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Sequencing of Production-Scale Synthetic Oligonucleotides by Enriching for Coupling Failures Using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

David Alazard,*[,1] Mark Filipowsky,* Joanna Raeside,† Maureen Clarke,† Mehrdad Majlessi,* James Russell,* and William Weisburg*

**Gen-Probe Incorporated, 10210 Genetic Center Drive, San Diego, California 92121-4362; and †Department of Chemistry, University of Strathclyde, Glasgow, Scotland G11XQ*

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A technique for sequencing oligonucleotides using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is described. The series of coupling failure species are extracted from the dimethoxytrityl-on, full-length oligonucleotide in crude synthetic material using C_{18} stationary-phase cartridges. These concentrated failure species can be easily detected by MALDI-TOF, which determines the mass difference between spectral ions to identify a particular base. The solid-phase extraction step greatly enhances ion signals and mass resolution, and sequencing information is generally obtained from the 5′ end up to the first three to four nucleotides at the 3′ end. Complete sequence can be generated in conjunction with snake venom phosphodiesterase digestion of purified material. This method eliminates difficulties associated with other mass spectrometric sequencing techniques involving oligonucleotide length; structure; and sugar, base, and backbone modifications. Examples of sequencing a 17-mer composed primarily of 2′-*O*-methylribonucleotides and a single nonnucleosidic linker and a mixed sugar backbone 51-mer with 2′-*O*-methylribonucleotides and a homopolymer tail are reported in this study.

Key Words: **oligonucleotide sequencing; MALDI-TOF mass spectrometry; crude synthetic oligonucleotide coupling failures; solid-phase extraction.**

Oligonucleotides play an important role in the diagnostic industry for the detection of transmittable diseases. Genetically based assays that target and amplify RNA or DNA sequences provide a fast, specific, and sensitive means for detecting etiologic agents of disease (1). Characterization of the oligonucleotides inherent to this type of assay includes purity, identity, and sequence confirmation. The conventional sequencing involves separation of DNA ladders, generated by Sanger dideoxy termination reactions (2) or Maxam–Gilbert cleavages (3), by polyacrylamide gel electrophoresis. However, this technique is lengthy in time and requires the use of fluorescent dyes or isotopes to label the DNA. More recently, mass spectrometry has been successfully used for sequence determination.

Four methodologies have been utilized to sequence oligonucleotides by mass spectrometry. The first involves the analysis of Sanger dideoxy termination reactions using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)[2] mass spectrometry (4–14). Progressively, longer regions are being sequenced by increasing amounts of termination groups using cycle sequencing (6), optimizing reaction conditions (12), purifying extension products (7), eliminating salt adducts (8), and utilizing delayed extraction technology for better resolution (6). Although Taranenko *et al.* (12) identified sequence of primer extensions greater than 100 bases, MALDI-TOF se-

[1] To whom correspondence should be addressed at Gen-Probe Inc., 10210 Genetic Center Drive, San Diego, CA 92121-4362. Fax: (858) 410-8871. E-mail: davidal@gen-probe.com.

[2] Abbreviations used: MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; BSP, bovine spleen phosphodiesterase; CSP, calf spleen phosphodiesterase; SVP, snake venom phosphodiesterase; ESI, electrospray ionization; CID, collision-induced dissociation.

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quencing using the Sanger method has been limited to only unmodified, phosphodiester deoxyribonucleotides in the literature.

A second commonly used method for mass spectrometric sequencing involves the generation of oligonucleotide ladders by exonuclease digests (15–27). With this procedure, DNA fragments are generated from the sequential hydrolysis of nucleotides using a 5′ → 3′ bovine/calf spleen phosphodiesterase (BSP/CSP) or a 3′ → 5′ snake venom phosphodiesterase (SVP). Sequencing ladders have also been generated via chemical cleavage experiments (28, 29). These truncated oligonucleotides have been analyzed using both MALDI-TOF (15, 17, 19–24) and electrospray ionization (ESI) (16, 18, 25–27) mass spectrometry. Again, improved techniques such as delayed extraction, sample cleanup, and optimization of enzyme, buffer pH, and matrices have enhanced resolution to sequence DNA strands up to 35 bases. Furthermore, it has been reported that a uridine can be distinguished from a cytidine based on peak intensities (22) and mass accuracies reaching 0.01% (30). Even though enzymatic sequencing of oligonucleotides using mass spectrometry is practical and basic to interpret, this technique does not work well for backbone, sugar, and terminus modifications. Phosphorothioate, labeled, 2′-*O*-methylribose, and non-nucleosidic linker-containing oligonucleotides are resistant to phosphodiesterase digestion, although some groups have reported success in sequencing these modified structures. Pieles *et al.* (15) detected a 2′-*O*-methyl group on the ribose of a single adenosine. Schuette *et al.* (19) described the sequencing of phosphorothioate oligonucleotides by oxidizing them into their phosphodiester form prior to exonuclease digestion. Wu *et al.* (26) reported sequencing oligonucleotides with both termini blocked using *Crotalus duressus terrificus* snake venom phosphodiesterase with an acidic buffer. Also BSP/CSP and SVP cleave only single-stranded DNA; oligonucleotide regions exhibiting high levels of intra-/intermolecular structure would also be resilient to their digestion.

A third sequencing approach involves the gas-phase fragmentation of oligonucleotides by collision-induced dissociation (CID) using tandem mass spectrometry coupled with ion trap (24, 31–33), Fourier transform ion cyclotron resonance (34–36), and triple-quadrupole (24, 27, 37–45) ESI analyzers. Gas-phase fragments can also be generated via post source decay (46) and prompt fragmentation (47–51) following MALDI. With CID, parental ions are fragmented by collisions with neutral gas molecules, resulting in a distribution of multiply charged states. Using deconvolution software, these ion series are converted to the molecular mass of the oligonucleotide fragments to provide sequence information. Accuracy and resolution are very important to identify and separate the large number of fragmentation patterns. However, interpretation of the data can be complicated because cleavage can occur at the phosphate, sugar, and base sites of the standard and modified DNA. Although this technique is very sensitive and can identify modified oligonucleotides, it is limited to smaller oligonucleotides with less than 25 nucleotides for complete sequence information.

A fourth, less documented methodology involves the detection of failure sequences from a crude synthesis (49, 52, 53). By utilizing the inefficiency of nucleotide coupling during the synthesis process, failure species (N-1, N-2, N-3, etc.) corresponding to successive 3′ → 5′ terminations of the full-length product can be directly detected by MALDI-TOF mass spectrometry. Sequence information is generated by a series of failure ions, each of which varies by the molecular mass of the specific nucleotide not coupled. Keough *et al.* verified the majority of the sequence of a methylphosphonate 18-mer using this technique (52, 53). Juhasz *et al.* obtained sequence information up to the 3′ trinucleotide of a crude 31-mer (49). This approach is beneficial for sequencing structural and modified oligonucleotides, which are often resilient to enzymatic digestion, chemical cleavage, or Sanger termination reactions. Also much longer regions can be verified from a single crude sample. However, as coupling efficiencies reach greater than 98% for large-scale syntheses, the amounts of failure species fall below the detection limit of current MALDI-TOF applications.

The sum of failure species available from an oligonucleotide preparation depends upon the coupling efficiency of the synthesis process (54). Therefore, the total fraction of failure sequences can be defined by the equation

$$\% \text{ failure sequences} = [1 - 0.98^{(N-1)}] \times 100,$$

whereby 0.98 is the average coupling efficiency per cycle for an oligonucleotide of length N. For example, a 51-mer would minimally generate 36.4% full-length material and 63.6% failures ($[1 - 0.98^{(51-1)}] \times 100$), distributed among 51 different species. Consequently, it is necessary to extract and concentrate the failure sequences from the predominant, full-length oligonucleotide of a typical synthesis.

In this study, we describe a new approach for sequencing oligonucleotides using crude synthetic material. Using C_{18} solid-phase extraction cartridges, failure sequences are purified from the full-length oligonucleotide, which binds stronger due to the hydrophobicity of its dimethoxytrityl protecting group (Fig. 1). Extracted failure species are subsequently desalted, concentrated, and prepared for MALDI-TOF analysis to verify sequence. Examples of sequencing a 17-mer composed primarily of 2′-*O*-methylribonucleotides and

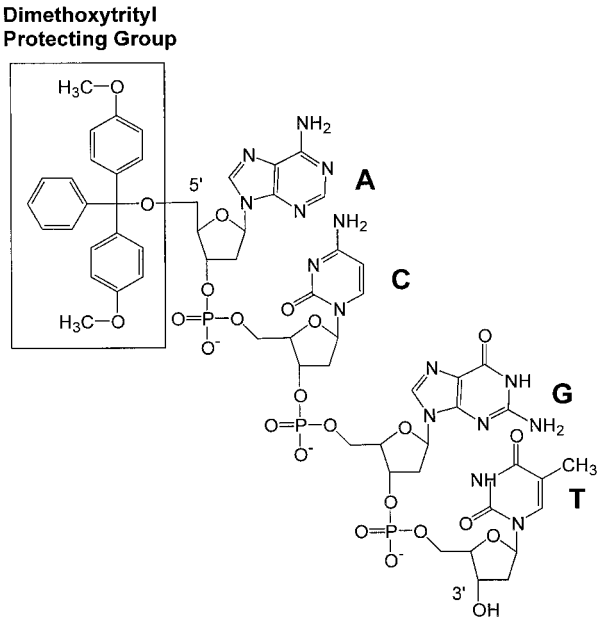

FIG. 1. Structure of a dimethoxytrityl protecting group attached to the 5′ base of an oligonucleotide.

a single nonnucleosidic linker (Fig. 2) (55) and a mixed sugar backbone 51-mer with 2′-*O*-methylribonucleotides and a homopolymer tail are reported.

MATERIALS AND METHODS

Synthesis of Crude Oligonucleotides

All oligonucleotides were synthesized at Gen-Probe Incorporated on an Oligo Pilot II (Amersham Pharmacia, Piscataway, NJ) utilizing standard phosphoramidite techniques. Synthesis scales ranged from 80 to 750 μmol. Synthetic strands (14-mers–55-mers) were produced using deoxyribose, 2′-*O*-methylribose, and/or linker phosphoramidites (Amersham Pharmacia) and cleaved from polystyrene phenoxyacetyl(dA) (Amersham Pharmacia) or control pore glass supports (Proligo, Boulder, CO) using 30% ammonium hydroxide (J. T. Baker, Phillipsburg, NJ). All other bulk reagents for synthesis were purchased from Amersham Pharmacia, except for acetonitrile (J. T. Baker). Concentrations of crude oligonucleotides were determined by UV absorbency at 260 nm using a HP 8453 UV/visible spectrophotometer (Hewlett-Packard, Palo Alto, CA).

Extraction and Concentration of Failure Sequences from Crude Material

A crude oligonucleotide (1–2 mg) was speed vacuumed overnight to remove ammonium hydroxide. Dried sample was redissolved by adding 2 ml of 0.1 M triethylamine acetate (Glen Research, Sterling, VA), heating at 95°C for 5–10 min, and vortexing. For purification, Sep-Pak Plus C_{18} cartridges (Waters Corporation, Milford, MA) were conditioned with 10 ml acetonitrile (Fisher Scientific, Pittsburgh, PA), followed by 5 ml of 2 M triethylamine acetate. The crude preparation was loaded onto the cartridges and washed with 10 ml water. Failure sequences were eluted using 2 ml of 17–18% acetonitrile in 0.1 M triethylamine acetate; the majority of the dimethoxytrityl-on, full-length oligonucleotide remained bound. The eluent was diluted with 5 ml of 0.1 M triethylamine acetate. For desalting, a new Sep-Pak cartridge was conditioned as before. The diluted failure elution was loaded onto the cartridge, washed with 10 ml water, and eluted with 1 ml of 100% acetonitrile. The extracted failure sequences were concentrated under vacuum to approximately 20 μl.

Sample Preparation for MALDI-TOF Analysis

Matrix, buffer, and cation-exchange beads were obtained from a Sequazyme oligonucleotide sequencing kit (PerSeptive Biosystems, Framingham, MA). Concentrated oligonucleotide failure sequences were diluted 1:10 with water. Two microliters of this dilution was mixed with 1 μl ammonium citrate buffer (50 mg/ml, pH 9.4) and 7 μl of 3-hydroxypicolinic acid (50 mg/ml). This sample was mixed with cation-exchange beads by pipetting for further desalting. Two microliters of the supernatant was spotted on the sample plate and allowed to dry at room temperature.

MALDI-TOF Mass Spectrometry

All DNA samples were analyzed using a linear Voyager DE mass spectrometer (PerSeptive Biosystems) equipped with a 337-nm laser. Two methods were employed depending on the oligonucleotide length. For

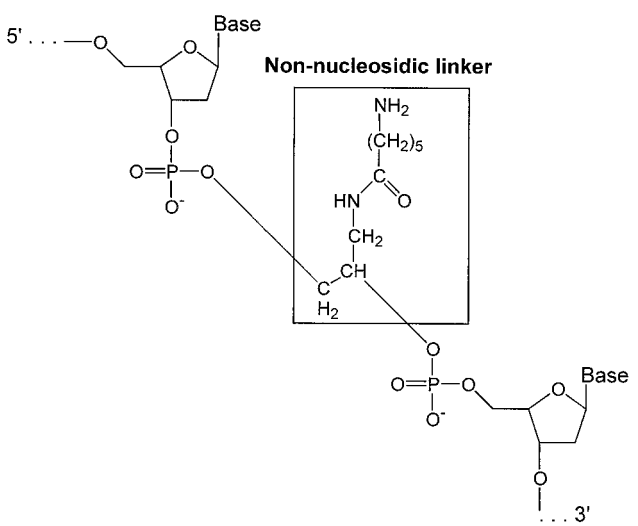

FIG. 2. Structure of a nonnucleosidic linker coupled between an oligonucleotide chain.

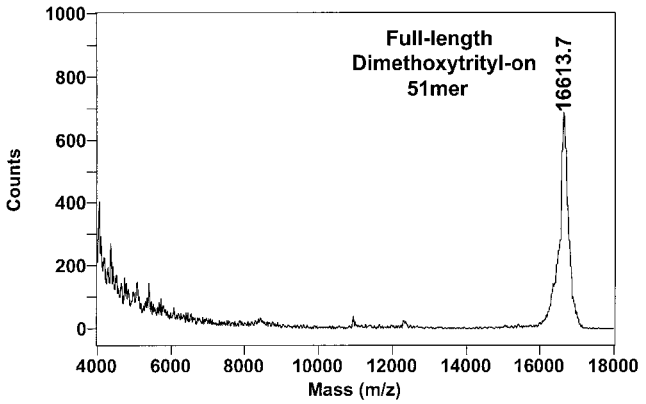

FIG. 3. Positive ion MALDI-TOF mass spectrum from the straight application of a crude synthesis of a mixed sugar backbone 51-mer.

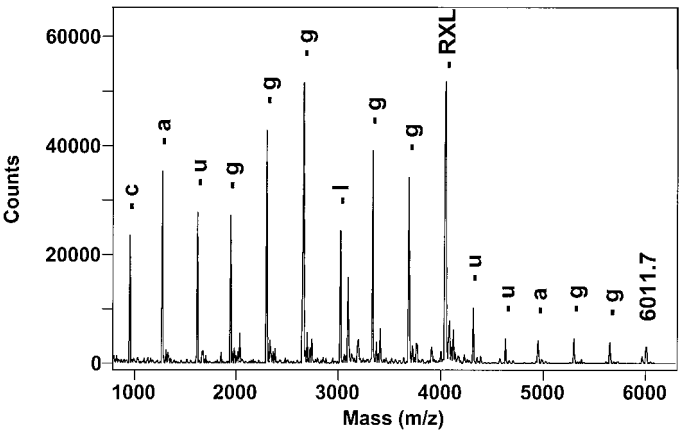

FIG. 4. Positive ion MALDI-TOF mass spectrum of extracted failure sequences from the crude synthesis of a 17-mer (5′ gga uu(RXL)g gIg ggu aca gT 3′) composed primarily of 2′-*O*-methylribonucleotides and a single nonnucleosidic linker. See Table 2 for molecular mass differences obtained for base assignment. Note: Uppercase letters denote deoxyribonucleotides, lowercase letters denote 2′-O-methylribonucleotides, and RXL denotes nonnucleosidic linker.

oligonucleotides less than 35 bases, the following instrument parameters were used: positive ion mode; 25,000 acceleration voltage; 92.5% grid voltage; 0.15% guide wire voltage; 250 ns delay time. For oligonucleotides greater than 35 bases, the following instrument parameters were used: positive ion mode; 25,000 acceleration voltage; 91.5% grid voltage; 0.15% guide wire voltage; 400 ns delay time. Spectra were obtained using laser intensities ranging from 2300 to 2600. Sequence was determined using PerSeptive Grams/386 software, version 3.04, which calculated the mass difference between ions for identification of a particular base.

RESULTS AND DISCUSSION

The synthesis procedure for oligonucleotides generates failure sequences, due to the inefficiency of nucleotide coupling. Analyzing the failure species from crude synthetic material, Keough *et al.* (52, 53) and Juhasz *et al.* (49) verified sequence of a methylphosphonate 18-mer and an unmodified 31-mer by MALDI-TOF. The advantage of this technique is that sequencing information can be determined for modified and structural oligonucleotides resilient to enzymatic hydrolysis, Sanger termination reactions, or chemical cleavage. However, sequencing using straight application of crude material is hindered by a highly efficient synthesis, which produces levels of failure sequences too low for detection (Fig. 3). Crude material contains predominantly the full-length oligonucleotide, which can suppress the MALDI-TOF signal when loaded at high concentrations. Using this new approach, failure species are extracted from the dimethoxytrityl-on, full-length oligonucleotide, resulting in a concentrated series of failure products readily detectable by MALDI-TOF. Sequence is verified by the mass difference between failure ions to identify a particular base (Table 1).

Figure 4 shows the MALDI-TOF mass spectrum of enriched failure sequences from a 17-mer, which con-

TABLE 1

Molecular Masses of Deoxyribonucleotides, 2′-*O*-Methylribonucleotides, and the Nonnucleosidic Linker

Nucleotide	Symbol	Molecular mass (Da)	Molecular mass criteria range for base assignment (Da)
Deoxyriboadenosine-5′-phosphate	A	313.2	309.0–314.0
2′-*O*-Methylriboadenosine-5′-phosphate	a	343.3	340.0–350.0
Deoxyribocytidine-5′-phosphate	C	289.2	280.0–295.0
2′-*O*-Methylribocytidine-5′-phosphate	c	319.2	316.7–319.6
Deoxyriboguanosine-5′-phosphate	G	329.2	325.1–339.9
2′-*O*-Methylriboguanosine-5′-phosphate	g	359.2	350.1–370.0
Deoxyribothymidine-5′-phosphate	T	304.2	295.1–308.9
2′-*O*-Methylribouridine-5′-phosphate	u	320.2	319.6–325.0
Deoxyriboinosine-5′-phosphate	I	314.2	314.0–316.7
Nonnucleosidic linker	RXL	266.1	255.0–279.0

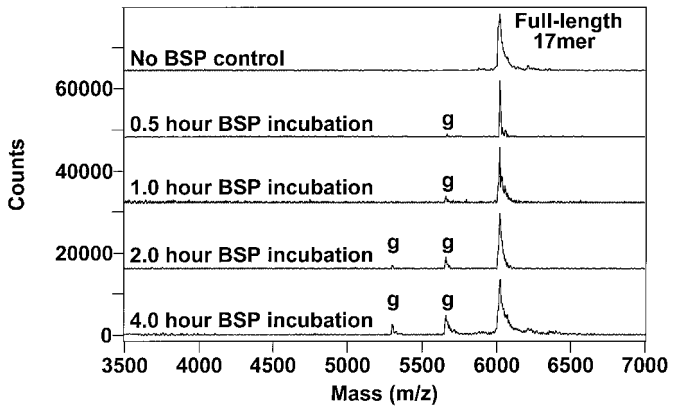

FIG. 5. Positive ion MALDI-TOF mass spectra of bovine spleen phosphodiesterase (BSP) digestions of a 17-mer composed primarily of 2′-*O*-methylribonucleotides and a single nonnucleosidic linker. Base hydrolysis was performed at 37°C using 1 μl BSP at the highest concentration according to the Sequazme oligonucleotide sequencing kit (PerSeptive Biosystems, Framingham, MA), 1 μl 0.1 M ammonium citrate buffer, pH 5.6, and 300 pmol oligonucleotide. Hydrolysis reactions were stopped by the addition of 3-hydroxypicolinic acid (50 mg/ml) matrix at the various time intervals. Note: Lowercase "g" denotes 2′-*O*-methylriboguanosine.

tains 15 2′-*O*-methylribonucleotides, two deoxyribonucleotides, and one nonnucleosidic linker located internally. With this oligonucleotide, enzymatic sequencing was extremely problematic due to the very slow hydrolysis of the 2′-*O*-methylribonucleotides and the inability to digest beyond the nonnucleosidic linker (Fig. 5). Results after the extraction procedure demonstrate removal of the dimethoxytrityl-on, full-length oligonucleotide, leaving only the failures species for sequencing. Excellent mass spectral signals (up to 50,000 counts) and resolution of the failure ions were observed. Sequence was verified for 13 2′-*O*-methylribonucleotides and one deoxyriboinosine, as well as the nonnucleosidic linker. As mass signals approach the smaller 3′ sequences (<1000 Da), doubly/triply charged ions and matrix peaks interfere with the detection of these failure species. Therefore, complete sequencing information for the remaining 3′ end was obtained using SVP under stringent incubation conditions. Table 2 summarizes the mass differences between observed failure ions by MALDI-TOF.

Figure 6 illustrates the MALDI-TOF mass spectrum of purified failure sequences from a much longer oligonucleotide, a 51-mer. This oligonucleotide contains a region of 2′-*O*-methylribonucleotides, which, as with the 17-mer above, greatly hindered phosphodiesterase sequencing. This oligonucleotide also contains a long polydeoxyadenosine 3′ tail susceptible to secondary intra/intermolecular structure. Similarly exonuclease sequencing would be deficient, since BSP/CSP and SVP cleave only single-stranded DNA. Again extraction resulted in a series of concentrated failure sequences and

TABLE 2

MALDI-TOF Mass Differences of Extracted Failure Sequences from the Crude Synthesis of a 17-mer Composed Primarily of 2′-*O*-Methylribonucleotides and a Nonnucleosidic Linker (see Fig. 4)

Theoretical mass $(M + H)^+$	Observed mass $(M + H)^+$	Molecular mass difference (Da)	Base assignment[a]
946.0	948.9		
1265.2	1268.2	319.3	c
1608.5	1611.9	343.7	a
1928.7	1932.4	320.5	u
2287.9	2291.9	359.5	g
2647.1	2651.4	359.5	g
3006.3	3010.6	359.2	g
3320.5	3324.9	314.3	I
3679.7	3684.2	359.3	g
4038.9	4043.7	359.5	g
4305.0	4309.8	266.1	RXL
4625.2	4629.9	320.1	u
4945.4	4949.9	320.0	u
5288.7	5294.0	344.1	a
5647.9	5652.7	358.7	g
6007.1	6011.7	359.0	g

[a] Uppercase letters denote deoxyribonucleotides, lowercase letters denote 2′-*O*-methylribonucleotides, and RXL denotes nonnucleosidic linker.

the removal of the dimethoxytrityl, full-length oligonucleotide. Strong mass spectral signals and resolution of failure ions were obtained. Sequence was verified for 47 of the 51 bases from the MALDI-TOF spectrum acquired by the application of purified material to a single well on a sample plate. Complete sequencing information for the remaining four bases on the 3′ end

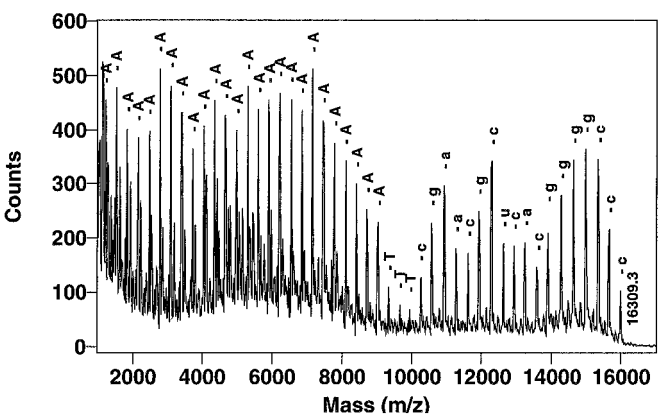

FIG. 6. Positive ion MALDI-TOF mass spectrum of extracted failure sequences from the crude synthesis of a mixed sugar backbone 51-mer (5′ ccc ggg gca cuc gca agc TTT AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA 3′). See Table 3 for molecular mass differences obtained for base assignment. Note: Uppercase letters denote deoxyribonucleotides, and lowercase letters denote 2′-*O*-methylribonucleotides.

TABLE 3

MALDI-TOF Mass Differences of Extracted Failure Sequences from the Crude Synthesis of a Mixed Sugar Backbone 51-mer (see Fig. 6)

Theoretical mass/charge $(M + H)^+$	Observed mass/charge $(M + H)^+$	Molecular mass difference (Da)	Base assignment[a]
1191.7	1196.5		
1504.9	1509.7	313.2	A
1818.1	1822.9	313.2	A
2131.3	2136.5	313.6	A
2444.5	2450.2	313.7	A
2757.7	2763.9	313.7	A
3070.9	3077.5	313.6	A
3384.1	3390.9	313.4	A
3697.3	3703.6	312.7	A
4010.5	4016.7	313.1	A
4323.7	4330.1	313.4	A
4636.9	4643.7	313.6	A
4950.1	4957.2	313.5	A
5263.3	5270.6	313.4	A
5576.5	5584.1	313.5	A
5889.7	5897.5	313.4	A
6202.9	6210.9	313.4	A
6516.1	6523.6	312.7	A
6829.3	6836.6	313.0	A
7142.5	7149.9	313.3	A
7455.7	7463.6	313.7	A
7768.9	7777.2	313.6	A
8082.1	8090.1	312.9	A
8395.3	8403.1	313.0	A
8708.5	8716.4	313.3	A
9021.7	9029.6	313.2	A
9334.9	9343.1	313.5	A
9639.1	9646.1	303.0	T
9943.3	9948.2	302.1	T
10247.5	10255.2	307.0	T
10566.7	10574.4	319.2	c
10925.9	10933.3	358.9	g
11269.2	11276.4	343.1	a
11612.5	11619.6	343.2	a
11931.7	11938.6	319.0	c
12290.9	12297.6	359.0	g
12610.1	12616.3	318.7	c
12930.3	12937.3	321.0	u
13249.5	13256.5	319.2	c
13592.8	13600.1	343.6	a
13912.0	13919.0	318.9	c
14271.2	14278.6	359.6	g
14630.4	14637.4	358.8	g
14989.6	14996.4	359.0	g
15348.8	15355.9	359.5	g
15668.0	15674.7	318.8	c
15987.2	15992.0	317.3	c
16306.4	16309.3	317.3	c

[a] Uppercase letters denote deoxyribonucleotides, and lowercase letters denote 2′-*O*-methylribonucleotides.

was easily acquired using SVP digestions. Table 3 summarizes the mass differences between failure ions by MALDI-TOF.

The extraction procedure utilizes the hydrophobicity of the dimethoxytrityl group present on the full-length oligonucleotide. Crude material is bound to a C_{18} stationary-phase cartridge conditioned with 2 M triethylamine acetate. Shorter failure sequences are eluted with 17% acetonitrile in 0.1 M triethylamine acetate, while longer failures (>35 bases) require 18% acetonitrile in 0.1 M triethylamine acetate. Acetonitrile percentages greater than 18% will begin to elute the dimethoxytrityl-on, full-length oligonucleotide with the failures. Reloading the purified failure products on a new C_{18} cartridge is helpful in eliminating the triethylamine acetate and other adducts prior to MALDI-TOF analysis. When concentrating the failures under speed vacuum, it is important not to allow the sample to dry completely; leaving a concentrate of approximately 20 μl negates any problems with dissolving the material back into solution. Also straight application of the concentrate for MALDI-TOF results in poor crystallization of the matrix due to the acetonitrile; a 1:10 dilution with water greatly enhances crystallization and the ionization of the failure sequences.

Mass spectrometry has emerged as a useful alternative for sequencing oligonucleotides. Various strategies (Sanger termination reactions, enzymatic digestion, chemical cleavage, gas-phase fragmentation) using MALDI-TOF and ESI are currently being employed, although each technique has its own limitation depending on the oligonucleotide length; structure; and base, sugar, and backbone modifications. MALDI-TOF analysis of crude synthetic material can confirm oligonucleotide sequence, but it relies on sufficient amounts of failure species for detection. By extracting and concentrating the failure products from the dimethoxytrityl-on, full-length material, a single MALDI-TOF analysis can verify sequence for the majority of an oligonucleotide, independent of its length and any structural modifications. This technique has been beneficial for characterization of oligonucleotides inherent to genetically based diagnostic assays and provides an additional tool for sequence identification in the biotechnology industry.

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