technical report

Mass spectrometry analysis of oligonucleotide syntheses

Contents

1. Introduction 1
2. MALDI-TOF technology 2
3. ESI technology 3
4. Comparison of MALDI-TOF and ESI technologies 4
5. Mass spectrometry oligonucleotide QC at Integrated DNA Technologies 5
6. Interpreting mass spectrometry results for oligonucleotide syntheses 5
    - 6.1 MALDI-TOF MS 5
    - 6.2 ESI MS 6
    - 6.3 Identifying irregular peaks 7
7. References 8

1. Introduction

Oligonucleotide synthesis is a complex process, one in which a single 25-base sequence requires successful completion of over 100 sequential chemical reactions. Still, current phosphoramidite synthesis chemistries are robust. Automated solid-phase synthesis platforms have improved reliability and increased the capacity of high-throughput oligo manufacturing. Even with the highest efficiency synthesis, it is crucial that synthesized oligos are evaluated for quality before use in molecular biology applications. Specifically, oligos need to be accurately assessed to ensure they contain precisely the correct sequence. The best method available to assess compound identity in a high-throughput environment is mass spectrometry (MS).

MS is used in a number of scientific fields with many variations. However, all MS analysis involves 2 sequential steps—ionization and mass measurement. In the ionization step, gaseous ions are generated from a small amount of sample and transported to a mass detector/analyzer for the second step, mass measurement. Within the context of oligo synthesis, the most common MS methods are MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) and ESI (electrospray ionization). Both methods have associated advantages and disadvantages that will vary according to the analytical environment in which they are used. The basic technologies, as well as their advantages and disadvantages, are presented below.

2. MALDI-TOF technology

MALDI, which more specifically refers to the ionization step of MALDI-TOF MS, was first introduced in 1987. Following the development of MALDI, a number of subsequent experiments verified its utility across many fields, and MALDI-TOF MS quickly evolved as a highly reliable tool for nucleic acid analysis [1–3].

In MALDI-TOF MS, a small amount (1–3 pmol) of test sample is mixed with a carrier or matrix solution, usually 3-hydroxypicolinic acid, and the mixture is deposited at a fixed position on a sample grid within the MALDI-TOF instrument. The oligo/matrix sample is then vaporized by a pulse of laser light, causing some oligo molecules to ionize through protonation (the oligo gains a proton and subsequent +1 charge relative to unprotonated molecules). Using an electrostatic field, the MALDI-TOF instrument accelerates the ionized oligo molecules of different sizes to a common kinetic energy (KE) and measures their TOF across a fixed distance. Equations 1–5 explain how this process is used to analyze the mass of a sample oligo [4].

When ions of the same KE travel through the TOF analyzer, they obey the rule:

$$KE = \tfrac{1}{2} mv^2 = zeV \qquad (1)$$

Where m is mass, v is velocity, z is the charge of the ion (+1), and eV is the applied voltage. Knowing that velocity (v) is a function of distance (d) and time (t),

$$v=\frac{d}{t} \qquad (2)$$

Equation 1 can be rearranged,

$$t=d\sqrt{\frac{m}{2(KE)}} \qquad (3)$$

and substituted, so that:

$$t=d\sqrt{\frac{m}{2(zeV)}} \qquad (4)$$

Because d and eV are constant within the TOF analyzer, and t is measured, equation 4 demonstrates that an ion's TOF (t) is directly proportional to its mass-to-charge (m/z) ratio:

$$t\propto\frac{m}{z} \qquad (5)$$

Therefore, it takes large ions more time to arrive at the mass detector, which is located at the end of the TOF analyzer. Similarly, small ions will travel faster, and arrive at the detector in less time. These principles allow the MALDI-TOF instrument to record the entire mass spectra of a sample oligo and compare it to the expected mass (based on the oligo's specific sequence).

3. ESI technology

ESI MS was developed around the same time as MALDI MS [5,6]. In this process, a sample in solvent is introduced into the ESI instrument through a stainless steel capillary as a fine spray in the presence of a strong electric field. Addition of nitrogen gas to the interface removes a portion of solvent from the droplets, and the remaining sample molecules are conveyed to a high-vacuum region of the instrument via a low-pressure transport system. In this high-vacuum, low-pressure region, a second stainless steel capillary further separates the molecules from any remaining solvent, a process known as "declustering." It is at this stage that the molecules become ionized via deprotonation. The removal of multiple protons from different sample molecules results in a variety of species with multiple negative charges.

Similar to MALDI, ESI refers only to the ionization step of the MS process. For the mass measurement step, a variety of techniques and instruments can be used in conjunction with ESI, including TOF and quadrupole mass analyzers. At IDT, we use quadrupole ion traps (ESI-ion trap) for all of our MS analyses (see below for more detail). In these devices, ions generated from ESI are transferred to a section of the instrument comprised of direct current (DC) and radio frequency (RF) electrodes. Fluctuating voltages applied to the RF electrodes create an electric field, which causes the ions to alternatively accelerate and decelerate in an oscillating pattern. This oscillation forms a stable electric environment where the ions are "trapped" and stored by the applied RF potentials. By varying the RF potentials, ions of specific m/z ratios can be ejected out of the trap and into a mass detector. This method is commonly referred to as mass selective ejection and can be used to generate a sensitive mass spectrum analysis of an oligo sample [7].

4. Comparison of MALDI-TOF and ESI technologies

In 2004, Hail et al. compared ESI and MALDI-TOF technologies for oligonucleotide analyses [8]. In their report, they mention that MALDI-TOF is relatively easy to operate, offers high sensitivity for oligos, and is well suited to a high-throughput environment. However, they also note, "MALDI-TOF is not particularly effective when analyzing long (>50 bases) and/or fragile oligonucleotides". Alternatively, ESI technology maintains mass accuracy, resolution, and sensitivity over a range of lengths (20–120 bases). The data in Table 1 demonstrates that ESI outperforms MALDI-TOF for both short and long oligos. While performance differences for shorter oligos are slight, there is a significant advantage seen when using ESI to evaluate oligos longer than 50 bases.

Table 1. Experimental mass accuracy for standard oligonucleotides analyzed by MALDI-TOF and ESI.*

Oligo length (nt)	Calculated mass (Da)	MALDI-TOF		ESI	
		Experimental mass (Da)	Error (ppm)	Experimental mass (Da)	Error (ppm)
10	2993.0	2992.1	–300.7	2993.1	33.4
20	6168.1	6167.4	–113.5	6167.6	–81.1
40	12300.0	12298.6	–113.8	12299.7	–24.4
60	18410.0	18414.6	249.9	18410.1	5.3
80	24661.1	24664.7	146.0	24661.0	–4.1
100	30951.1	30978.3	878.8	30950.6	–16.2
120	37031.0	37121.6	2446.6	37030.0	–27.0

* Table source: Hail et al. [8]

5. Mass spectrometry oligonucleotide QC at Integrated DNA Technologies

As a global leader in high-quality oligo synthesis, IDT was a pioneer in using MS for quality control (QC). For years, we performed MALDI-TOF MS assessment for all standard probes and primers, as well as for more complex, modified, and HPLC- and PAGE-purified oligos. However, in 2002, we began a transformation towards ESI, specifically with 3D-trap (LCQ) and linear-trap (LTQ) instruments. Today, ESI-ion trap instruments are used for all MS analyses at IDT, and due to the previously mentioned advantages of ESI technology, this transformation has since allowed us to provide customers with greater mass accuracy for oligos up to 200 bases. Importantly, ESI-MS analysis is provided for every oligo we synthesize and this data can be accessed online at any time, free of charge.

6. Interpreting mass spectrometry results for oligonucleotide syntheses

Since IDT provides MS data for every synthesized oligo, it is important that customers know how to interpret MS results. Although we now assess all of our oligos using ESI MS, some customers may still have access to historical MALDI-TOF traces. Therefore, the following section presents a tutorial for interpreting both types of MS data.

6.1 MALDI-TOF MS

To begin, it is important to remember that both MALDI-TOF and ESI MS do not measure mass directly. Instead, they measure a m/z ratio. Ideally, for MALDI-TOF, a single ionized species is present (molecules that gain a single proton are measured as $M–H^+$), however some molecules gain two protons in the desorption phase ($M–2H^+$). Such multiple ionized species are rare and can be distinguished from the single-ionized species due to their altered m/z ratio. A mass spectrum is a useful QC tool that helps verify accurate synthesis of a custom oligo. More specifically, it confirms whether or not an oligo has the expected molecular weight based upon the specific sequence requested (see Figures 1, 3).

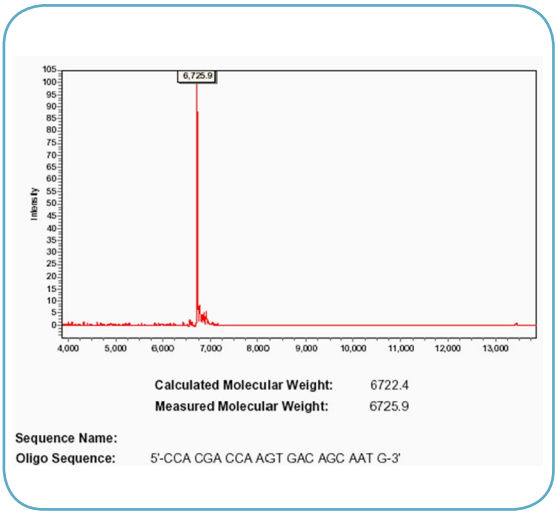

Figure 1. MALDI-TOF mass spectrogram of a 22 nt oligonucleotide. The synthesized sequence is shown at the bottom and the expected and observed molecular weights (Da) are presented above the sequence.

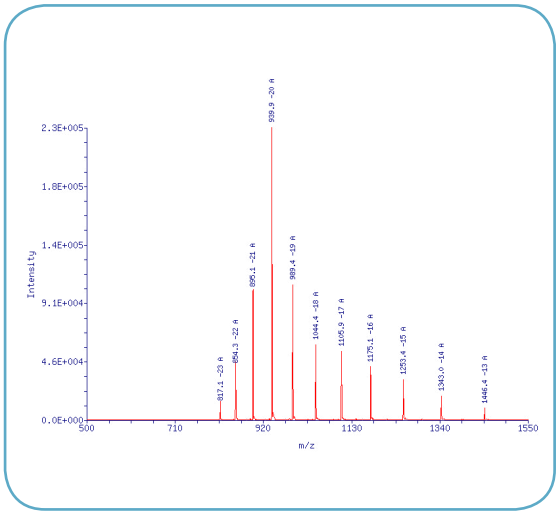

Figure 2. ESI mass spectrometry trace (raw data) of an HPLC-purified, 60 nt oligonucleotide with a 5′ spacer modification. Multiple peaks are seen at different m/z ratios, representing various amounts of deprotonation.

Even with good machine calibration, accurate measurement by MALDI-TOF is limited to oligos less than 50 bases in length. This is because oligos greater than 50 bases in length "fly" poorly in a MALDI-TOF instrument, resulting in an insufficient number of ionized particles reaching the detector and subsequent low-resolution spectra. While measurements of longer oligos are sometimes within a few daltons (Da) of expectation, mass estimates can only be guaranteed to be accurate within ± 0.2%. For example, a 50 nt oligo having a calculated molecular weight of 15,130 Da might have a measured molecular weight of 15,150 Da and "pass" MALDI-TOF QC, since this measurement lies within the resolution range of the instrument. However, as shown in Figure 1, measurements for shorter oligos are typically much more precise, with errors of ± 0.1% being commonly achieved for 22 nt oligos.

6.2 ESI MS

In ESI MS, the parent ions are not observed. Instead, multiple negatively charged species are present. The raw data trace of a purified, 60 nt oligo is shown in Figure 2, where the mass-to-charge measurement range is 550 to 1550 m/z. All of the peaks labeled with "A" are from the same oligo. The differences among these peaks represent the various m/z ratios generated by different amounts of deprotonation. The m/z ratio is calculated by taking the measured molecular weight of an oligo, subtracting a number of H^+ (mass = 1.0079 amu), and then dividing by the number of H^+ removed. For example, the peak labeled 20A at 939.9 m/z can be calculated as:

$$\frac{18{,}817.5 - (20 \times 1.0079)}{20}$$

Similarly, the peak labeled 19A at 989.4 m/z can be calculated as:

$$\frac{18{,}817.5 - (19 \times 1.0079)}{19}$$

Based on the computations on the previous page, a deconvolution algorithm can be used to convert raw data (like that seen in Figure 2) into a new ESI trace. Figure 3 presents an ESI trace produced after such deconvolution analysis. When examining raw data, the deconvolution program looks for groups of peaks that add up to the same parent peak and removes all peaks that do not. In doing so, the deconvolution analysis removes noise, and this is why a final deconvoluted ESI trace has a smooth baseline compared to a final MALDI-TOF trace. As seen in Figure 3, the expected error in ESI analyses of oligos over 14,000 Da is ≤0.02%.

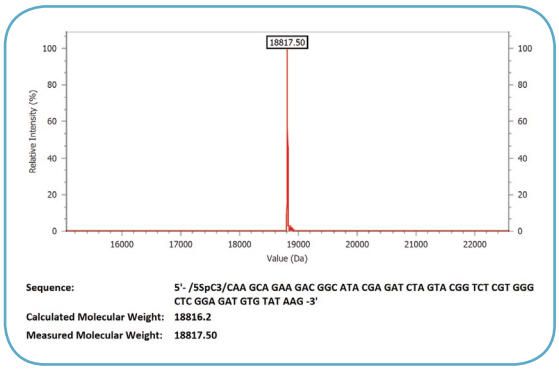

Figure 3. ESI mass spectrogram of an HPLC-purified, 60 nt oligonucleotide with a 5′ spacer modification. The synthesized sequence is shown at the bottom and the expected and observed molecular weights (Da) are presented above the sequence.

6.3 Identifying irregular peaks

In both MALDI-TOF and ESI-MS results, there will be a main peak representing the synthesized oligo. However, there can be, and often are, additional peaks present in the final traces. During MALDI-TOF analysis, oligo depurination can occur as a result of heat (laser ionization) in the acidic environment (the matrix). Similarly, in ESI, depurination can occur during analysis due to added heat in the transport region of the instrument. In either method, if depurination occurs, it can result in secondary peaks having 135 or 151 Da mass units less than the major peak.

Another cause of irregular peaks can be attributed to the phosphoramidite chemistries involved in the oligo synthesis process. To prevent branching and other undesired side reactions during synthesis, manufacturers often use nucleotides which are fixed with multiple protecting groups, including dimethoxytrityl (DMT). In normal manufacturing, these protecting groups are cleaved off post-synthetically during the final "deprotection" and "desalting" steps. However, if protecting group removal is deficient, the side groups will remain on the oligo. In these instances, MS data will show resulting oligo mass to be higher than expected.

Finally, it is important to note that oligo modifications add mass to an oligo, and are taken into account in the final mass spectrograms produced. A list of mass contributions for the most common DNA and RNA modifications is listed on the IDT modifications page. Additionally, the anhydrous molecular weight of both unmodified and modified oligos can be calculated using IDT's OligoAnalyzer® 3.1.

7. References

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