Sequencing Oligonucleotides by Enrichment of Coupling Failures Using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

This unit describes a procedure for sequencing production-scale synthetic oligonucleotides. Coupling failure sequences in a crude synthesis are isolated from the full-length 5′-*O*-DMTr-oligonucleotide using a DMTr-selective C18 purification cartridge (see *UNIT 10.7*). The extraction is subsequently analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to determine the mass difference between failure ions for identification of a particular base or structural modification. The methods presented here are based on MALDI-TOF-MS procedures in *UNIT 10.1*, which should be consulted for additional details.

EXTRACTION, DESALTING, AND CONCENTRATION OF FAILURE SEQUENCES FROM A CRUDE OLIGONUCLEOTIDE

BASIC PROTOCOL 1

This protocol describes the extraction of coupling failure sequences from the full-length 5′-*O*-DMTr-oligonucleotide of a crude synthesis. A crude synthesis is first prepared for loading onto a C18 reversed-phase purification cartridge. Coupling failures are then eluted from the full-length oligonucleotide, which binds more strongly to the cartridge due to the hydrophobicity of its attached DMTr protecting group. The eluate is then desalted and concentrated, resulting in a series of failure products readily detectable by MALDI-TOF analysis. Refer also to *UNIT 10.7* regarding DMTr-selective cartridge purification and affinity cartridge desalting of detritylated oligonucleotides.

Materials

Crude 5′-*O*-(4,4′-dimethoxytrityl)-protected (DMTr) oligonucleotide (1 to 2 mg from an 80- to 750-μmol scale synthesis), ammoniacal solution
0.1 and 2.0 M triethylammonium acetate (TEAA), pH 7.0, HPLC grade
Acetonitrile (MeCN), HPLC grade
Ultrapure (e.g., Milli-Q) water
17% or 18% (v/v) MeCN in 0.1 M TEAA, pH 7.0 (see recipe)
Vacuum centrifugation system (Speedvac)
Temperature-controlled heating block
5-mL disposable plastic syringes with exposed Luer tips
C18 reversed-phase oligonucleotide purification cartridges (e.g., Sep-Pak Plus, Waters Corporation)

Prepare crude oligonucleotide

1. Evaporate the ammoniacal solution of a crude 5′-*O*-DMTr-oligonucleotide synthesis to dryness under vacuum using a Speedvac overnight.
2. Add 2 mL of 0.1 M TEAA to the dried oligonucleotide.
3. Heat sample at 95°C for 10 min, then vortex into solution.

   The sample may contain residual material that does not go into solution. Do not overheat, as this can result in removal of the DMTr group from the full-length oligonucleotide.

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Extract failure sequences

4. Using a 5-mL disposable plastic syringe, pass 5 mL MeCN and then 5 mL of 2 M TEAA through a C18 reversed-phase oligonucleotide purification cartridge into a waste container.

   All reagents should be eluted from the cartridge in a dropwise fashion.

   Multiple cartridges can optionally be used with a vacuum manifold system (Analytichem or National Scientific; optional) with an in-line trap for waste and a water aspirator pump or small vacuum pump.

5. Slowly pass the dissolved oligonucleotide solution at approximately 1 drop/second through the cartridge to the waste.

6. Pass 10 mL of ultrapure water through the cartridge to the waste.

7. Elute and collect failure sequences with 2 mL of 17% or 18% (v/v) MeCN in 0.1 M TEAA, pH 7.0.

   Use 17% MeCN in 0.1 M TEAA for shorter sequences (≤35 bases) and 18% MeCN in 0.1 M TEAA for longer sequences (>35 bases).

8. Dilute the eluate with 5 mL of 0.1 M TEAA.

Desalt and concentrate failure sequences

9. Pass 5 mL MeCN and then 5 mL of 2 M TEAA through a new purification cartridge into a waste container.

10. Slowly pass the diluted eluate (containing failure sequences) at approximately 1 drop/second through the new cartridge to the waste.

11. Pass 10 mL ultrapure water through the cartridge to the waste.

12. Elute and collect desalted failure sequences with 1 mL MeCN.

13. Concentrate extracted failure sequences under vacuum using a Speedvac to ~20 to 50 μL.

    Do not allow the sample to dry completely.

BASIC PROTOCOL 2

PREPARATION OF OLIGONUCLEOTIDE FAILURE SEQUENCES FOR MALDI-TOF ANALYSIS

This protocol describes the preparation of extracted failure sequences for MALDI-TOF analysis. Diluted oligonucleotide coupling failure sequences are mixed with ammonium citrate buffer and 3-hydroxypiconilic acid for spotting on a sample plate well. Refer also to *UNIT 10.1* for alternative procedures for matrix/co-matrix/analyte preparation.

Materials

Concentrated oligonucleotide failure sequences (see Basic Protocol 1)
Ultrapure (e.g., Milli-Q) water
50 mg/mL ammonium citrate buffer, pH 9.4 (from Sequazyme Oligonucleotide Sequencing Kit, PerSeptive Biosystems, or see recipe)
50 mg/mL 3-hydroxypiconilic acid (3-HPA) matrix (see recipe)
Ammonium-activated cation-exchange resin beads (see *UNIT 10.1*)
Parafilm

1. Dilute concentrated oligonucleotide failure sequences 1:10 with ultrapure water.

2. On a small piece of Parafilm, combine 2 μL of diluted failure sequences, 1 μL of 50 mg/mL ammonium citrate buffer (pH 9.4), and 7 μL of 3-HPA matrix.

3. Add approximately 0.1 mg of ammonium-activated cation-exchange resin beads (a small spatula tip full) to cover the bottom of the solution droplet.

   Do not overload the beads, which can completely absorb the droplet.

4. Mix droplet and beads by withdrawing and expelling the solution 10 times with a pipettor.

5. Allow beads to settle for 30 sec and then spot 2 μL of the supernatant on a sample plate well.

6. Allow spot to crystallize at room temperature for approximately 10 min.

   Do not load the sample plate until the spot has crystallized to dryness.

MALDI-TOF ANALYSIS

BASIC PROTOCOL 3

Experimental Conditions

Conditions for MALDI-TOF analysis are based on operation of the linear Voyager-DE mass spectrometer (PerSeptive Biosystems) equipped with a 337-nm laser. Instrument parameters depend in part on sequence length.

For shorter oligonucleotides (≤35 bases):

Positive ion mode
25,000 acceleration voltage
92.5% grid voltage
0.15% guide wire voltage
250 nsec delay extraction time.

For longer oligonucleotides (>35 bases):

Positive ion mode
25,000 acceleration voltage
91.5% grid voltage
0.15% guide wire voltage
400 nsec delay extraction time.

Spectra are obtained using laser intensities ranging from 2300 to 2600 μJ, or just above the threshold of ion detection. Typically, 50 to 100 scans are collected. Smoothing is performed using 19 points and a polynomial order of 2. Instrument parameters may need optimization according to the particular mass spectrometer configuration

Table 10.10.1 Characteristic Molecular Masses for Deoxyribonucleotides, 2′-*O*-Methylribonucleotides, and a Non-Nucleosidic Linker[a]

Nucleotide	Symbol	Mass (Da)
2′-Deoxyadenosine-5′-phosphate	A	313.2
2′-Deoxycytidine-5′-phosphate	C	289.2
2′-Deoxyguanosine-5′-phosphate	G	329.2
2′-Deoxythymidine-5′-phosphate	T	304.2
2′-*O*-Methyladenosine-5′-phosphate	a	343.3
2′-*O*-Methylcytidine-5′-phosphate	c	319.2
2′-*O*-Methylguanosine-5′-phosphate	g	359.2
2′-*O*-Methyluridine-5′-phosphate	u	320.2
Non-nucleosidic linker	X	266.1

[a]Adapted from Alazard et al. (2002) with permission from Elsevier.

(e.g., continuous extraction or a reflected pathlength). Manufacturer specifications and recommendations from *UNIT 10.1* (e.g., negative ion mode) may also influence the sensitivity and resolution.

Interpretation of Mass Spectrometry Data

After obtaining the mass of individual oligonucleotide coupling failures, the mass difference between successive spectral ions is calculated. The identification of a particular

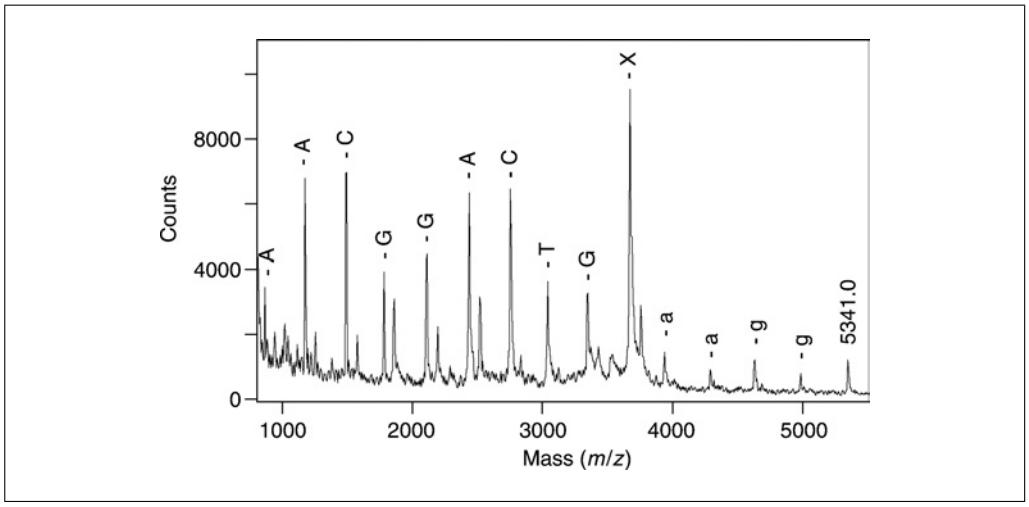

Figure 10.10.1 Positive-ion MALDI-TOF mass spectrum of extracted failure sequences from the crude synthesis of a 16-mer (5′ gga aXG TCA GGC AAC AT 3′). Uppercase letters denote deoxyribonucleotides, lowercase letters denote 2′-*O*-methylribonucleotides, and X denotes the non-nucleosidic linker. See Table 10.10.2 for molecular mass differences obtained for base assignment.

Table 10.10.2 Molecular Mass Differences of Enriched Failure Sequences from a 16-mer Composed of Deoxyribonucleotides, 2′-*O*-Methylribonucleotides, and a Non-Nucleosidic Linker

Observed mass $(M + H)^+$	Molecular mass difference (Da)	Base assignment[a]
852.4		
1166.8	314.4	A
1481.1	314.3	A
1771.4	290.3	C
2101.5	330.1	G
2431.4	329.9	G
2745.3	313.9	A
3035.3	290.0	C
3340.5	305.2	T
3669.3	328.8	G
3936.6	267.3	X
4281.9	341.9	a
4623.8	345.1	a
4981.0	357.2	g
5341.0	360.0	g

[a]Uppercase letters denote deoxyribonucleotides, lowercase letters denote 2′-*O*-methylribonucleotides, and X denotes the non-nucleosidic linker.

nucleotide or structural modification is determined based on its characteristic mass (Table 10.10.1). Sequencing information is read from the 5′→3′ direction, as demonstrated for a sample 16-mer with a mixed sugar backbone and non-nucleosidic linker (Table 10.10.2 and Fig. 10.10.1).

REAGENTS AND SOLUTIONS

Use ultrapure (e.g., Milli-Q-purified) water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acetonitrile (MeCN) in 0.1 M triethylammonium acetate (TEAA), pH 7.0, 17% or 18% (v/v)

Measure 1.7 or 1.8 mL of MeCN in a 15-mL centrifuge tube. Add 0.1 M TEAA, pH 7.0 (see recipe) to a volume of 10 mL and mix. Prepare fresh prior to use and keep at room temperature.

Storage for greater than 3 days is not recommended.

Ammonium citrate, pH 9.4, 50 mg/mL

Dissolve 5.0 g dibasic ammonium citrate in 100 mL ultrapure water. Adjust pH to 9.4 with ammonium hydroxide. Store up to 1 month at 4°C.

3-Hydroxypiconilic acid (3-HPA), 50 mg/mL

Weigh 0.05 g of 3-HPA in a 1.5-mL microcentrifuge tube. Add 1 mL ultrapure water, then vortex into solution. Store up to 1 month at 4°C.

Triethylammonium acetate (TEAA), pH 7.0, 0.1 M

Measure 5.0 mL of 2.0 M TEAA, pH 7.0 (commercially available), in a 100-mL storage bottle. Add ultrapure water to a volume of 100 mL and mix. Prepare fresh prior to use and keep at room temperature.

Storage for greater than 3 days is not recommended.

COMMENTARY

Background Information

Since the early 1990s, mass spectrometry has emerged as a successful alternative for the sequence determination of oligonucleotides. Various strategies—such as collision-induced dissociation (McLuckey et al., 1992; Little et al., 1996; Ni et al., 1996), Sanger termination reactions (Köster et al., 1996; Roskey et al., 1996; Taranenko et al., 1998), chemical cleavage (Polo et al., 1997; Isola et al., 1999), and enzymatic digestion (Limbach et al., 1994; Wu and Aboleneen, 2000) using electrospray ionization (*UNIT 10.2*) and MALDI-TOF (*UNIT 10.1*)—are currently being employed. However, each technique has its own limitation dependent on the interpretation of data, the oligonucleotide length, and structural modifications.

The generation of oligonucleotide fragments using exonucleases is the more commonly used approach for MALDI-TOF sequencing (Pieles et al., 1993; Schuette et al., 1995; Smirnov et al., 1996). With this procedure, "mass ladders" (*UNIT 10.1*) are created by the partial, sequential hydrolysis of nucleotides using a 5′→3′ calf spleen phosphodiesterase (CSP) or a 3′→5′ snake venom phosphodiesterase (SVP). The mass difference between successive fragment ions is calculated to identify a particular base. This technique is practical and easy to interpret. However, backbone, sugar, and terminal modifications are resilient to phosphodiesterase digestion. Also CSP and SVP cleave only single-stranded oligonucleotides; regions with a high degree of intra- or intermolecular structure will also resist hydrolysis.

Another straightforward approach to MALDI-TOF sequencing utilizes crude synthetic oligonucleotides (Juhasz et al., 1996; Keough et al., 1996; Alazard et al., 2002). Similar to exonuclease digestion, the mass

difference between spectral ions is calculated, but the coupling failures generated during synthesis are used for the calculation. However, with a highly efficient synthesis, the amount of failure species can fall below the detection limit of MALDI-TOF analysis, especially with the predominant, full-length product still present. Therefore, enrichment of the failure sequences using DMTr-selective C18 purification cartridges is quite beneficial, since it enhances the signal and resolution of failure ions, allowing for an accurate confirmation of an oligonucleotide sequence.

This technique is useful for routine quality control testing or as a process verification. It is helpful for sequencing modified oligonucleotides, since it depends on the coupling efficiency of the synthetic process and not structural alterations affecting enzymatic digestion. Modified and non-nucleosidic amidites will, if anything, have a reduced coupling efficiency, thus increasing their failure signal.

Critical Parameters and Troubleshooting

After the crude ammoniacal solution of an oligonucleotide synthesis is evaporated overnight, 0.1 M TEAA is added and the sample is heated slightly to help dissolve the dried material. Overheating must be avoided since it will cause detritylation, which in turn will cause a large amount of the full-length material to elute with the coupling failures during extraction. The ensuing MALDI-TOF analysis will result in an excess of the full-length signal, suppressing the ions of the desired sequencing failures. Some insoluble material may remain after vortexing. It should be allowed to settle, and only the dissolved solution should be loaded on the cartridge to prevent clogging.

The extraction procedure utilizes the increased hydrophobicity of the full-length 5′-*O*-DMTr-protected oligonucleotide. Failure sequences are eluted using 17% or 18% MeCN in 0.1 M TEAA, pH 7.0, depending on their length. MeCN percentages greater than 18% will co-elute the DMTr-on product with the failures. Highly hydrophobic modifications may require a stepwise increase in the MeCN percentage to elute failure species.

When concentrating extracted failure sequences under vacuum centrifugation, it is important to prevent the solution from drying completely. A residual concentrate of approximately 20 to 50 μL will avoid difficulty dissolving a dried sample back into solution. A 1:10 dilution of the concentrate with water will improve crystallization with the matrix, enhancing ionization of the coupling failure sequences during MALDI-TOF analysis.

Sequence information is obtained from the 5′ end to the beginning of the 3′ end. As mass signals approach the smaller 3′ sequences (<1000 Da), matrix and doubly or triply charged ions interfere with their detection. Complete sequencing at the 3′ end can be obtained using SVP hydrolysis if applicable (*UNIT 10.1*). Also, adduct peaks can complicate the reading of sequencing failures; desalting with a C18 cartridge and cation-exchange beads will help reduce the formation of these additional ions.

Anticipated Results

Following the above protocols, strong mass spectral signals and resolution of failure sequence ions by MALDI-TOF mass spectrometry are obtained. Sequencing information for oligonucleotides (≤60 bases) from the 5′ end up to three to four nucleotides at the 3′ end is expected. Backbone, sugar, and terminal (e.g., phosphorothioate, 2′-*O*-methylribose, fluorescent labeling) modifications can also be identified using this procedure.

Time Considerations

The evaporation of an ammoniacal solution of a crude oligonucleotide synthesis is best done overnight prior to the extraction procedure and MALDI-TOF analysis, since vacuum centrifugation can take up to 6 to 8 hr depending on the sample volume. The extraction and desalting of the oligonucleotide failure sequences typically requires 1 hr. The concentration of these purified coupling failure sequences to approximately 20 to 50 μL requires ~2 hr. The sample preparation for MALDI-TOF analysis and data interpretation generally takes 30 to 60 min.

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