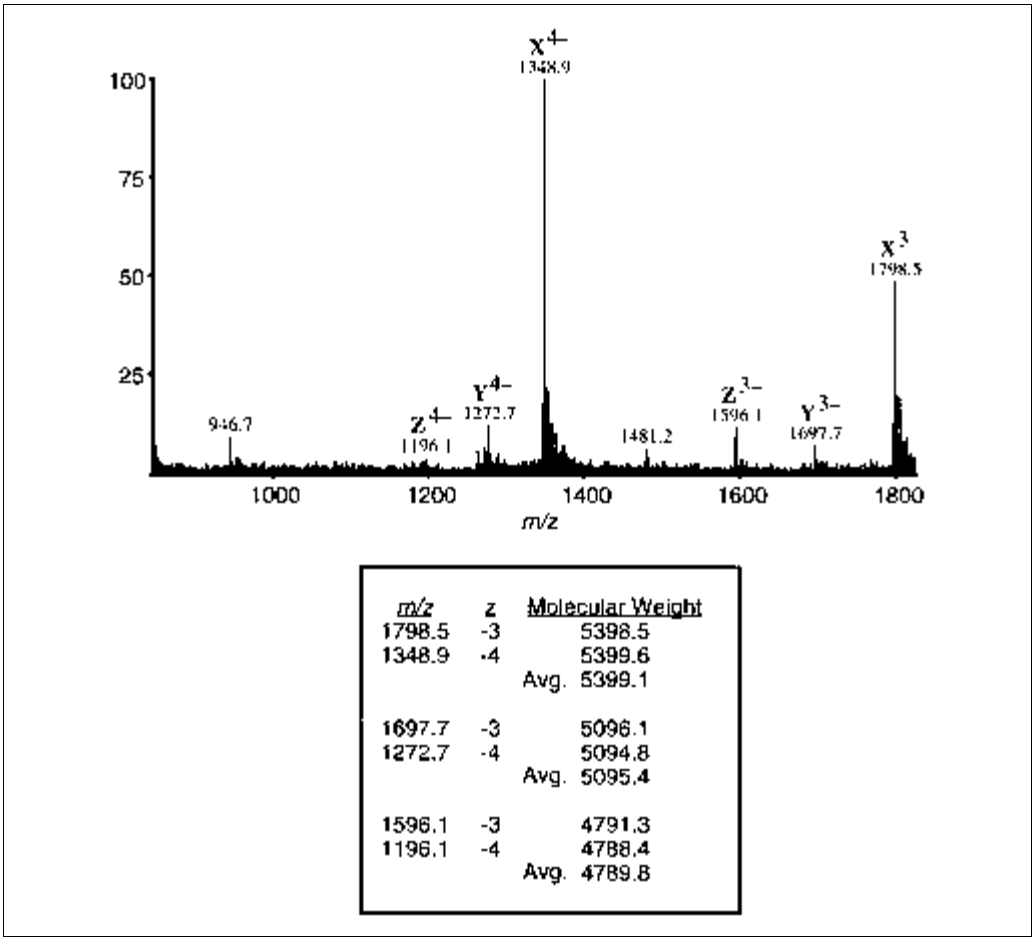
# Analysis of Oligonucleotides by Electrospray Ionization Mass Spectrometry

Because of the high molecular weights and thermal lability of biomolecules such as nucleic acids and proteins, the analysis of these molecules by mass spectrometry is a rather difficult process. Two major problems in such analyses are the need for a "soft" ionization method capable of generating intact molecular ions and the limited upper mass range of most mass analyzers. In the mid 1980s, Fenn and co-workers demonstrated that electrospray ionization (ESI) could be used to analyze molecules with molecular weights ($M_r$) larger than the mass-to-charge ratio ($m/z$) limit of the mass analyzer (Fenn et al., 1989). Later work on oligonucleotides opened the door to accurate, high-resolution analysis of these compounds by ESI-MS (Covey et al., 1988).

ESI allows for the analysis of high-molecular-weight compounds through the generation of multiply charged ions in the gas phase. Because the basis of the mass spectrometric measurement is the $m/z$ value of the molecule, the presence of multiple charges on the molecule will result in a decrease in the $m/z$ values and allow characterization using mass analyzers with limited $m/z$ ranges. A typical example of the electrospray mass spectra one obtains for oligonucleotides is shown in Figure 10.2.1. The sample contains three unique




| m/z | z | Molecular Weight |
|---|---|---|
| 1798.5 | -3 | 5398.5 |
| 1348.9 | -4 | 5399.6 |
| | Avg. | 5399.1 |
| 1697.7 | -3 | 5096.1 |
| 1272.7 | -4 | 5094.8 |
| | Avg. | 5095.4 |
| 1596.1 | -3 | 4791.3 |
| 1196.1 | -4 | 4788.4 |
| | Avg. | 4789.8 |

**Figure 10.2.1** Representative electrospray mass spectrum of a mixture of oligonucleotides. X = 17-mer, Y = 16-mer, and Z = 15-mer. The characteristic feature of an electrospray mass spectrum is the presence of multiply charged ions of each analyte.

**Contributed by Lenore M. Polo and Patrick A. Limbach**
*Current Protocols in Nucleic Acid Chemistry* (2000) 10.2.1-10.2.20

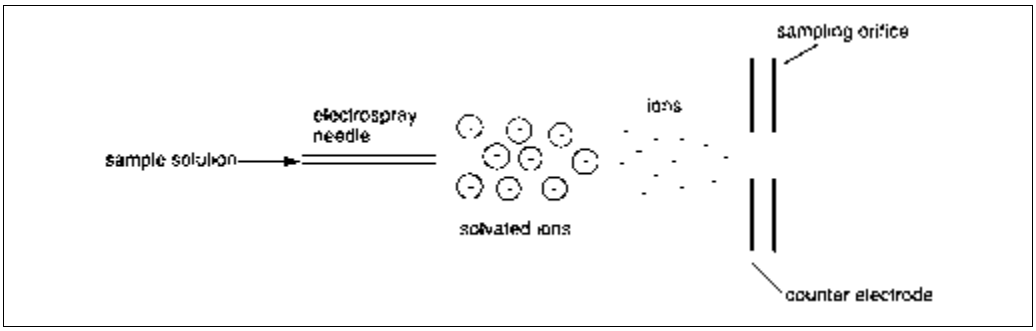



**Figure 10.2.2** Diagram of a typical electrospray source.

oligonucleotides—a 17-mer, a 16-mer, and a 15-mer—designated X, Y, and Z, respectively. As is illustrated in this spectrum, each of the oligonucleotide species are detected as multiply charged negative ions. Thus, even though the molecular weights of each oligonucleotide are greater than the *m/z* range of the mass analyzer (here 2000), the sample can still be identified due to the presence of the multiply charged peaks. A discussion on identification of oligonucleotide-ion charge state and molecular weight will be presented later in this unit.

A necessary requirement for ESI is that the analyte molecules be charged in solution. The negatively charged phosphate backbone of oligonucleotides allows for negative-ion-mode analysis of ESI-generated ions. A diagram of a typical electrospray source is shown in Figure 10.2.2. Transfer of ions from solution phase to the gas phase is accomplished by generating an electric field between a spraying needle that is held at a high negative potential and a counter-electrode held at ground or a positive potential some distance from the needle. The solution being sprayed exits the needle as a conical distribution of droplets ("Taylor cone"), each containing excess negative charge. A heated drying gas, such as nitrogen, is typically used to assist evaporation of the solvent sheath from the ion. The desolvated, multiply charged ion is then introduced into the mass spectrometer for analysis (Gaskell, 1997).

A number of applications of ESI to oligonucleotide and nucleic acid analysis have been reported since the introduction of this technique. Among the applications that will be discussed here are molecular mass measurement, sequence identification, and analysis of noncovalent complexes. In addition, the ESI source can be readily coupled with a number of separation techniques such as liquid chromatography (Apffel et al., 1997a,b; Glover et al., 1995), capillary electrophoresis (Barry et al., 1996), and capillary electrochromatography (Ding and Vouros, 1997) for the analysis of mixtures of oligonucleotides.

## ESI-MS VERSUS MALDI-MS FOR OLIGONUCLEOTIDE ANALYSIS

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) for oligonucleotide analysis is discussed in *UNIT 10.1*. Here, a brief comparison of the two approaches for oligonucleotide analysis is presented; a summary is provided in Table 10.2.1. The advantages of ESI-MS include higher mass accuracies, ease of coupling to on-line separation methods, and the ability to characterize noncovalent interactions; it is also the preferred method for gas-phase MS sequencing (tandem MS). MALDI-MS is more tolerant of sample contaminants, can handle complex mixture analysis, and is the preferred method for sequencing by an exonuclease digestion protocol. In general, both methods are capable of providing molecular weight and sequence information from oligonucleotides, and the choice of ionization method depends on the available instrumentation, type of analyses desired, and user preference.

**Table 10.2.1** Summary of the Characteristics of MALDI-MS Versus ESI-MS

| Parameter or characteristic | MALDI-MS | ESI-MS |
|---|---|---|
| Mass errors | 1.0–0.05% | 0.1–0.005% |
| Upper mass limit | ~36,000 | ~40,000 |
| Feasibility of: | | |
| Mixture analysis | Yes | Difficult |
| Easy coupling to separation methods | No | Yes |
| Exonuclease sequencing | Preferred | Possible |
| Sequencing via gas-phase dissociation (MS/MS) | Possible | Preferred |
| Characterization of noncovalent interactions | No | Yes |

## CONVENTIONAL VERSUS NANOELECTROSPRAY CONFIGURATION

ESI is inherently a solution-based ionization technique. The sample solution flows through the charged ESI needle, resulting in the formation of the Taylor cone mentioned above. Currently, there are two different flow-rate/needle configurations utilized in ESI-MS: conventional electrospray, and micro- or nanoelectrospray. Conventional electrospray is performed using stainless steel needles (with 0.15 to 0.41-mm-i.d. orifices) and the sample solution is delivered at a flow rate of 1 to 10 μL/min. Many of the original ESI-MS oligonucleotide analysis results were obtained using this configuration.

Recently it has been shown that spraying capillaries with a 1- to 2-μm-i.d. spraying orifice have several advantages over the traditional ESI needle, particularly in biomolecular analyses (Wilm and Mann, 1996). This "nanoelectrospray" source is essentially a capillary that has been pulled to a fine tip. The small spraying orifice requires low flow rates (nL/min), which aids in sample conservation. In addition, the overall efficiency of desolvation, ionization, and transfer is increased. The small size of the droplets generated means that one analyte molecule is present per droplet. Because the size of the droplets is significantly reduced, desolvation is easily achieved without the need of a drying gas. The overall charge-to-volume ratio of the droplets is also higher than in conventional ESI, which improves ionization. Transfer of ions into the mass analyzer is improved because the spraying tip can be placed close to the orifice of the analyzer (1 to 2 mm away). Another important advantage of the nanoelectrospray source is that it has a higher tolerance to salt adduction than conventional ESI. In addition, it can operate at high pH. As described in the section on Sample Preparation, below, both of these characteristics can be useful in nucleic acid analysis.

## MASS ANALYZER CONFIGURATION

ESI can be coupled with a large variety of mass analyzers. Table 10.2.2 lists the analyzers most often coupled to the ESI source. Commercial versions of these instruments are available for each of the different mass analyzers listed. Quadrupole mass analyzers are the most popular configuration due to their ease of operation and low cost. However, they suffer from poor resolution as well as a limited *m/z* range (~3000). Triple-quadrupole instruments can be used to perform tandem MS experiments, and therefore are suitable when gas-phase sequencing experiments are of interest.

Two common mass analyzers that are useful for several types of analysis of oligonucleotides are the quadrupole ion-trap and the Fourier-transform ion-cyclotron resonance mass spectrometer (FTICR-MS; see Table 10.2.3 for definitions of the instrument configurations discussed in this unit). These instruments have an added advantage over

**Table 10.2.2** Mass Analyzers Typically Coupled to ESI Sources[a]

| Analyzer[b] | Resolution | Sensitivity | Mass accuracy | Upper $m/z$ limit | Tandem MS capability |
|---|---|---|---|---|---|
| TOF | Good | High | High | ~40,000 | No |
| Quadrupole | Fair | Good | Fair | 3,000 | No |
| Triple-quadrupole | Fair | High | Fair | 3,000 | Yes |
| Double-focusing sector | High | Fair | High | 10,000 | Yes |
| Ion-trap | Fair | Good | Fair | 3,000 | Yes |
| FTICR | Very high | Good | Very high | 10,000 | Yes |

[a]The relative performance aspects of each analyzer are also included.
[b]See Table 10.2.3 for definitions of instrument configurations.

the other mass analyzers listed in Table 10.2.2 that they operate on the principle of ion trapping. With trapped-ion mass analyzers, the analyte ions are confined to a particular region of space and their subsequent analysis is performed as a function of time. In contrast, analysis of ions with quadrupole, triple-quadrupole, sector, and time-of-flight mass analyzers is performed by separating the analysis regions in space. Thus, trapped-ion instruments are ideal for performing multiple stages of tandem mass spectrometry because no additional hardware is needed (McLuckey and Habibi-Goudarzi, 1993; Little et al., 1994a; Habibi-Goudarzi and McLuckey, 1995; Little and McLafferty, 1995). Another advantage is that these instruments permit alternative dissociation schemes—be-

**Table 10.2.3** Glossary of Mass Spectrometry Terms Used in This Unit

| | |
|---|---|
| Collision-induced dissociation (CID) | Process in which an ion is dissociated as a result of interaction with a target neutral species |
| Double-focusing mass spectrometer | Mass spectrometer that uses direction and velocity to focus ions of the same mass and charge |
| Fourier-transform ion-cyclotron resonance mass spectrometer (FTICR-MS) | Mass spectrometer in which ions are confined by an electric and magnetic field and detected on the basis of their cyclotron frequency at a particular excitation energy in the high magnetic field |
| Ion-trap mass spectrometer | Mass spectrometer in which ions can be confined for extended periods of time by the use of a quadrupolar electric field |
| Quadrupole mass spectrometer | Mass spectrometer in which ions with a desired $m/z$ are focused towards the detector by the use of a static and a high-frequency electric field |
| Tandem mass spectrometer (MS/MS) | Mass spectrometer in which ions of selected $m/z$ pass through two or more stages of analysis |
| Time-of-flight mass spectrometer (TOF-MS) | Mass spectrometer which operates on the principle that ions of different $m/z$ with the same initial energy require different times to travel through a field-free region |
| Triple-quadrupole mass spectrometer | Mass spectrometer composed of three sets of quadrupole rods, the first and last set operated in a manner similar to the quadrupole mass spectrometer and the second (middle) set used as a collision cell to allow for MS/MS experiments |

sides the common collision-induced dissociation (CID) method—to be employed (Little et al., 1994b, 1996). Quadrupole ion-trap MS typically gives poorer resolution and mass accuracy than FTICR-MS. FTICR-MS offers the highest resolution and mass accuracy, but the instrumentation is not only more expensive, but also more difficult to operate.

Two additional mass analyzers can be used with ESI. A promising combination is the use of a time-of-flight (TOF) mass analyzer with an ESI source. At the time of writing, only first-generation ESI-TOF mass spectrometers are available and the ultimate effectiveness of the combination is not yet known. However, their high sensitivity, extended upper *m/z* range, adequate resolution and mass accuracy, and high duty cycle suggests that ESI-TOF mass analyzers may soon replace quadrupole mass analyzers as the instruments of choice for molecular weight determinations using ESI-MS. Double-focusing sector instruments are suitable for use with electrospray sources, have high sensitivities and mass resolution, and have tandem MS capabilities. However, their complexity and the small number of vendor offerings has limited their use for oligonucleotide analysis by ESI-MS.

## SAMPLE PREPARATION

The success of oligonucleotide analyses using ESI depends largely on sample preparation. When dealing with nucleic acids, the two key factors in sample preparation are reduction of cation adducts (oligonucleotide purification and desalting) and selection of a proper solvent system.

### Purification Methods

The greatest difficulty in achieving accurate mass measurement of oligonucleotides and nucleic acids is the formation of cation adducts. The negative charge on the nucleic acid phosphate backbone results in a large degree of Coulombic strain. In solution, solvent molecules help reduce these Coulombic interactions. In the gas phase, where solvent molecules are absent, relief of the strain is achieved by neutralization or cation adduction. Figure 10.2.3 is a representative electrospray mass spectrum of an oligonucleotide with a large number of cation adducts. The presence of nonvolatile cation adducts such as sodium and potassium results in spectral peaks broadened by the adduction. In addition, the ion current is shared over a larger number of peaks, thereby decreasing sensitivity. As illustrated in Figure 10.2.3b, a number of cation-adduct combinations may be present for a particular charge state, and a complex mass spectrum results.

A number of purification techniques have been applied to nucleic acid samples (Stults and Marsters, 1991; Emmett and Caprioli, 1994; Little et al., 1994a; Potier et al., 1994; Greig and Griffey, 1995; Limbach et al., 1995; Liu et al., 1996, 1997; Muddiman et al., 1996a). Although these methods differ in protocol, the objective and end result are the same—to replace the nonvolatile (e.g., sodium and potassium) adducts with volatile ones. Most commonly, ammonium salts are exchanged for the nonvolatile adducts.

Purification techniques that reduce or eliminate the presence of cation adducts can be classified into two groups: off-line and on-line methods (Table 10.2.4). The former include ammonium acetate precipitation , dialysis, and HPLC purification. The latter include on-line microdialysis, on-line micro-HPLC, and solvent additives.

By far the most common purification method is ammonium acetate precipitation (see Basic Protocol 1; Stults and Marsters, 1991; Limbach et al., 1995). In this procedure, the oligonucleotide is mixed with ammonium acetate and the oligonucleotide in its ammonium salt form is precipitated from ethanol. This procedure can be repeated a number of times, and it is advisable to do so, as multiple precipitations increase the overall amount of salt exchange.

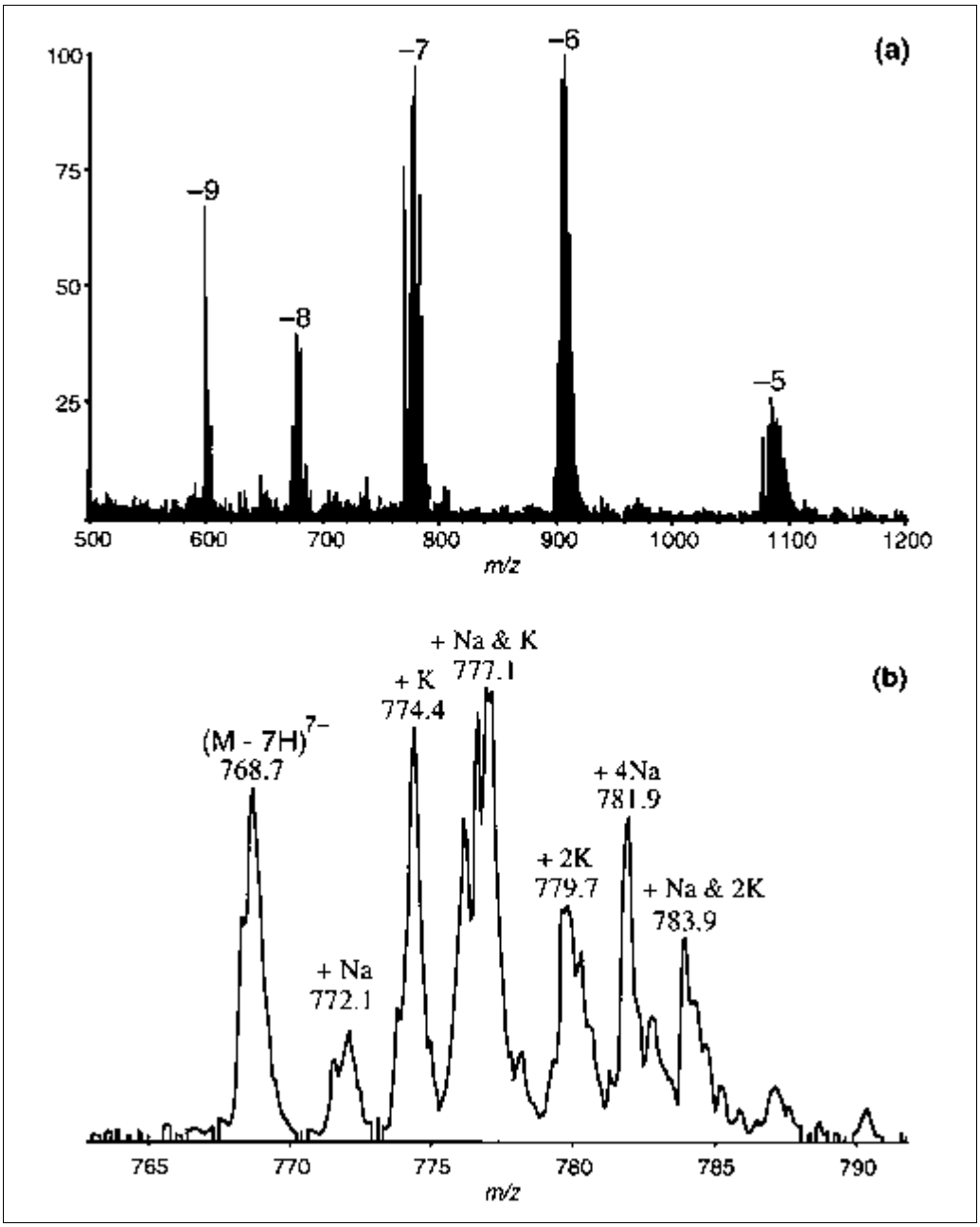



**Figure 10.2.3** Representative electrospray mass spectrum used to illustrate the detrimental effects of cation adduction on mass spectral quality.

Another routine approach to reducing salt adduct formation is to sequester the adducts using chelating agents and/or organic bases (see Basic Protocol 2; Potier et al., 1994; Greig and Griffey, 1995; Limbach et al., 1995; Muddiman et al., 1996a). The strategy here is to add chelating agents such as *trans*-1,2-diaminocyclohexane-*N,N,N′N′*-tetraacetic acid (CDTA; Limbach et al., 1995) or organic bases (Greig and Griffey, 1995; Muddiman et al., 1996a) such as triethylamine (TEA) that have a higher cation affinity than the nucleic acid. The cations will bind preferentially to these additives, leaving the nucleic acid in its free acid form. It is important to note that none of these purification techniques alone can remove all cation adducts, and using a combination of techniques—for example, ammonium acetate precipitation followed by CDTA and TEA addition—will improve results (Greig and Griffey, 1995; Limbach et al., 1995).

**Table 10.2.4** Techniques for Purifying Nucleic Acid Samples Prior to ESI-MS Analysis

| Method | Reference |
|---|---|
| *Off line* | |
| Ammonium acetate precipitation | Stults and Marsters (1991); Limbach et al. (1995) |
| Reversed-phase HPLC | Little et al. (1994a) |
| Microdialysis | Liu et at. (1997) |
| *On line* | |
| Addition of co-matrix and/or chelating agent | Potier et al. (1994); Greig and Griffey (1995); Limbach et al. (1995) |
| Microdialysis | Liu et al. (1996) |
| Microscale liquid chromatography | Emmett and Caprioli (1994) |

***BASIC PROTOCOL 1***

## Removal of Nonvolatile Cation Adducts from Oligonucleotide or Nucleic Acid Samples by Ammonium Acetate Precipitation

The amount of salt adduction in nucleic acid samples can be reduced by exchanging the nonvolatile cations with the volatile ammonium cation (see section on Purification Methods, above). The effectiveness of the cation exchange increases with repetition of the precipitation procedure (Stults and Marsters, 1991; Limbach et al., 1995). This protocol should only be used for oligonucleotides larger than 25 bases—smaller oligonucleotides are difficult to precipitate and large sample losses will occur.

### *Materials*

Oligonucleotide or nucleic acid sample, dry
10 M ammonium acetate
Absolute and 70% ethanol, ice cold

*CAUTION:* RNA samples are easily degraded by adventitious nucleases. If RNA is to be purified or analyzed, all laboratory equipment must be sterilized prior to use. Use of gloves is recommended during preparation of nucleic acid samples and solutions to avoid contamination of glassware and other equipment. Autoclaved distilled water and the highest available grade of salts must be used during solution preparation.

1. Dissolve the nucleic acid in 40 to 70 µL ultrapure water to generate a 250 to 400 µM solution.
2. Add ⅓ volume of 10 M ammonium acetate.
3. Add 2.5 vol ice-cold absolute ethanol to precipitate the nucleic acid. Store at least 3 hr at −20°C.
4. Centrifuge the suspension 15 min at 12,500 rpm, 25°C.
5. Decant the supernatant, working carefully to avoid disturbing the pellet.
6. Wash the pellet with 70% aqueous ethanol. Store the suspension at least 2 hr at −20°C.
7. Centrifuge the suspension 15 min at 12,500 rpm.
8. Decant the supernatant and lyophilize the pellet.
9. Dissolve the pellet in ultrapure water to a final concentration of 300 to 1000 µM.

*BASIC PROTOCOL 2*

## Removal of Nonvolatile Cation Adducts from Oligonucleotide or Nucleic Acid Samples with Chelating Agents and/or Organic Bases and ESI-MS Sample Preparation

This protocol describes the steps for preparing oligonucleotide and nucleic acid solutions for analysis by ESI-MS. The addition of chelating agents or organic bases serves to sequester cations, thereby reducing the extent to which adducts are observed in the mass spectra (see section on Purification Methods, above). Each of these agents will reduce the amount of adduct formation, but the results are usually better when both materials are used. This protocol is the recommended choice for analyzing small oligonucleotides (≤25 bases). For larger oligonucleotides, this procedure should be used in conjunction with ammonium acetate precipitation (see Basic Protocol 1).

### *Materials*

Oligonucleotide or nucleic acid sample
*trans*-1,2-Diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid (CDTA)
Ethylenediaminetetraacetic acid (EDTA)
0.1% aqueous triethylamine
Appropriate solvent: 30% to 70% aqueous acetonitrile, isopropanol, or methanol (see section on Solvent Selection, below, for discussion of how to choose an appropriate solvent.)

*CAUTION:* RNA samples are easily degraded by adventitious nucleases. If RNA is to be purified or analyzed, all laboratory equipment must be sterilized prior to use. Use of gloves is recommended during preparation of nucleic acid samples and solutions to avoid contamination of glassware and other equipment. Autoclaved distilled water and the highest available grade of salts must be used during solution preparation.

1. For oligonucleotides >25 bases, carry out ammonium acetate precipitation (see Basic Protocol 1).
2. Prepare a 1 to 10 μM solution of oligonucleotide in the appropriate organic solvent.
3. Add 5 μL of 0.1% triethylamine to each 100 μL of oligonucleotide solution.
4. If analyzing intact nucleic acids, add CDTA or EDTA to the sample so as to obtain a final CDTA or EDTA molar concentration three times that of the nucleic acid sample.
5. This solution is now ready for immediate analysis by ESI-MS.

### Solvent Selection

A second aspect of sample preparation is the selection of an appropriate solvent for electrospray. Two important criteria are (1) that the solvent readily evaporate to facilitate the transfer of the ion from the liquid to the gas phase, and (2) that the solvent allow the generation of a large number of ions. It has been shown that as the organic composition of the solvent increases, the signal intensity increases as well (Bleicher and Bayer, 1994). In that particular study, the best ion signals were seen with acetonitrile, although other solvents such as isopropanol or methanol have been used. Typically, the aqueous oligonucleotide solution is mixed in a 1:1 ratio with the organic solvent. This allows the oligonucleotide to remain solubilized while assisting the ESI process by improving evaporation.

The second consideration in solvent selection is that the solvent must allow the ready formation of ions. The main factor in this case is solution pH: as pH increases, more negative ions are produced and the signal intensity rises (Bleicher and Bayer, 1994). This

increase in pH can be achieved by adding an organic base such as TEA to the nucleic acid solution, which also reduces cation adduction, as previously discussed. Finally, the pH can be manipulated to change the charge-state distribution of the peaks observed in the spectra (Stults and Marsters, 1991; Cheng et al., 1995; Griffey et al., 1997). As the pH increases, the charge-state distribution shifts to higher charge states or lower *m/z*. Thus, when using a mass analyzer with a limited *m/z* range, it is preferable to work at high solution pH.

The typical procedure for preparing samples for ESI-MS analysis is as follows. After purification of the oligonucleotide or nucleic acid sample by one of the off-line techniques mentioned above, the oligonucleotide sample is dissolved in ultrapure (e.g., Nanopure) water to a stock concentration of 50 to 100 μM. An aliquot of the stock solution is then diluted into the organic solvent of interest to a final concentration of 1 to 10 μM. Chelating agents or organic bases such as TEA can be added to this solution prior to analysis.

The deciding criteria for final concentration and organic content of solvent depend on the particular type of information desired from the mass spectrometric step. When the molecular weight or sequence of single-stranded oligonucleotides or intact nucleic acids are to be determined, the percentage organic solvent should be between 50% and 90%; smaller oligonucleotides are more soluble at the higher percentages of organic solvent. The best concentration will vary by instrument. When noncovalent interactions of double-stranded oligonucleotides or intact nucleic acids are to be investigated, the organic content should remain as low as possible while still generating a stable electrospray (typically 10% to 35%). Analyte concentrations in these cases are typically higher (~25 μM).

## MOLECULAR WEIGHT DETERMINATION USING ESI-MS

Molecular weight determination of oligonucleotides and intact nucleic acids is one of the more common applications of ESI, as well as one of the simplest. This approach can be used to verify the expected base composition of an oligonucleotide by comparing the measured mass to the mass corresponding to the predicted composition. The analysis of small oligonucleotides (<10,000 Da) has become routine. In fact, if constraints can be placed on the number of A, C, G, or U/T residues allowed in the molecule, then the base composition of an oligonucleotide up to the 14-mer level can be determined unambiguously by accurate mass measurement alone (Pomerantz et al., 1993). For larger oligonucleotides and nucleic acids, however, extremely high mass accuracies are required to confirm base composition assignments.

The most impressive molecular weight measurements have been obtained using FTICR-MS (Chen et al., 1995; Cheng et al., 1996a). With this high-resolution mass analyzer, DNA molecules in the 110-MDa range have been detected with a 10% uncertainty in the molecular weight value (Chen et al., 1995). Other relatively large oligonucleotides and nucleic acids in the molecular weight region between 15,000 and 40,000 Da have also been analyzed. Among the applications of ESI-MS to these molecules are the analysis of intact tRNA and rRNA (Limbach et al., 1995) and PCR products (Naito et al., 1995; Muddiman et al., 1996b, 1997). In these cases, the errors in mass measurement are typically ~0.01% or less.

Because ESI produces multiply charged ions, the resulting spectra may be complicated by the presence of several ions of the same mass but different charge and their respective cation adducts. Determination of the charge state of a particular peak may be accomplished using mathematical equations. For a negatively charged peak, $p_1$, one may write Equation 10.2.1:

$$p_1 z_1 = M_r - M_a z_1 = M_r - 1.0079\ z_1$$

**Equation 10.2.1**

where $p_1$ is the *m/z* value of the peak, $z_1$ is the charge of the peak, $M_r$ is the molecular weight of the sample, and $M_a$ is the molecular weight of the charge-carrying species. Typically, the charge-carrying species is a proton; therefore, $M_a = 1.0079$. Similarly, for a second peak, $p_2$, that is *j* peaks away from $p_1$, one may write Equation 10.2.2:

$$p_2(z_1 - j) = M_r - M_a(z_1 - j) = M_r - 1.0079(z_1 - j)$$

**Equation 10.2.2**

Solving Equations 10.2.1 and 10.2.2, Equation 10.2.3 is obtained for $z_1$:

$$z_1 = -j(p_2 + 1.0079)/|p_1 - p_2|$$

**Equation 10.2.3**

Once the charge state is known, Equation 10.2.1 can be used to determine the molecular weight of the sample being analyzed. The data in Figure 10.2.1 will serve to illustrate how charge-state and molecular weight determinations are made. To determine the charge state, for example, of the peak at 1348.9, the following values can be substituted into Equation 10.2.3: $p_1 = 1348.9$, $p_2 = 1798.5$, and $j = 1$. By substituting these values into Equation 10.2.3, a value of $z_1$ of −4 is obtained for the charge state. Using Equation 10.2.1, $M_r$ is found to be 5399.6. To obtain a more accurate value for $M_r$, it should be calculated from the values measured for a number of adjacent peaks and the calculated values then averaged, as shown in Figure 10.2.1. The value one obtains from solving Equation 10.2.3 should be an integer; if it is not, a different pair of *m/z* values should be chosen. For example, if one chooses $p_1 = 1348.9$, $p_2 = 1697.7$, and $j = 1$, a value of $z_1 = -4.87$ is obtained for the charge state. Because this value is not an integer, one can conclude that the *m/z* values of 1348.9 and 1697.7 do not compose an oligonucleotide of the same mass.

In some cases, multiple peaks are not present in the electrospray series or are difficult to discern (such as in tandem MS experiments), and some other means of determining the charge state of the ion is necessary. On a high-resolution instrument such as a sector or FTICR mass spectrometer, the ability to resolve isotope peaks is helpful in determining the charge state of a particular peak. If the spacing between a peak A and its consecutive isotope peak A+1 is 1 (e.g., for $^{13}C$), then this peak will correspond to the singly charged species. If, for example, the difference between the A and A+1 peaks detected in the mass spectrum is 0.20 u, then the A peak corresponds to the species carrying a −5 charge (i.e., $\frac{1}{5} = 0.20$ u). In general, if the difference between the A and A+1 peaks is 1/*x* u, the A peak will correspond to the −*x* charge state.

Low-resolution instruments typically cannot resolve $^{13}C$ isotopes for multiply charged peaks where $z > 3$. Often, the charge state of the particular ion can be determined by the mass shift of a cation adduct. For example, a single sodium adduct will appear at an *m/z* value 22 u higher than the unadducted *m/z* value for singly charged ions, $\frac{22}{2} = 11$ u higher for doubly charged ions, $\frac{22}{3} = 7.3$ u higher for triply charged ions, and so forth (see Fig. 10.2.3b).

## OLIGONUCLEOTIDE AND NUCLEIC ACID SEQUENCING USING ESI-MS

A powerful application of ESI-MS in the analysis of oligonucleotides and nucleic acids is sequence determination. The two most common techniques for sequencing are tandem MS and indirect sequencing using enzyme digestions (Limbach, 1996). In tandem MS,

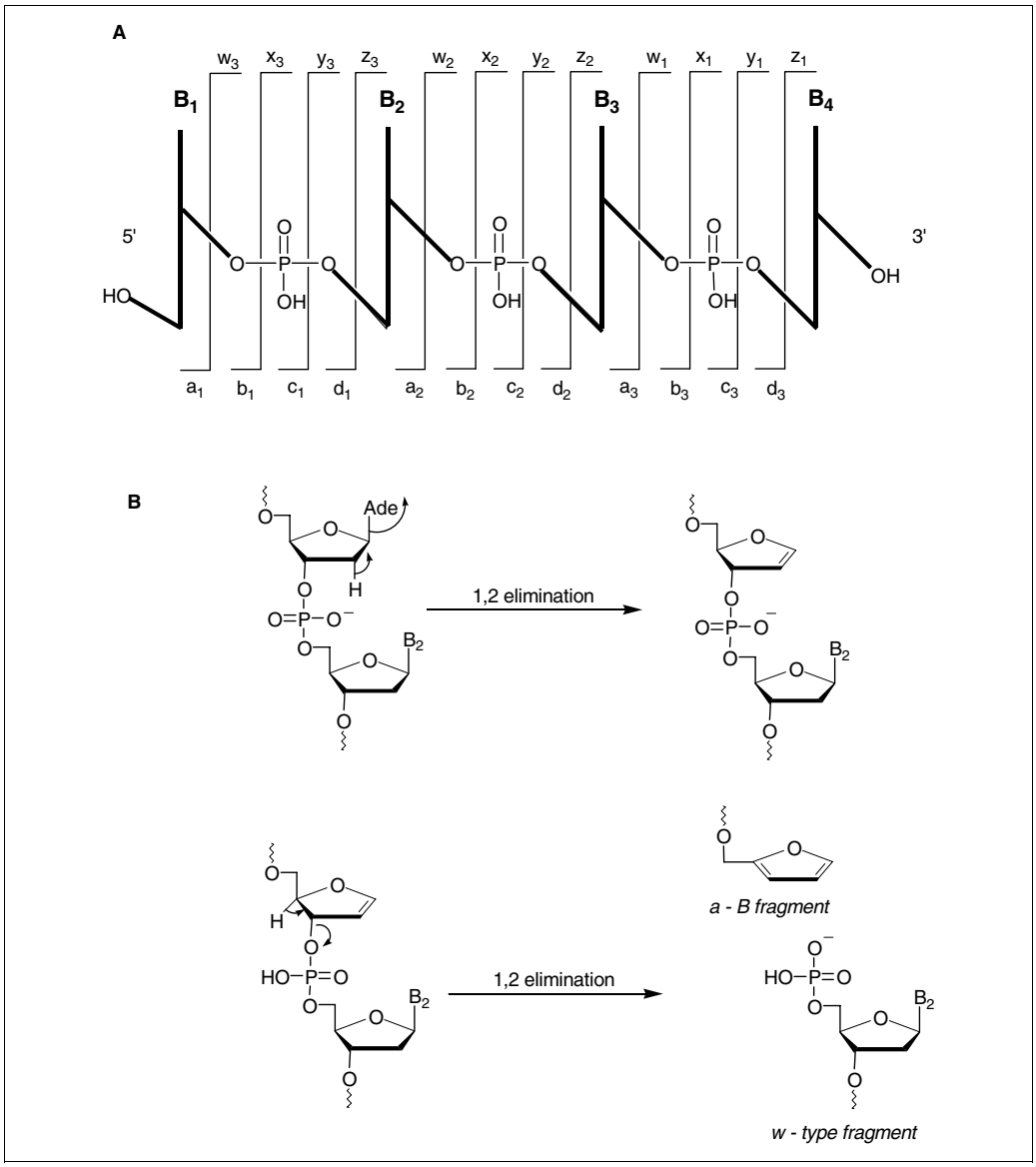



**Figure 10.2.4** (**a**) Nomenclature for the dissociation products of oligonucleotides. (**b**) Prevalent oligonucleotide fragmentation mechanism, especially with ion-trap and FTICR-MS instruments (McLuckey and Habibi-Goudarzi, 1993). The mechanism involves two elimination reactions, the first resulting in loss of the base and the second resulting in cleavage at the sugar to yield (a-B)-type and w-type fragments.

molecules are allowed to dissociate in the gas phase, and analysis of the resulting fragmentation pattern yields insight into the structure and, in the case of oligonucleotides, sequence of the molecule. Figure 10.2.4a gives the nomenclature for assigning dissociation products of oligonucleotides. As can be seen in the figure, a large number of fragmentation pathways are possible. This large number of pathways results in highly complicated spectra, and as oligonucleotide length increases, so does the spectral complexity. Figure 10.2.4b shows the most common fragmentation pathway oligonucleotides are found to follow during tandem MS experiments. Generally, the resulting mass spectrum will be composed primarily of a-B fragments and the w-type fragments, although the other dissociation products may also be present (McLuckey and Habibi-Goudarzi, 1993). Tandem MS analysis of oligonucleotides using ESI have been carried out using triple-quadrupole (Barry et al., 1995, 1996; Wolter and Engels, 1995; Boschenok

and Sheil, 1996; Ni et al., 1996a), ion-trap (McLuckey et al., 1992, 1995; McLuckey and Habibi-Goudarzi, 1993; Habibi-Goudarzi and McLuckey, 1995), and FTICR mass analyzers (Little and McLafferty, 1995; Little et al., 1995, 1996).

Although the techniques and the information generated by each of these mass analyzers are similar, there do exist substantial differences, primarily in the preferred order of base loss. For instance, in ion-trap and FTICR-MS studies, adenine is usually lost, and this leads to cleavage of the 3′ C-O bond of the sugar, generating w-type and (a-B)-type fragments (Fig. 10.2.4; McLuckey et al., 1992). In contrast, triple-quadrupole studies show no preference for loss of any one base over another (Barry et al., 1995; Boschenok and Sheil, 1996; Ni et al., 1996a).

Recently, McCloskey and co-workers (Ni et al., 1996b) developed an algorithm for the sequence identification of oligonucleotides using a triple-quadrupole mass spectrometer equipped with an ESI source. The algorithm is based on the analysis of relative ion abundances and charge-state distributions of ions generated using low-energy collisionally induced dissociation (CID). In their preliminary study, the sequence of an "unknown" 15-mer was determined. In a subsequent study, the algorithm was employed to determine the sequence of oligonucleotides in combinatorial libraries composed of mixtures of 8-mers or 12-mers containing two and three unknown nucleotides in the sequence, respectively (Pomerantz et al., 1997). The greatest advantage presented by this technique for this application is the ability to sequence isomeric oligonucleotides. When analyzing mixtures using tandem MS, components of a mixture are analyzed by selecting (one at a time) each molecular ion in the mass spectrum. Once isolated, it is allowed to undergo CID, and the fragment-ion spectrum is analyzed to determine the sequence. If, however, the molecular ion selected in the primary mass spectrum represents two or more components of equal mass, the CID spectrum becomes more difficult to interpret. By employing the sequencing algorithm, the isomeric oligonucleotides can be distinguished and sequenced.

The algorithm is not without its limitations, however. In the case of isomeric mixtures, as the number of components represented by the molecular ion in the primary mass spectrum increases, it becomes nearly impossible to determine the individual oligonucleotides unambiguously without a previous fractionation step. The task becomes even more difficult if the number of components in the mixture is unknown. In addition, as one of the factors used for sequence determination is relative ion abundance, the analysis of minor components becomes difficult in a mixture where one or more components are in a molar excess.

**Table 10.2.5** Characteristic Mass Losses During Exonuclease Digestion for Naturally Occurring Deoxynucleotides (dX) and Ribonucleotides (rX)[a]

| Nucleotide | Δ mass (singly charged) | Δ mass (doubly charged) | Δ mass (triply charged) |
|---|---|---|---|
| dA | 313.27 | 156.64 | 104.42 |
| dG | 329.27 | 164.64 | 109.76 |
| dC | 289.25 | 144.63 | 96.417 |
| dT | 304.26 | 152.13 | 101.42 |
| rA | 329.27 | 164.64 | 109.76 |
| rG | 345.27 | 172.64 | 115.09 |
| rC | 305.25 | 152.62 | 101.75 |
| ru | 306.26 | 153.13 | 102.09 |

[a]The particular values are listed for several different charge states, and will be determined by the charge states of the two oligonucleotides used to find Δ mass. All values are atomic weight based.

Apart from these specific drawbacks in analyzing mixtures, it is uncertain whether the algorithm will be applicable when other mass analyzers or high-energy CID are employed. Nonetheless, its development represents a large step forward in the simplification of tandem MS analyses of oligonucleotides. Although it is questionable whether this technique will be applicable to larger molecules, it can clearly be used to analyze oligonucleotides up to the 15-mer range. In addition, the sequence location as well as the nature (i.e., sugar or base) of modifications can be determined.

The second approach for sequencing using ESI-MS involves the partial enzymatic digestion of oligonucleotides or nucleic acids followed by mass-spectral analysis of the digestion products (see Basic Protocol 3). Enzymatic digestion may consist of either exonuclease or endonuclease digestion. Sequence identification using exonuclease digestion is a relatively straightforward, simple method, particularly for smaller oligonucleotides (Limbach et al., 1994; Glover et al., 1995). Exonuclease digestions generate consecutive backbone cleavages which, when analyzed using mass spectrometry, create a "mass ladder." The sequence of the molecule is then determined from the mass difference between adjacent peaks in the spectrum. Table 10.2.5 lists the expected mass difference for loss of each of the deoxy- and ribonucleotides. 5′ and 3′ exonucleases can be used to generate 5′ and 3′ sequence information, respectively. Modified nucleosides can be easily identified with this method from the anomalous mass shifts caused by the modifications.

***BASIC PROTOCOL 3***

## Oligonucleotide or Nucleic Acid Sequencing Using Sequential Exonuclease Digestion and ESI-MS

This protocol describes the procedure for on-line sequencing of oligonucleotides or nucleic acids using ESI-MS. To generate 3′ sequence information, phosphodiesterase I should be used, as it cleaves oligonucleotides in the 3′-to-5′ direction. Similarly, if 5′ sequence information is required, phosphodiesterase II should be used. During the first few minutes of analysis, information can be gathered on the first five or so nucleotides in the sequence. After longer reaction times, sequence information can be obtained for nucleotides further along the chain (Limbach et al., 1994). This protocol is most effective for oligonucleotides <30-mers.

### *Materials*

Oligonucleotide or nucleic acid sample, dry
Phosphodiesterase I (from snake venom, SVP; Sigma) or phosphodiesterase II (from calf spleen, CSP; Sigma or Worthington) dissolved to 0.1 U/μL in water
5.0 M ammonium acetate (APPENDIX 2A), pH 9.8 to 10.4 for phosphodiesterase I *or* 6 to 7 for phosphodiesterase II
Organic solvent (see section on Solvent Selection, above)
Variable-flow-rate syringe pump

1. Mix ~1.2 nmol dry oligonucleotide, 10 μL (1.0 U) phosphodiesterase, and 0.5 M ammonium acetate at the appropriate pH to adjust the pH for optimum enzyme digestion.

2. Dilute the solution in an appropriate percentage of organic solvent to a concentration of 10 pmol/μL.

3. Draw the digestion mixture into a syringe and infuse the mixture into the ESI-MS at a flow rate of ~2 μL/min using a syringe pump.

*Digestion will occur in the syringe as the data is acquired. Data can be acquired continuously or every 2 min until digestion is complete.*

4. The syringe may be cooled (to 0°C to 10°C) or heated (to 30°C to 45°C) in a water bath in order to decrease or increase digestion times, respectively.

*Although direct analysis of the digestion mixtures can be advantageous because the sequence information is obtained from a difference in mass rather than an absolute value, it is sometimes preferable to separate the digestion products before MS analysis (Glover et al., 1995). For example, one may wish to separate digestion products when dealing with oligonucleotides ≥30-mers, since the large number of peaks generated can decrease sensitivity.*

### Analysis of RNA Modifications Using Endonuclease Digestion and ESI-MS

For larger nucleic acids, the generation of smaller fragments using endonucleases facilitates sequence assignments. One of the more significant advances in this area has been achieved by McCloskey and co-workers. This group has developed a protocol based on site-specific endonuclease digestions to determine modifications in transfer RNA (tRNA) and ribosomal RNA (rRNA; Crain, 1990; Bruenger et al., 1993; Kowalak et al., 1993, 1994, 1995, 1996). The steps involved in this sequencing method are outlined in Figure 10.2.5, and the enzymatic digestions used in this method are outlined in the protocols below. The purified RNA sample is divided in two. One of the samples is digested with nuclease P1 and alkaline phosphatase to generate its component nucleosides (see Basic Protocol 4). Liquid chromatography mass spectrometry (LC-MS) is then used to characterize the nucleosides present in the mixture, as well as to determine if any modifications are present (evidenced by a difference in mass from the "naturally" occurring nucleosides;

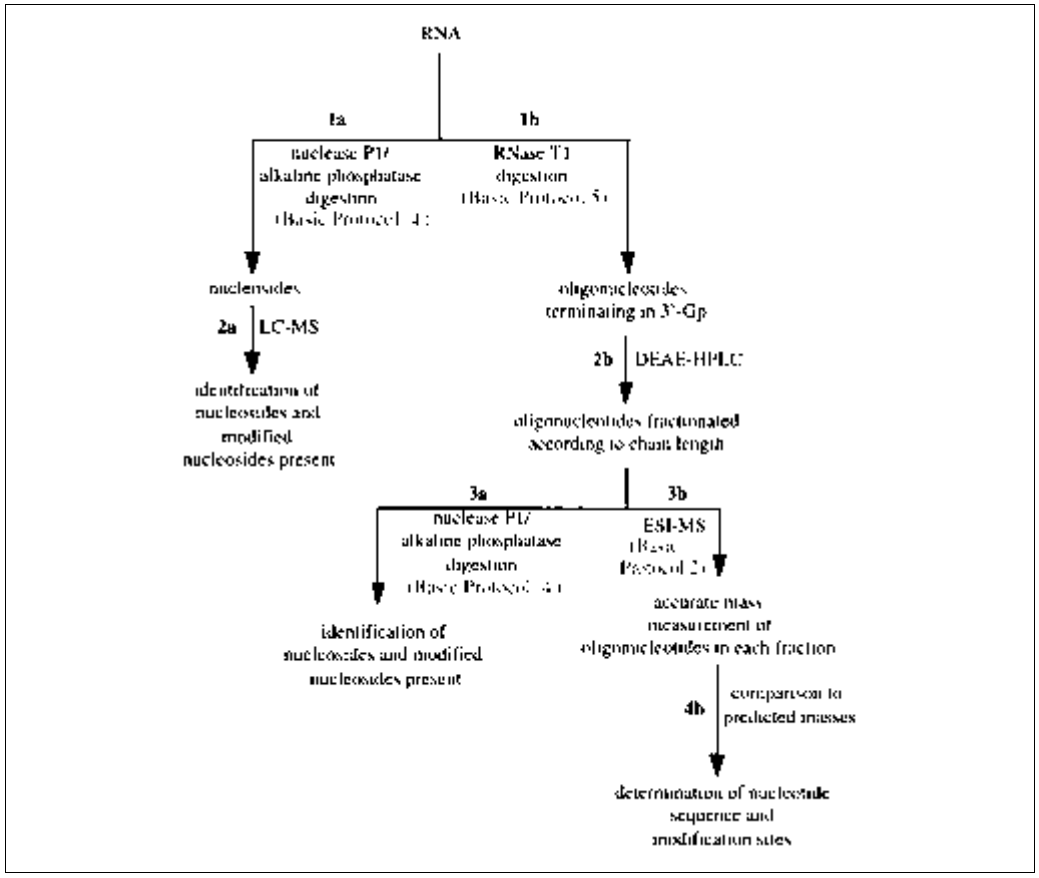



**Figure 10.2.5** Endonuclease-based RNA sequencing protocol.

step 1a). The other RNA sample is subjected to RNase T1 digestion (see Basic Protocol 5) to generate oligonucleotide fragments terminating in a 3′-Gp (step 1b). The resulting oligonucleotides are then separated based on their chain length using DEAE-HPLC (step 2b). Each of the collected fractions (which typically contain more than one oligonucleotide) is then separated into two batches. One set is subjected to nuclease P1 and alkaline phosphatase digestion as in step 1a and the nucleosides and modified nucleosides present in each fraction are determined (step 3a). Each oligonucleotide fraction in the second set is analyzed using ESI-MS (step 3b). The accurate mass measurement obtained is then compared to the predicted masses from the known gene sequence from which the nucleotide sequence, as well as sites of modifications, can be determined (step 4b).

Because the number of RNase T1 fragments that are generated can be quite large, an alternative procedure is to isolate regions of interest selectively by hybridizing complementary oligodeoxynucleotides to the region and using mung bean nuclease to digest the unhybridized sections of RNA (Kowalak et al., 1995). The purified oligoribonucleotides remaining may then be subjected to the protocol detailed in Figure 10.2.5.

***BASIC PROTOCOL 4***

## Total Nucleoside Digestion of RNA

The following procedure can be used to digest RNA to its component nucleosides. This is a necessary step in the determination of post-transcriptional modifications using the method developed by McCloskey and co-workers. The amounts of enzyme specified are those required to completely digest ~20 µg (0.5 $A_{260}$ units) of RNA (Crain, 1990).

### *Materials*

RNA sample, dry
Nuclease P1 (Sigma), dissolved to 2 U/µL in 0.05 M ammonium acetate, pH 5.3
Snake venom phosphodiesterase I (SVP) *or* bovine intestinal phosphodiesterase (both from Sigma; the former is preferred), dissolved to 0.001 U/µL in water
Bacterial alkaline phosphatase (Sigma), suspended in 2.5 M ammonium sulfate
1 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
0.1 M ammonium acetate, pH 5.3
1 M ammonium bicarbonate, pH 7.8
37° to 100°C water bath

*CAUTION:* RNA samples are easily degraded by adventitious nucleases. If RNA is to be purified or analyzed, all laboratory equipment must be sterilized prior to use. Use of gloves is recommended during preparation of nucleic acid samples and solutions to avoid contamination of glassware and other equipment. Autoclaved distilled water and the highest available grade of salts must be used during solution preparation.

1. Dissolve the RNA sample in 1 mM Tris·Cl, pH 7.4, to a concentration of 1 to 3 µg/µL.
2. Heat the solution 3 min at 100°C to denature the nucleic acid, and immediately chill on ice.
3. Add $^1/_{10}$ vol of 0.1 M ammonium acetate, pH 5.3.
4. Add 2 U nuclease P1 and incubate the solution 2 hr at 45°C.

   *If thiolated nucleosides are present, include $^1/_{10}$ vol of 1 mM dithiothreitol in the buffer to reduce sulfur loss.*

5. Add $^1/_{10}$ vol of 1 M ammonium bicarbonate, pH 7.8, and 0.002 U phosphodiesterase I, and incubate 2 hr at 37°C.

   *The pH of ammonium bicarbonate will increase with time, so the pH of this buffer should be checked, and adjusted if necessary by addition of acetic acid, prior to use.*

6. Add 0.5 U alkaline phosphatase and incubate 1 hr at 37°C.

   *Bacterial alkaline phosphatase from Sigma is provided as a suspension in 2.5 M ammonium sulfate. The ammonium sulfate does not typically interfere with subsequent LC/MS analysis. If dry alkaline phosphatase is used, dissolve the enzyme to 0.5 U/μL in 2.5 M ammonium sulfate.*

7. The solution is ready for immediate analysis by LC/MS.

   *For details of LC-MS analysis of RNA, consult Pomerantz and McCloskey (1990).*

   *The digest can be stored at –20°C before use; in this case it must first be neutralized with acetic acid.*

***BASIC PROTOCOL 5***

## RNase T1 Digestion of RNA

Total nucleoside digestion of RNA will yield information on the kinds of post-transcriptional modifications present in RNA, but analysis of RNase T1 products is required to identify the sequence location of these modifications. The following is the procedure for generating RNase T1 fragments of RNA (Kowalak et al., 1993).

### *Materials*

RNA sample, dry
RNase T1 (Sigma or Ambion), suspended in 3.2 M ammonium sulfate, pH ~6
1 mM EDTA/50 mM Tris·Cl, pH 7.5 (see *APPENDIX 2A* for preparation of EDTA and Tris·Cl stock solutions)
37°C water bath

*CAUTION:* RNA samples are easily degraded by adventitious nucleases. If RNA is to be purified or analyzed, all laboratory equipment must be sterilized prior to use. Use of gloves is recommended during preparation of nucleic acid samples and solutions to avoid contamination of glassware and other equipment. Autoclaved distilled water and the highest available grade of salts must be used during solution preparation.

1. Dissolve 8 nmol RNA in 1 mM EDTA/50 mM Tris·Cl, pH 7.5, to a concentration of 0.2 nmol/μL.
2. Heat the solution 3 min at 100°C to denature the nucleic acid, and immediately chill on ice.
3. Add 2000 U RNase T1 and incubate 30 min at 37°C.

   *RNase T1 from Sigma is supplied as a suspension in 3.2 M ammonium sulfate. The ammonium sulfate does not typically interfere with subsequent HPLC analysis. If dry RNase T1 is to be used, dissolve the enzyme to 1000 U/μL in 3.2 M ammonium sulfate.*

4. The solution is ready for immediate analysis by HPLC.

   *For details of HPLC analysis of RNA, consult Kowalak et al., 1993.*

   *The digest can be stored at –20°C prior to HPLC, although long-term storage (>6 months) is not recommended as it may lead to RNA degradation.*

## ANALYSIS OF NONCOVALENT COMPLEXES USING ESI-MS

Among the advantages of ESI in nucleic acid analysis is that this is what is termed a "soft" ionization technique in the sense that the ionization process itself does not cause molecular fragmentation. There are several classes of noncovalent complexes that can be studied using ESI-MS (Przybylski and Glocker, 1996; Loo, 1997). Among these are specific complex stoichiometry, competition of complex components, specificity of solution conditions for complex formation, and gas-phase stability of complex ions. Each of these properties can be studied by varying certain ESI parameters. For example, studies on complex stoichiometry can be achieved by changing solution concentrations, ESI temperature, and ESI voltage. Competitions between components in a complex can be studied by inducing modifications in the components and monitoring the changes effected on the ESI spectra. Solution requirements are determined by changing such variables as buffer pH. Lastly, the gas-phase stability of the complexes may be determined using CID experiments (Przybylski and Glocker, 1996). In addition, ESI-MS has several advantages over the other techniques traditionally used to study noncovalent complexes (e.g., NMR, X-ray crystallography), including the ability to accurately measure molecular mass and the smaller amounts of sample required.

The earliest studies of nucleic acid complexes were carried out on oligonucleotide duplexes. Typically, the observation of oligonucleotide duplexes is achieved with samples dissolved in 10 M ammonium acetate. The duplex is usually observed at low charge states (high $m/z$), requiring the use of an analyzer with an extended mass range. Besides small oligonucleotide duplexes, double-stranded PCR products have also been observed (Wunschel et al., 1996). The complexes studied consisted of 100 to 105 bp. However, accurate mass measurement of such large complexes is difficult to obtain even on a high-resolution instrument because of the large amount of cation adduction present in these samples. Quadruplex structures of DNA have also been observed using ESI-MS (Goodlett et al., 1993). However, formation of these complexes requires the presence of high concentrations of monatomic cations (e.g., $Na^+$) which, as previously discussed, degrade spectral quality.

In addition to base-paired nucleic acid hybridizations, ESI-MS has also been used to analyze the binding of proteins to DNA and RNA. Among the studies that have been conducted on protein-nucleic acid complexes are determination of gas-phase stability (Gale and Smith, 1995), recognition studies (Sannes-Lowery et al., 1997), measurement of dissociation constants (Greig et al., 1995), determination of binding stoichiometry (Cheng et al., 1996b) and specificity (Gao et al., 1995), and competitive binding studies (Cheng et al., 1996c).

ESI-MS for the study of noncovalent complexes is still a developing technique. Apart from the fundamental studies of noncovalent complexes, this methodology may have more far-reaching applications. Among the proposed applications (Przybylski and Glocker, 1996) are sequence determination by hybridization, analysis of antisense oligonucleotide complexes, and analysis of intercalation complexes.

## SUMMARY

The speed and sensitivity of mass spectrometric analyses make this technique an attractive approach to analyzing oligonucleotides and nucleic acids. With the advent of "soft" ionization techniques such as MALDI and ESI, the analysis of these molecules in their intact form has brought mass spectrometry into the forefront as a viable method for characterization of nucleic acids. Although MALDI has a greater tolerance for salt adducts than ESI, the latter technique has the advantage that the generation of multiply charged ions allows the analysis of these molecules using more routine analyzers such as

quadrupoles that may have a limited *m/z* range. In addition, ESI-MS allows the analysis of intact noncovalent complexes. This characteristic permits the study of these complexes in the gas-phase with minimal sample amounts, and provides complementary information to the classic solution phase methods.

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