

Identification of RNA Linkage Isomers by Anion-Exchange Purification with ESI-MS of Automatically-Desalted Phosphodiesterase-II Digests

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ABSTRACT

Among the possible contaminants unique to RNA are linkage isomers that are difficult to identify by MS and LC-MS techniques. Using nonporous and monolithic polymer anion exchangers for purification, we demonstrated a method to identify the presence of these isomers. We also suggested a confirming technique employing an exonuclease incapable of cleaving 2'-5' linkages. We now present a method identifying the location of the linkage in the RNA isomer by anion-exchange purification and ESI-MS of the digestion products. Because the IP-RPLC desalting methods typically employed do not effectively separate oligonucleotides less than 6 bases, we employed a direct reversed-phase method to automatically desalt the digestion products, and couple the purification to ESI-MS.

RNA LINKAGE ISOMERS: RNA FRAGMENT DESALTING

At TIDES 2006 we documented that the DNAPac[®] PA200 column could resolve several RNA oligos of identical sequence, but differing only in the presence of aberrant 2'-5' linkages at specific positions in the sequence (See Figures 1, 2). Treatment of those RNA oligos with

phosphodiesterase-II, a 5' exonuclease considered incapable of cleaving 2'-5' linkages, resulted in fragments of uncertain lengths. These were discernable by chromatography on the DNAPac PA200 because digestion of each linkage isomer produced fragment(s) unique to the position(s) of the 2'-5' linkage in the sequence (See Figures 3, 4).

This work was subsequently published: Thayer et. al, *Anal. Biochem.* **2007**, *361*, 132-139

At the TIDES 2008 Oligonucleotide Analysis Workshop we presented a simple, automated method for desalting DNAPac-separated nucleic acids for subsequent ESI-MS (See Figures 5-7). We now find the IP-RPLC protocol developed for that work fails to resolve oligonucleotides from the salt when the oligos are < 6 bases, and update that report (See Figures 8-10).

Here we employ a direct RPLC method to retain and resolve short, or long, oligonucleotides away from salt during the desalting step. This allows ESI-MS identification of the short RNA fragments produced by the phosphodiesterase digestion (See Table 1).

Using a simple gradient, the DNAPac PA200 column retains and resolves several RNA oligonucleotides having identical lengths and sequences, but harboring aberrant 2'-5' linkages at various positions in the sequence.

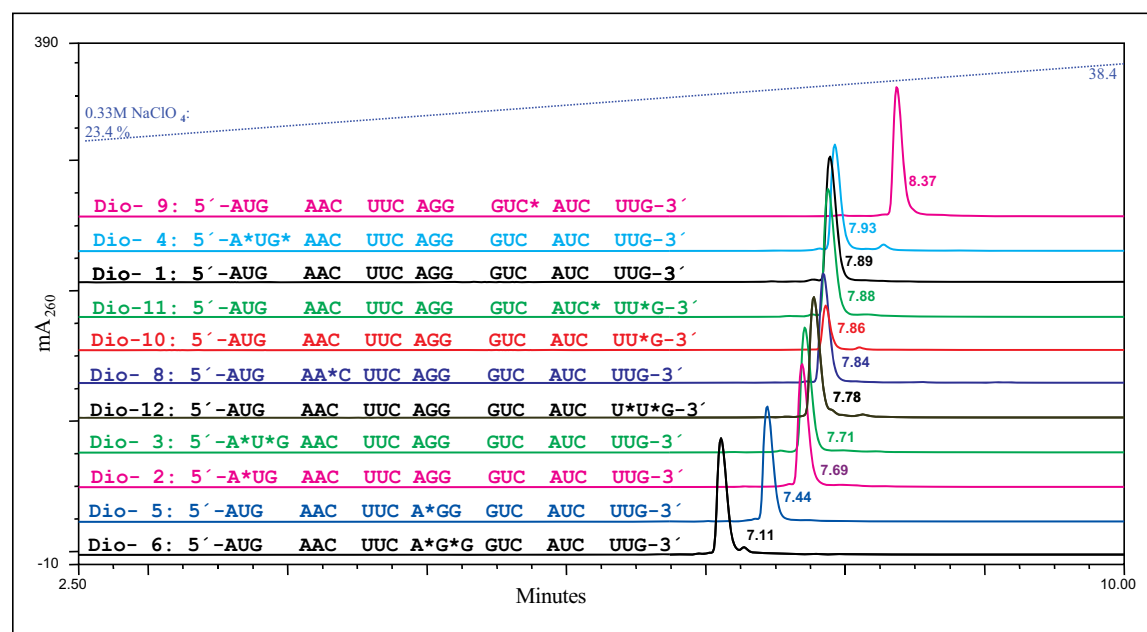


Figure 1. Resolution of same-sequence mixed-base 21 mers with 2'-5' linkages. Column: DNAPac PA200, Gradient: 66-132 mM NaClO₄/10 min, 1 mL/min, 30 °C, pH 7.

The new DNASwift™ SAX-1S monolith, a high capacity column for nucleic acid purification, exhibits resolution for these isomers similar to the DNAPac column (Figure 2).

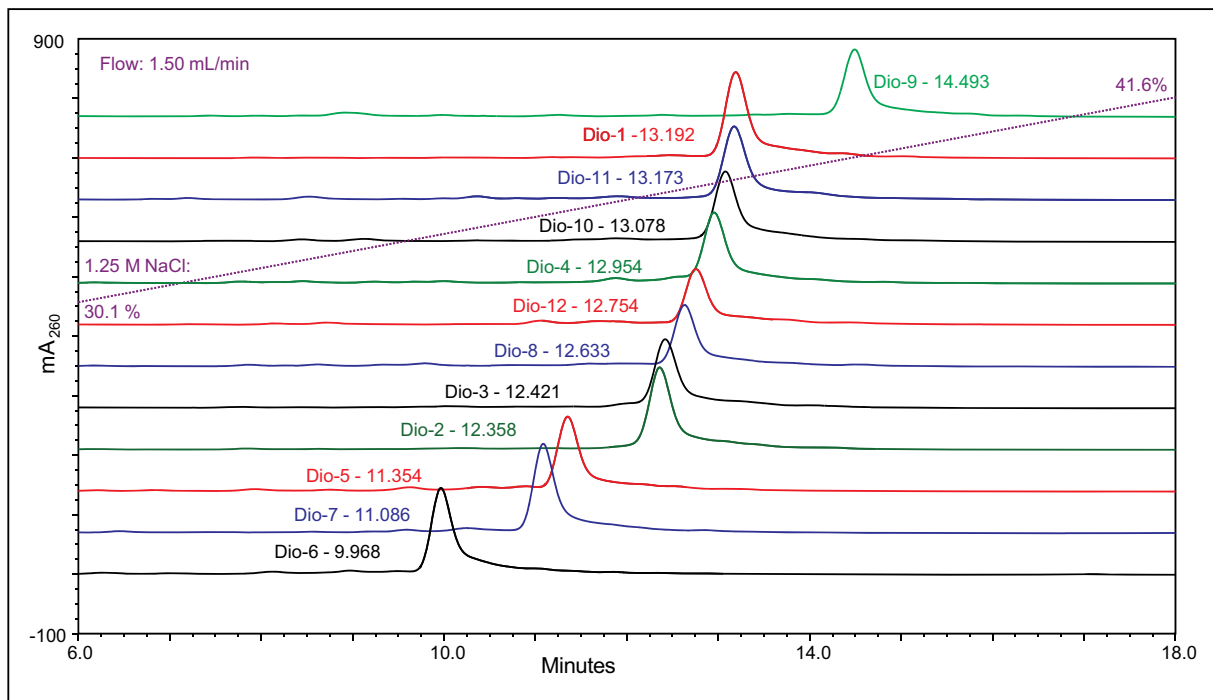


Figure 2. DNASwift preparative resolution of RNA linkage isomers. 325–575 mM NaCl in 10 column volumes (CV), 1.5 mL/min, pH 7, 30 °C.

Figures 3 and 4 show that each linkage site produces a unique set of phosphodiesterase-II (PDAse-II) digestion fragments.

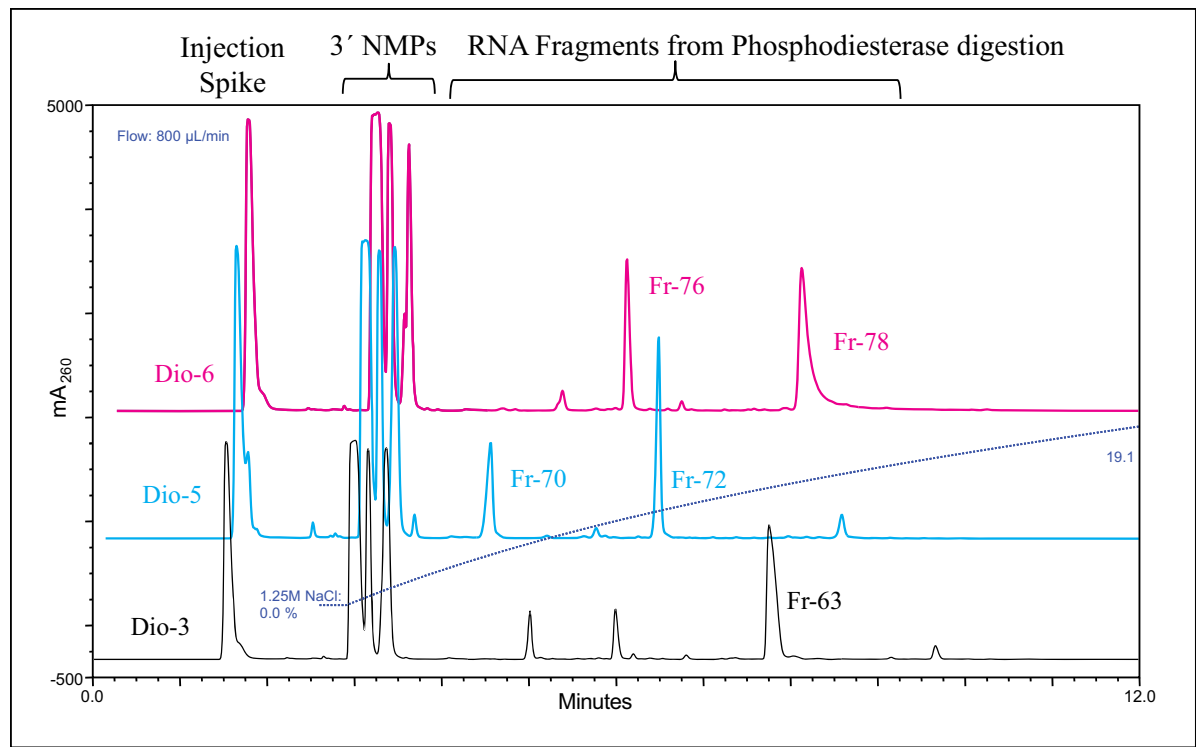


Figure 3. Fractions collected from Dio-3, -5, and -6, separated using the DNAPac PA200 column.

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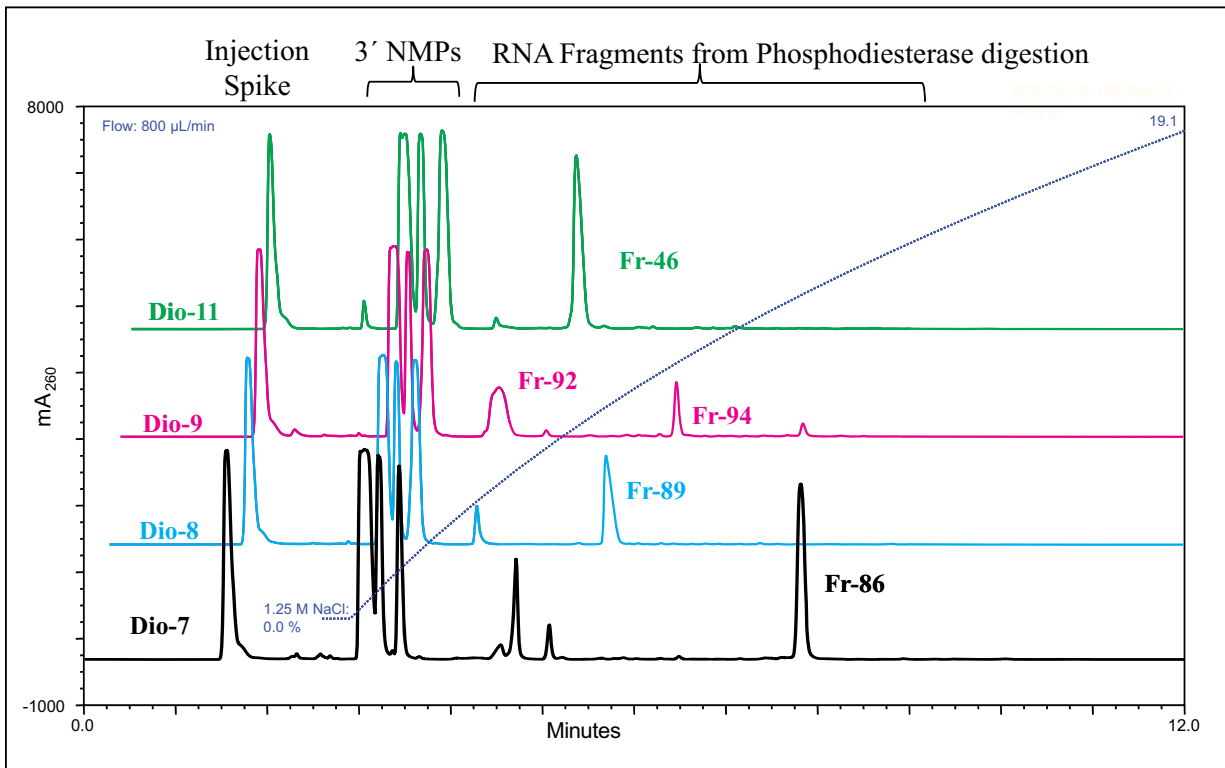


Figure 4. Fractions collected from Dio-7, -8, -9, and -11, separated using the DNAPac PA200 column.

The UltiMate 3000 system, equipped with the autosampler/fraction-collection option can be configured to both purify oligonucleotides and automatically desalt them under program control. A schematic diagram of the system is provided below.

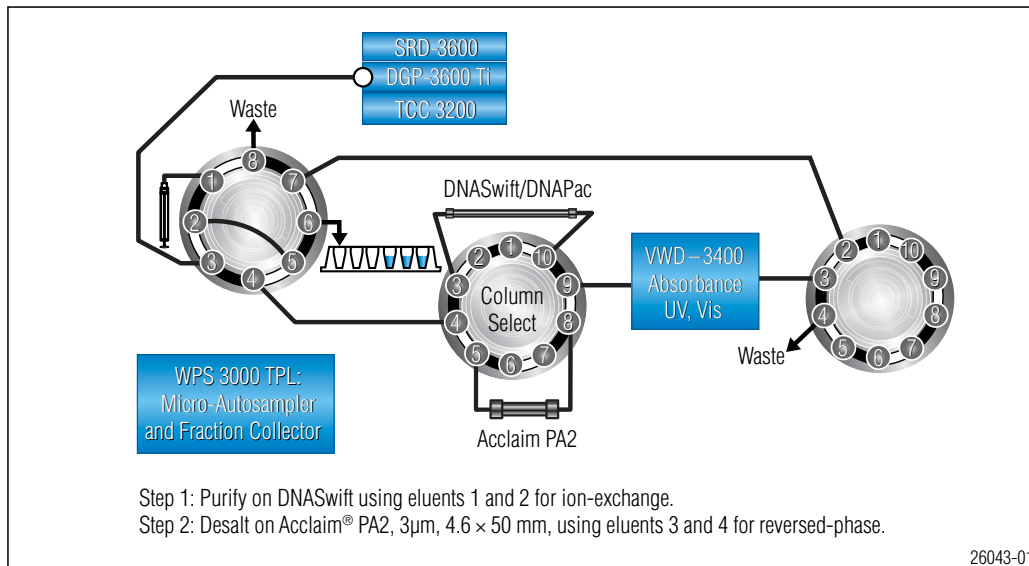


Figure 5. Simple, automated purification and desalting using the UltiMate® 3000 Titanium system.

An example of RNA purification and desalting for ESI-MS is shown in Figures 6 and 7. In Figure 6, 125 µg of a 21-base RNA linkage isomer is purified on the DNASwift monolith. The insets of this figure show the purification to enrich the target 21-mer RNA from 78% to 97%.

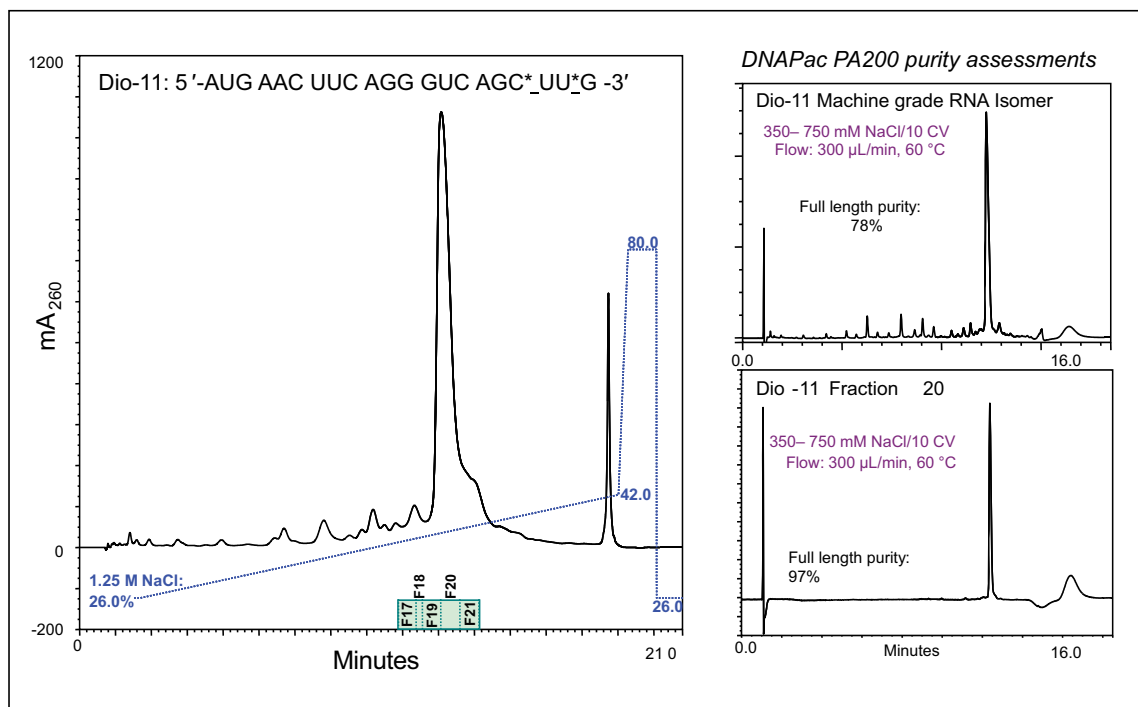


Figure 6. DNASwift purification of RNA linkage isomer Dio-11. 325–525 mM NaCl in 10 CV, pH 7, 30 °C, 1.5 mL/min, 125 µg sample.

Figure 7 shows the automated desalting of this 21-base RNA linkage isomer, and the deconvoluted mass spectrum of the desalted RNA. This reveals the correct mass for the oligonucleotide, and the presence of minor amounts of sodium adducts.

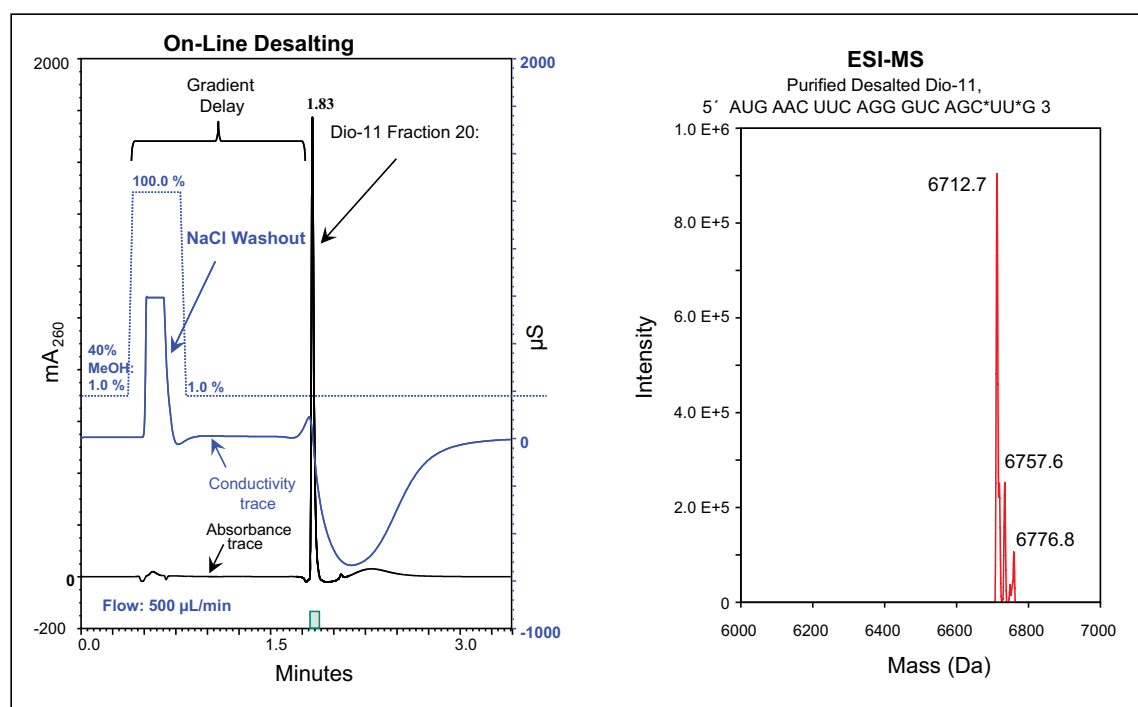


Figure 7. Automated RNA isomer desalting on Acclaim PA2: ion-pair desalting of purified fraction 11 and resulting deconvoluted ESI-MS spectrum.

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The two panels at the left of Figure 8 show the successful ion-pair mode desalting of a similar 21-base RNA isomer, and that this approach fails to successfully desalt the short RNA fragments produced by PDAse-II treatment of the RNA. The right panel shows that this fragment is successfully desalted using a direct reversed-phase method with 20 mM ammonium formate and a step from 1 to 40% methanol.

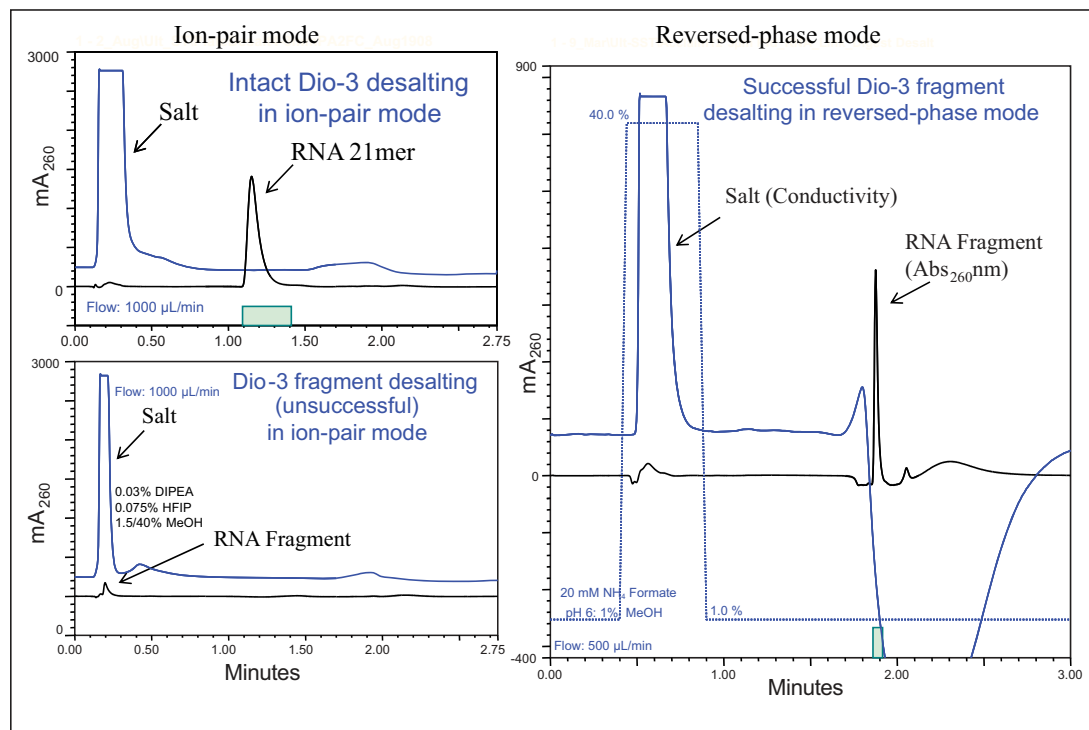


Figure 8. Oligonucleotide fragment desalting: Acclaim PA2, ion-pair vs reversed-phase modes.

Figure 9 shows an example mass spectrum before and after deconvolution and correction for monoisotopic mass on a Thermo LTQ mass spectrometer.

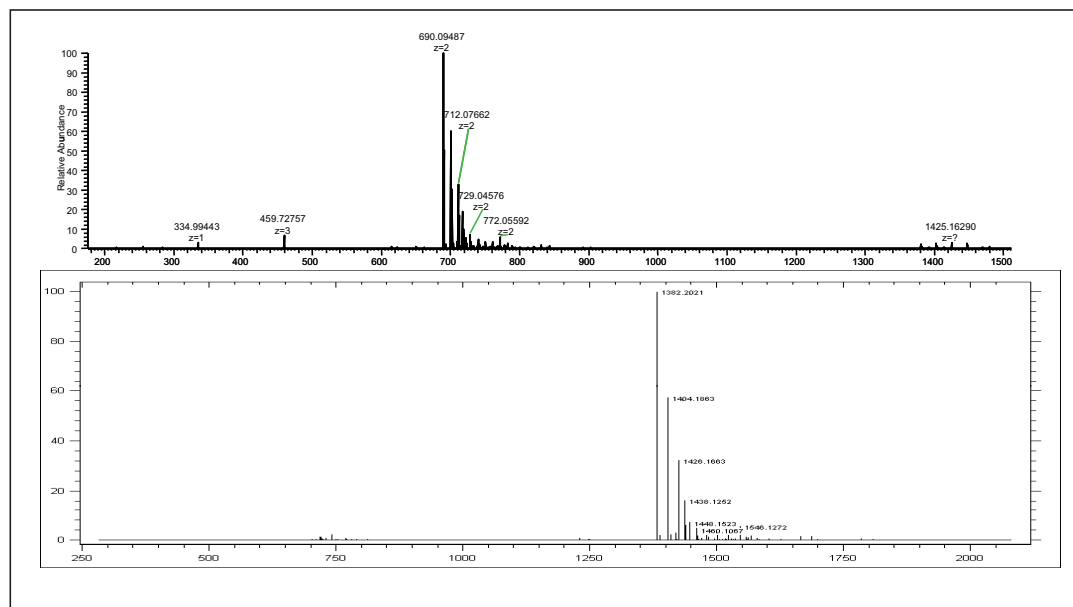


Figure 9. ESI-MS identification of fragment 86 from Dio-7. The deisotoped spectrum reveals the uncharged monoisotopic mass. The mass is consistent with rRGrGrGp (i.e., one terminal phosphate). The expected mass for this species is 1382.2 (~2 ppm error).

Figure 9 displays the raw spectra of two other RNA isomer fragments produced by PDAse-II digestion (of Dio-3 and Dio-5), after automated desalting.

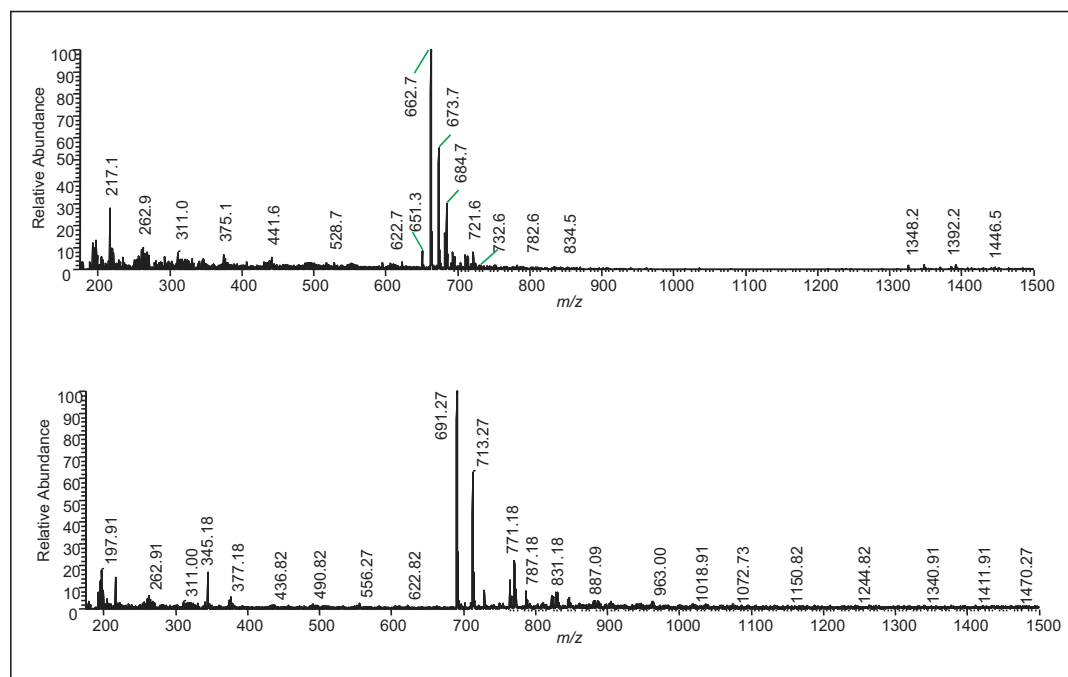


Figure 10. Examples of ESI-MS spectra of aberrant-linkage RNA fragments.
 Top: Negative ion mass spectrum of Dio-3, fragment 63. The peak at m/z 662.7 is a doubly-charged peak, consistent with rArUrGrA-p (MW=1327.2).
 Bottom: Negative ion mass spectrum of Dio-5, fragment 70. The peak at m/z 691.2 is consistent with rArG-p (MW 692.1).

Table 1 summarizes the results of the RNA isomers studied here.

Table 1. Identification of Phosphodiesterase-II RNA Fragments Based on original sequence, resulting mass, and elution on DNAPac PA200				
eGFP				
Antisense 5'-AUG AAC UUC AGG GUC AGC UUG-3'				
Modifications: All underlined residues have a 2'-5' linkages				
Name	Fraction	Sequence and Position of Digest Fragment	Fragment ID	Mass
Dio-1	none	5'-AUG AAC UUC AGG GUC AGC UUG-3'	None (only NMPs)	—
Dio-3	63	5'- <u>A*U</u> *G AAC UUC AGG GUC AGC UUG-3'	rArUrGrA-p	1327.2
Dio-5	70	5'-AUG AAC UUC <u>A*GG</u> GUC AGC UUG-3'	rArG-p	691.2
Dio-5	72	5'-AUG AAC UUC <u>A*GG</u> GUC AGC UUG-3'	rArGrG-p	1037.2
Dio-6	76	5'-AUG AAC UUC <u>A*G</u> *G GUC AGC UUG-3'	rArGrG-p ?	1037.2
Dio-6	78	5'-AUG AAC UUC <u>A*G</u> *G GUC AGC UUG-3'	p-rArGrG ?	1037.2
Dio-7	86	5'-AUG AAC UUC <u>A*GG*</u> GUC AGC UUG-3'	rArGrGrG-p	1382.2
Dio-8	89	5'-AUG <u>AA</u> *C UUC AGG GUC AGC UUG-3'	rArCrU-p	958.1
Dio-9	92	5'-AUG AAC UUC AGG GUC <u>*</u> AGC UUG-3'	rCrA-p	652.1
Dio-9	94	5'-AUG AAC UUC AGG GUC <u>*</u> AGC UUG-3'	p-rArG	692.1
Dio-11	46	5'-AUG AAC UUC AGG GUC AGC* <u>UU</u> *G-3'	rCrUrUrG	1200.2

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Observations Based on This Data:

1. Phosphodiesterase-II fails to cleave 2'-5' linked RNA, but will cleave endonucleolytically, at 3'-5' linkages one or two bases to the 3' side of the aberrant linkage. This enzyme requires a 5'-OH and leaves 3'- (or 2'-) phosphonucleotides, unless the last base is the 3' terminus where it leaves the 3'-hydroxide (Table 1, Dio-3, -5, -6, -7, -8, -11 and -9 [Fr 92]).
2. Two products from Dio-6 that are resolved by ~ 2 minutes by DNAPac chromatography (Figure 3) have the same mass (Table 1). This is consistent with phosphorylated AGG RNA representing the 3' phosphorylated form, and either a 5' phosphorylated 3'-hydroxyl form, or a short (3-nucleotide) tetrad ladder (Table 1, Dio-6 [Fr 78]).
3. Also of interest is the second product of Dio-9 (phosphorylated AG), that seems to indicate cleavage of the 2'-5' linkage, but not the adjacent (3'-5') linkage (Table 1, Dio-9 [Fr-94]).

CONCLUSIONS

The DNAPac PA200 Column

- Resolves many mixed-base 21-mer linkage isomers (Figure 1).
- Retains incomplete PDAse-II degradation products of each of the mixed-base 2'-5' linkage isomers in unique positions (Figure 3-4).
- Partially resolves the complete degradation products (3' NMPs) of both normal and aberrantly-linked isomers (Figures 3-4).
- Delivers purified oligos and RNA fragments fractions high in salt concentration.
- Designed for high resolution analyses for samples up to ~100 µg on-column.

The DNASwift SAX-1S Monolith

- Resolves RNA linkage isomers (Figure 2)
- Delivers purified oligos and RNA fragment fractions high in salt concentration
- Designed for high-capacity, high-yield oligonucleotide purification (up to 10 mg on column)

Automated Acclaim PA2 Desalting

- In ion-pair mode, desalts oligos and RNA fragments >6 bases long (Figures 5-7)
- In RP mode (NH₄HCO₂H), desalts oligos and fragments from 2 bases up (Figure 8)
- Allows direct ESI-MS identification of phosphodiesterase-II digestion products of RNA linkage isomers (Figures 9-10 Table 1).

ORDERING INFORMATION

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DNAPac PA200 Guard (2 × 50 mm)	063423
DNAPac PA200 Guard (4 × 50 mm)	062998
DNAPac PA200 Analytical (9 × 250 mm)	063421
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DNAPac PA200 Guard (22 × 50 mm)	SP6731
DNASwift Monolith SAX-1S (5 × 150 mm)	066766
Acclaim PA2 C18, 3 µm, 120 Å (4.6 × 50 mm)	063189

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LPN 2292-01 6/09
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