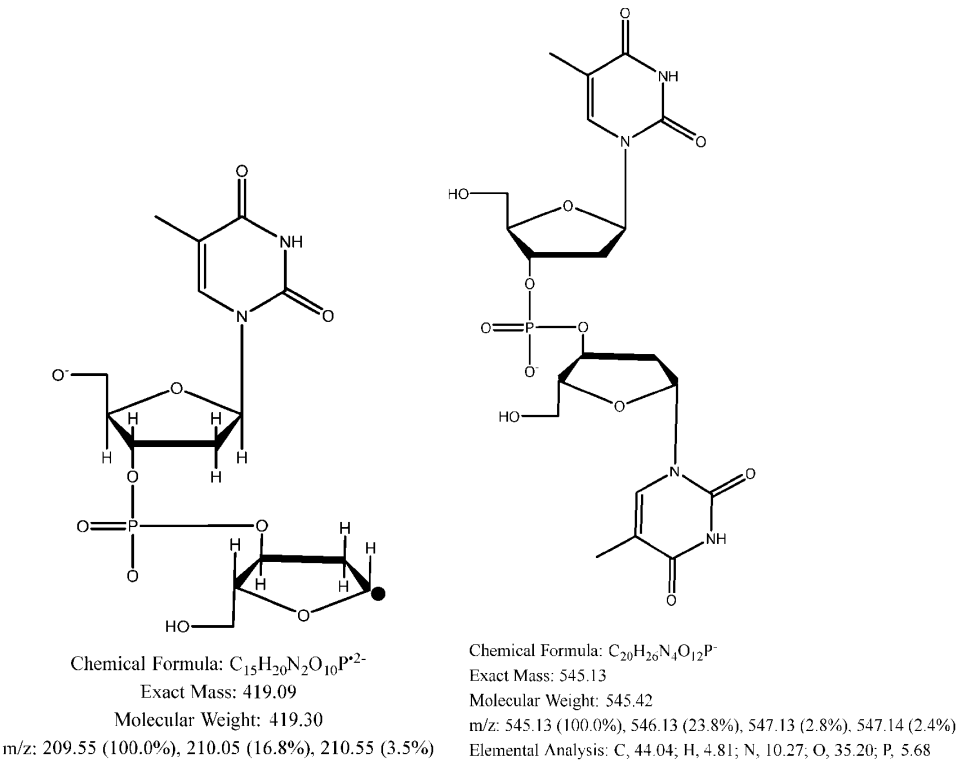
Elemental Analysis: C, 40.97; H, 4.81; O, 43.66; P, 10.56

Chemical Formula:  $C_{10}H_{14}N_2O_8P^-$ 

Exact Mass: 321.05

Molecular Weight: 321.20

m/z: 321.05 (100.0%), 322.05 (11.9%), 323.05 (1.7%)



[0078] The resulting fragment ions (in the provided example 125.0, 195.0, 293.0, 321.1, and 419.1,  $m/z$  of the parent ion 545.1  $m/z$ ) can be used to qualify the mass axis calibration and linearity of the MS/MS system. This is demonstrated by the results shown in Table 3 and Figures 5 to 7.

Table 3

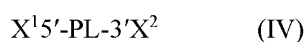
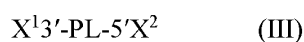
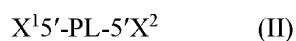
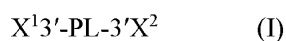
| Conc (mg/ml) | Area (419.09m/z) | Area (125.04 m/z) | Area (195.01 m/z) |
|--------------|------------------|-------------------|-------------------|
| 0.0001       | 4788             | 63911             | 4470              |
| 0.00025      | 9978             | 131201            | 11498             |
| 0.0005       | 16242            | 238481            | 21041             |
| 0.001        | 37544            | 490689            | 45216             |
| 0.0025       | 73995            | 1017894           | 100404            |
| 0.005        | 177746           | 2242341           | 218631            |
| 0.01         | 405919           | 5432318           | 478678            |

|       |         |          |         |
|-------|---------|----------|---------|
| 0.025 | 1331378 | 20797822 | 1604954 |
| 0.05  | 2915650 | 47034429 | 3460893 |

### Exemplary Embodiments

[0079] Exemplary embodiments provided in accordance with the presently disclosed subject matter include, but are not limited to, the following:

[0080] Embodiment 1. A method of evaluating performance of a mass spectrometer instrument comprising: providing a mass spectrometer (MS) instrument; introducing a standard to the MS instrument, wherein the standard comprises an oligonucleotide having a structure of Formula I, Formula II, Formula III, or Formula IV:



wherein

PL is a phosphate linkage comprising  $\text{-PO}_4\text{-}$ ,  $\text{-PO}_3\text{-O-PO}_3\text{-}$ ,  $\text{-PO}_3\text{S}$ ,  $\text{-PO}_3\text{BH}_2\text{-}$  or  $\text{-PO}_3\text{-O-PO}_2\text{-O-PO}_3\text{-}$ ;

$X^1$  and  $X^2$  are independently a nucleoside, a nucleotide, or an oligonucleotide; and evaluating one or more mass spectrometry signals corresponding to the oligonucleotide from the mass spectrometry instrument.

[0081] Embodiment 2. The method of embodiment 1, wherein the evaluating comprises identifying one or more mass peaks corresponding to the oligonucleotide, and comparing the identified one or more mass peaks to one or more expected mass peaks for the oligonucleotide.

**[0082]** Embodiment 3. The method of embodiment 1 or embodiment 2, further comprising adjusting the MS instrument based on the evaluating of the one or more mass spectrometry signals.

**[0083]** Embodiment 4. The method of any of embodiments 1 to 3, wherein the mass spectrometry instrument has a negative ionization mode, and wherein the standard is introduced for a negative mode mass spectrometry analysis.

**[0084]** Embodiment 5. The method of embodiment 4, further comprising performing a qualification of a negative ionization mode of the MS instrument.

**[0085]** Embodiment 6. The method of embodiment 5, wherein the qualification comprises: analyzing the oligonucleotide in a plurality of amounts; and determining whether the one or more mass spectrometry signals are substantially linear relative to the plurality of amounts.

**[0086]** Embodiment 7. The method of embodiment 6, wherein the analyzing is a non-linear regression analysis relative to the plurality of amounts used to calibrate or qualify a mass spectrometer.

**[0087]** Embodiment 8. The method of any of the preceding embodiments, further comprising performing a mass-axis calibration.

**[0088]** Embodiment 9. The method of any of the preceding embodiments, wherein the standard is a continuous reference standard introduced to the MS instrument on a continuous basis during sample analysis.

**[0089]** Embodiment 10. The method of any of the preceding embodiments, wherein the standard is an internal standard introduced to the MS instrument simultaneously with a sample for analysis.

[0090] Embodiment 11. The method of any of the preceding embodiments, wherein  $X^1$  and  $X^2$  are the same nucleoside.

[0091] Embodiment 12. The method of any of embodiments 1 to 10, wherein  $X^1$  and  $X^2$  are different nucleosides.

[0092] Embodiment 13. The method of any of embodiments 1 to 10, wherein the oligonucleotide of Formula I is deoxythymidine-3'-PL-3'-deoxythymidine.

[0093] Embodiment 14. The method of any of the preceding embodiments, wherein the oligonucleotide is in solution.

[0094] Embodiment 15. The method of any of the preceding embodiments, wherein the oligonucleotide is solubilized from a solid.

[0095] Embodiment 16. The method of any of the preceding embodiments, wherein the oligonucleotide is from 2 to 100 nucleobases in length.

[0096] Embodiment 17. The method of any of the preceding embodiments, wherein the oligonucleotide is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length.

[0097] Embodiment 18. The method of any of embodiments 1 to 16, wherein the oligonucleotide is 25-30, 30-35, 35-40, 40-45, 45-50, 50-55, 55-60, 60-65, 65-70, 70-75, 75-80, 80-85, 85-90, 90-95, 95-100 nucleobases in length.

[0098] Embodiment 19. The method of any of the preceding embodiments, wherein the oligonucleotide comprises MOE, 2'fluoro, 2'Ome, 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3 -methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-

propynyl-uridine, C5 -propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, 6-O-methylguanine, LNA, UNA, C(et), 2-thiocytidine, methylated bases, intercalated bases, a nucleotide comprising a modified sugar (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose), or any combination thereof.

**[0099]** Embodiment 20. The method of embodiment 1, wherein the oligonucleotide further comprises TBDMS, MOM, MEM, DMT, TOM, ACE, TC, PMB, Methylthiomethyl phosphate, ether, Pivaloyl, ether, silyl ether, methyl ester, and trityl.

**[00100]** Embodiment 21. The method of any of the preceding embodiments, wherein the oligonucleotide further comprises an alkyl spacer.

**[00101]** Embodiment 22. The method of embodiment 21, wherein the alkyl spacer is selected from dodecyl, undecyl, decyl, nonyl, octyl, heptyl, hexyl, pentyl, butyl, propyl, ethyl, and methyl.

**[00102]** Embodiment 23. The method of any of the preceding embodiments, wherein the oligonucleotide further comprises a GalNAc and/or PEG residue.

**[00103]** Embodiment 24. The method of any of the preceding embodiments, wherein the oligonucleotide further comprises a 2', 3', and/or 5' synthesis protecting group.

**[00104]** Embodiment 25. The method of embodiment 24, wherein the 2', 3' and/or 5' synthesis protecting group is selected from TBDMS, MOM, MEM, TOM, ACE, TC, DMT, PMB, Methylthiomethyl ether, Pivaloyl, ether, silyl ether, methyl ester, and trityl.

**[00105]** Embodiment 26. The method of any of the preceding embodiments, wherein the oligonucleotide is composed of 3' and/or 5' linkages.

- [00106] Embodiment 27. The method of any of the preceding embodiments, wherein the oligonucleotide comprises a synthesis protecting group.
- [00107] Embodiment 28. The method of embodiment 27, wherein the synthesis protecting group is selected from the group consisting of acetyl, isobutyl, TAC, dmf, and benzoyl.
- [00108] Embodiment 29. The method of any of the preceding embodiments, wherein the oligonucleotide comprises a phosphate with a CNET protecting group.
- [00109] Embodiment 30. The method of any of the preceding embodiments, further comprising calibrating, standardizing, or qualifying an MS/MS mode of the MS instrument using the evaluated one or more mass spectrometry signals.
- [00110] Embodiment 31. The method of embodiment 30, wherein the standard is used to calibrate the MS/MS mode of a mass spectrometer.
- [00111] Embodiment 32. The method of embodiment 30, wherein the standard is used to standardize the MS/MS mode of a mass spectrometer.
- [00112] Embodiment 33. The method of embodiment 30, wherein the standard is used to qualify the MS/MS mode of a mass spectrometer.
- [00113] Embodiment 34. The method of embodiment 30, wherein the standard is infused into the MS instrument with a sample, and a known mass of the standard is used to dynamically or continuously correct or adjust the MS instrument's  $m/z$  calibration.
- [00114] Embodiment 35. The method of any of the preceding embodiments, further comprising loading the standard into a standard vessel; and the standard is introduced to the MS instrument by flowing from the standard vessel.

[00115] Embodiment 36. The method of embodiment 35, wherein the standard is continuously flowed to the MS instrument for a selected or predetermined period.

[00116] Embodiment 37. The method of any of the preceding embodiments, further comprise fluidically connecting the MS instrument to an oligonucleotide synthesis reaction chamber, and receiving a sample from the oligonucleotide synthesis reaction chamber.

[00117] Embodiment 38. The method of embodiment 37, wherein the standard is introduced to the MS instrument on a continuous basis during analysis of the sample from the oligonucleotide synthesis reaction chamber.

[00118] Embodiment 39. The method of embodiment 37, wherein the sample from the oligonucleotide synthesis reaction chamber comprises an oligonucleotide comprising a synthesis protecting group.

[00119] Embodiment 40. The method of embodiment 39, wherein the synthesis protecting group is selected from acetyl, isobutyl, TAC, dmf, benzoyl, TBDMS, MOM, MEM, DMT, PMB, Methylthiomethyl ether, Pivaloyl, ether, silyl ether, methyl ester, and trityl.

[00120] Embodiment 41. The method of embodiment 37, wherein the sample from the oligonucleotide synthesis reaction chamber comprises an oligonucleotide comprising a phosphate with a CNET protecting group.

[00121] Embodiment 42. The method of embodiment 37, wherein the sample from the oligonucleotide synthesis reaction chamber comprises an oligonucleotide comprising a GalNAc and/or PEG residue.

[00122] Embodiment 43. The method of any of the preceding embodiments, wherein the oligonucleotide of the standard comprises a plurality of oligonucleotides having a structure of Formula I, II, III, or IV.

[00123] Embodiment 44. The method of embodiment 43, wherein the two or more oligonucleotides have different molecular weights.

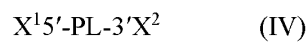
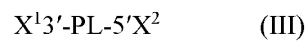
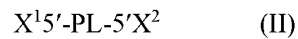
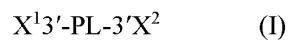
[00124] Embodiment 45. The method of embodiment 43, wherein the two or more oligonucleotides have different lengths.

[00125] In view of this disclosure it is noted that the methods and compositions can be implemented in keeping with the present teachings. Further, the various components, materials, structures and parameters are included by way of illustration and example only and not in any limiting sense. In view of this disclosure, the present teachings can be implemented in other applications and components, materials, structures and equipment to implement these applications can be determined, while remaining within the scope of the appended claims.

## CLAIMS

We claim:

1. A method of evaluating performance of a mass spectrometer instrument comprising:  
providing a mass spectrometer (MS) instrument;  
introducing a standard to the MS instrument, wherein the standard comprises an oligonucleotide having a structure of Formula I, Formula II, Formula III, or Formula IV:



wherein

PL is a phosphate linkage comprising  $-\text{PO}_4-$ ,  $-\text{PO}_3\text{-O-PO}_3-$ ,  $-\text{PO}_3\text{S}$ ,  $-\text{PO}_3\text{BH}_2-$  or  $-\text{PO}_3\text{-O-PO}_2\text{-O-PO}_3-$ ;

$X^1$  and  $X^2$  are independently a nucleoside, a nucleotide, or an oligonucleotide; and  
evaluating one or more mass spectrometry signals corresponding to the oligonucleotide from the mass spectrometry instrument.

2. The method of claim 1, wherein the evaluating comprises identifying one or more mass peaks corresponding to the oligonucleotide, and comparing the identified one or more mass peaks to one or more expected mass peaks for the oligonucleotide.

3. The method of claim 1, further comprising adjusting the MS instrument based on the evaluating of the one or more mass spectrometry signals.

4. The method of claim 1, wherein the mass spectrometry instrument has a negative ionization mode, and wherein the standard is introduced for a negative mode mass spectrometry analysis.

5. The method of claim 4, further comprising performing a qualification of a negative ionization mode of the MS instrument.

6. The method of claim 5, wherein the qualification comprises:  
analyzing the oligonucleotide in a plurality of amounts; and  
determining whether the one or more mass spectrometry signals are substantially linear relative to the plurality of amounts.

7. The method of claim 6, wherein the analyzing is a non-linear regression analysis relative to the plurality of amounts used to calibrate or qualify a mass spectrometer.

8. The method of claim 1, further comprising performing a mass-axis calibration.

9. The method of claim 1, wherein the standard is a continuous reference standard introduced to the MS instrument on a continuous basis during sample analysis.

10. The method of claim 1, wherein the standard is an internal standard introduced to the MS instrument simultaneously with a sample for analysis.
11. The method of claim 1, wherein  $X^1$  and  $X^2$  are the same nucleoside.
12. The method of claim 1, wherein  $X^1$  and  $X^2$  are different nucleosides.
13. The method of claim 1, wherein the oligonucleotide of Formula I is deoxythymidine-3'-PL-3'-deoxythymidine.
14. The method of claim 1, wherein the oligonucleotide is in solution.
15. The method of claim 1, wherein the oligonucleotide is solubilized from a solid.
16. The method of claim 1, wherein the oligonucleotide is from 2 to 100 nucleobases in length.
17. The method of claim 1, wherein the oligonucleotide is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length.

18. The method of claim 1, wherein the oligonucleotide is 25-30, 30-35, 35-40, 40-45, 45-50, 50-55, 55-60, 60-65, 65-70, 70-75, 75-80, 80-85, 85-90, 90-95, 95-100 nucleobases in length.

19. The method of claim 1, wherein the oligonucleotide comprises MOE, 2'fluoro, 2'Ome, 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3 -methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5 -propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, 6-O-methylguanine, LNA, UNA, C(et), 2-thiocytidine, methylated bases, intercalated bases, a nucleotide comprising a modified sugar (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose), or any combination thereof.

20. The method of claim 1, wherein the oligonucleotide further comprises TBDMS, MOM, MEM, DMT, TOM, ACE, TC, PMB, Methylthiomethyl phosphate, ether, Pivaloyl, ether, silyl ether, methyl ester, and trityl.

21. The method of claim 1, wherein the oligonucleotide further comprises an alkyl spacer.

22. The method of claim 21, wherein the alkyl spacer is selected from dodecyl, undecyl, decyl, nonyl, octyl, heptyl, hexyl, pentyl, butyl, propyl, ethyl, and methyl.

23. The method of claim 1, wherein the oligonucleotide further comprises a GalNAc and/or PEG residue.

24. The method of claim 1, wherein the oligonucleotide further comprises a 2', 3', and/or 5' synthesis protecting group.

25. The method of claim 24, wherein the 2', 3' an/or 5' synthesis protecting group is selected from TBDMS, MOM, MEM, TOM, ACE, TC, DMT, PMB, Methylthiomethyl ether, Pivaloyl, ether, silyl ether, methyl ester, and trityl.

26. The method of claim 1, wherein the oligonucleotide is composed of 3' and/or 5' linkages.

27. The method of claim 1, wherein the oligonucleotide comprises a synthesis protecting group.

28. The method of claim 27, wherein the synthesis protecting group is selected from the group consisting of acetyl, isobutyl, TAC, dmf, and benzoyl.

29. The method of claim 1, wherein the oligonucleotide comprises a phosphate with a CNET protecting group.

30. The method of claim 1, further comprising calibrating, standardizing, or qualifying an MS/MS mode of the MS instrument using the evaluated one or more mass spectrometry signals.

31. The method of claim 30, wherein the standard is used to calibrate the MS/MS mode of a mass spectrometer.

32. The method of claim 30, wherein the standard is used to standardize the MS/MS mode of a mass spectrometer.

33. The method of claim 30, wherein the standard is used to qualify the MS/MS mode of a mass spectrometer

34. The method of claim 30, wherein the standard is infused into the MS instrument with a sample, and a known mass of the standard is used to dynamically or continuously correct or adjust the MS instrument's  $m/z$  calibration.

35. The method of claim 1, further comprising loading the standard into a standard vessel; and the standard is introduced to the MS instrument by flowing from the standard vessel.

36. The method of claim 35, wherein the standard is continuously flowed to the MS instrument for a selected or predetermined period.

37. The method of claim 1, further comprise fluidically connecting the MS instrument to an oligonucleotide synthesis reaction chamber, and receiving a sample from the oligonucleotide synthesis reaction chamber.

38. The method of claim 37, wherein the standard is introduced to the MS instrument on a continuous basis during analysis of the sample from the oligonucleotide synthesis reaction chamber.

39. The method of claim 37, wherein the sample from the oligonucleotide synthesis reaction chamber comprises an oligonucleotide comprising a synthesis protecting group.

40. The method of claim 39, wherein the synthesis protecting group is selected from acetyl, isobutyl, TAC, dmf, benzoyl, TBDMS, MOM, MEM, DMT, PMB, Methylthiomethyl ether, Pivaloyl, ether, silyl ether, methyl ester, and trityl.

41. The method of claim 37, wherein the sample from the oligonucleotide synthesis reaction chamber comprises an oligonucleotide comprising a phosphate with a CNET protecting group.

42. The method of claim 37, wherein the sample from the oligonucleotide synthesis reaction chamber comprises an oligonucleotide comprising a GalNAc and/or PEG residue.

43. The method of claim 1, wherein the oligonucleotide of the standard comprises a plurality of oligonucleotides having a structure of Formula I, II, III, or IV.

44. The method of claim 43, wherein the two or more oligonucleotides have different molecular weights.

45. The method of claim 43, wherein the two or more oligonucleotides have different lengths.