An ESI-MS method for characterization of native and modified oligonucleotides used for RNA interference and other biological applications

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RNA interference (RNAi) has become a powerful tool for investigating gene function, and, in addition, shows potential for the development of therapeutic agents. RNAi can be triggered in a variety of eukaryotic cells using small interfering RNA (siRNA), their double-stranded precursors (double-stranded RNA) and short hairpin precursors (shRNA). Here, we describe a protocol for analyzing these RNAs and their modifications using electrospray ionization mass spectrometry (ESI-MS). This protocol involves the desalting of nucleic acids using ammonium acetate precipitation, followed by characterization using ESI-MS. This protocol has been chiefly used for analyzing siRNAs and their chemical modifications, but it has also been used and can be applied to the analysis of a wide range of native and modified oligonucleotides. This protocol provides accurate information on molecular weight for a range of nucleic acids and can be completed in less than a day.

INTRODUCTION

RNAi is a naturally occurring cellular phenomenon whereby short double-stranded RNA (dsRNA) molecules trigger the degradation of complementary mRNAs in a homology-dependent manner[1–3]. Since the demonstration of the effectiveness of siRNAs in specific silencing of gene expression in mammalian cells[4], siRNAs have been used in a number of studies investigating gene function and have shown great promise as a tool in functional genomics, *in vitro* and *in vivo* target validation and as therapeutics for silencing aberrant gene expression[5–8]. Several research groups are working to expand the utility of siRNAs by exploring nucleic acid modifications that can improve the cellular uptake, nuclease stability, potency and target specificity of siRNAs, and increase our understanding of the mechanistic details of RNAi and of other cellular processes[9–23].

Because of the importance of siRNAs as tools in studying gene function, in aiding drug discovery and as therapeutic agents, a robust and effective technique for quickly identifying and characterizing siRNAs and chemically modified siRNAs can be extremely useful in a number of studies. Although methods such as HPLC, capillary electrophoresis and polyacrylamide gel electrophoresis are useful for assessing purity and obtaining an approximate estimation of the length of oligonucleotides, mass spectrometry data are irreplaceable as they allow the determination of exact molecular weight of a species. We have developed an ESI-MS-based method for characterizing the modification of siRNAs with an array of photolabile groups[22,23]. One such modification reaction of siRNA with the 1-(4,5-dimethoxyl-2-nitrophenyl)diazoethane caging group is shown in **Figure 1** (see ref. 23). We have subsequently demonstrated that this method can be used for characterizing single-stranded oligonucleotides, shRNAs and double stranded precursors of siRNA (dsRNA) that are processed by the Dicer endonuclease to generate active siRNAs[22]. In addition, we have shown that this method can be used to

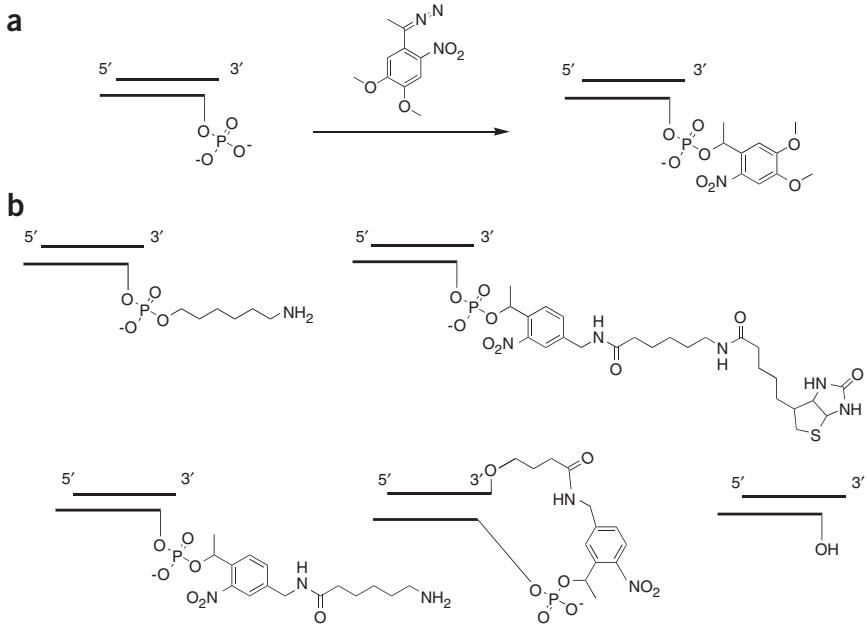

Figure 1 | Examples of modified and unmodified siRNAs and dsRNAs examined with mass spectrometry using this protocol. (**a**) Modified siRNA formed through the reaction of DMNPE with the 5′-antisense phosphate of siRNA. (**b**) Other examined siRNAs, dsRNAs and shRNAs. The typical length of siRNAs and dsRNAs examined using this protocol varies from 21 mer duplexes to 27 mer duplexes. The protocol has also been used for characterizing 50–60 mer shRNAs and a range of modified and unmodified single-stranded oligonucleotides (unpublished results).

quantify the amount of a specific oligonucleotide in the presence of a known quantity of a second structurally related oligonucleotide[22]. In addition to their use in triggering RNAi, synthetic oligonucleotides have been useful as primers for PCR, site-directed mutagenesis and sequencing reactions, as probes in cDNA hybridization studies and as antisense oligonucleotides. The ESI-MS method described in this protocol should be useful in identifying and characterizing a range of these single-stranded oligonucleotides, in addition to the siRNAs and chemically modified siRNAs described explicitly here.

The analysis of oligonucleotides by ESI-MS requires the preliminary removal of Na^+ ions from the oligonucleotide. The binding of Na^+ ions to the negatively charged oligonucleotide backbone disperses the oligonucleotide ion current among multiple Na^+-containing species within each charge state, thereby reducing the sensitivity of the mass measurement[24]. In addition, if the instrument used for analysis lacks sufficient peak-resolving power, the presence of multiple sodium adducts can result in broad peaks and erroneous mass measurements when analyzing larger oligonucleotides[24,25]. Stults and Marsters[26] and Potier *et al.*[27] have demonstrated that treatment with ammonium acetate aids in the removal of sodium ions and that the ammonium ions bound to the oligonucleotide are removed during the ionization process. We have used this method for the characterization of siRNAs and have demonstrated that the present desalting procedure combined with the sensitivity of a hybrid triple quadrupole ion trap mass spectrometer results in accurate and reproducible characterization of a wide range of oligonucleotides[22,23] (**Fig. 1**).

We have used an AB Sciex API 2000 LC/MS/MS system for analyzing the oligonucleotides, but this protocol could be useful for analyzing oligonucleotides using a range of mass spectrometers. In addition to the hybrid triple quadrupole ion trap analyzer we used, this method could be adapted to the characterization of oligonucleotides using a single quadrupole, triple quadrupole, ion trap, Fourier-transform ion cyclotron resonance, time-of-flight or quadrupole time-of-flight mass analyzer. Using these analyzers, available from different vendors such as Thermo Finnigan, Waters, Bruker Daltonics and Agilent, may require further optimization of the parameters described in the protocol, which have been optimized for the AB/MDS Sciex triple quadrupole ion trap analyzer. However, the same desalting procedure can be used for the removal of Na^+ ions and this should be independent of the instrument. The desalting procedure and the mass spectrometry conditions described here should be useful for analyzing a range of different oligonucleotides and siRNAs and for characterizing modification of the termini or internal positions of these oligonucleotides.

MATERIALS

REAGENTS

- siRNA/dsRNA duplexes or single-stranded oligonucleotides in water or buffer
- Water: RNase-free, diethylpyrocarbonate-treated (USB, cat. no. 70783)
- Ammonium acetate (Fisher Scientific, cat. no. A637)
- Ethanol 200 proof (absolute) for molecular biology (Sigma-Aldrich, cat. no. E7023)
- Glycogen (optional, see Step 2; USB, cat. no. 16445)
- Acetonitrile (HPLC; Fisher Scientific, cat. no. A998-4)
- Triethylamine (Sigma-Aldrich, cat. no. 471283)

EQUIPMENT

- Refrigerated microcentrifuge (Eppendorf 5417R microcentrifuge)
- Microliter syringe (250-µl Hamilton gas-tight syringe)
- UV spectrophotometer (Ocean Optics USB2000 spectrometer)
- Q TRAP LC/MS/MS system (AB/MDS Sciex API 2000 system, equipped with Analyst and BioAnalyst software)

REAGENT SETUP

siRNA/oligonucleotide Use RNase-free water to dissolve the chemically synthesized siRNA in water or buffer to a final concentration of 25 µM. We generally store our siRNA stock solutions in RNase-free water or Tris-acetate-EDTA buffer (12.3 mM Tris, 12.3 mM acetate, 0.3 mM EDTA, pH 8.3). 30 µl of the 25-µM solution is typically used for each analysis. The present protocol can be adapted to perform analyses on smaller samples, but, in our experience, using less than 10 µl (25 µM) of an siRNA solution results in a low signal-to-noise ratio. siRNA stock solutions can be stored for 2 months at −20 °C or for a year at −80 °C.

7.5-M ammonium acetate solution. Add 0.289 g of ammonium acetate to 500 µl RNase-free water and shake for 20 min. This solution can be stored for 2 months at 2–8 °C. Allow the solution to return to room temperature before use. ▲ **CRITICAL** Do not autoclave this solution as ammonium acetate decomposes in hot H_2O via the loss of ammonia.

20 mg ml^{-1} glycogen solution Add 10 mg of glycogen to 500 µl of RNase-free water in a safe-lock biopur Eppendorf tube and shake gently for 20 min. The resulting solution can be stored for 6 months at −20 °C. Allow the solution to return to room temperature before use.

Wash solution Add 49.5 µl of acetonitrile to 49.5 µl water and 1 µl triethylamine to make a final solution of water/acetonitrile (50:50, 100 µl) containing 1% triethylamine.

PROCEDURE

1| To the siRNA solution (25 µM, 30 µl) in water or buffer, add 15 µl of 7.5 M ammonium acetate solution to achieve a final concentration of 2.5 M ammonium acetate. Gently vortex and allow the mixture to stand for 1 h at room temperature to enhance the displacement of sodium ions.

2| (Optional) Add 0.75–1 µl of 20 µg $µl^{-1}$ glycogen solution to the mixture to help visualize the pellet formed and improve precipitation efficiency. Addition of glycogen solution is optional, but it is harder to visualize and handle the pellet without it.

3| Add 115 µl (~2.5 volumes) of ethanol. Place the sample for at least 4–7 h in a −80 °C freezer. Please note that a pellet can also be obtained with shorter incubation times and by incubating at −20 °C. However, we have observed that the precipitation efficiency tends to be lower and less reproducible when a shorter incubation time or different temperature is used.

4| Set the microcentrifuge temperature to 0 °C before using the microcentrifuge for precipitation. After the temperature stabilizes to 0 °C, remove the sample from the −80 °C freezer and centrifuge for 30 min at 17,400*g* and 0 °C to precipitate the siRNA.

? TROUBLESHOOTING

5| Immediately after obtaining the pellet, carefully decant the supernatant using a pipette.
▲ **CRITICAL STEP** A delay in removing the supernatant can lead to the solubilization of the siRNA and to a reduced precipitation efficiency.

6| To remove residual salt, wash the pellet with ice-cold 70% ethanol: add 100 µl of ice-cold 70% ethanol to the pellet. Ensure that the pellet is completely covered with 70% ethanol and shake gently for 1 min. Use a vortexer set at the lowest setting to shake the mixture thus prepared.
▲ **CRITICAL STEP** Do not simply add the 70% ethanol and decant, as it does not appear to efficiently remove the salts, which in turn can lead to signal suppression during electrospray ionization.

7| Set the microcentrifuge temperature to 0 °C and again centrifuge for 20 min at 17,400*g* and 0 °C to ensure that the siRNA remains precipitated or reprecipitates, in case the pellet detaches from the wall of the tube during the washing process.

8| Immediately after centrifuging the pellet, carefully decant the supernatant, using a pipette and allow the pellet to air-dry for 10–15 min. Avoid leaving the pellet to air-dry for longer as excessive drying can make the pellet harder to redissolve in water.

9| Add 32 µl of RNase-free water to the pellet and gently shake for 15 min at room temperature as in Step 6. Measure the absorbance of a very small portion of the sample at 260 nm to confirm that the siRNA is obtained. Using 0.5–1 µl of the stock in 70 µl of RNase-free water is generally sufficient to determine the concentration of the siRNA solution using the extinction coefficient and absorbance of the oligonucleotide species at 260 nm. Please note that we discard the sample used for absorbance studies and do not use it for the mass spectrometry analysis.

10| Through the mentioned procedure, we typically obtain a 60–80% precipitation efficiency, indicating a final siRNA concentration of 15–20 µM. Use 29.7 µl of this final amount for the mass spectrometric analysis. To the 29.7 µl of final siRNA solution, add 29.7 µl of acetonitrile and 0.6 µl of triethylamine to make a final solution of water/acetonitrile (50:50, 60 µl) containing 1% triethylamine. Gently shake the resulting solution for 5 min with a vortexer (see Step 6) before analyzing the sample.

11| To implement the direct infusion method for analysis, and before running the siRNA sample, introduce the wash solution (1% triethylamine in equal parts of water and acetonitrile, 100 µl) into the turbo ion spray source at a flow rate of 10 µl min^{-1} using the microliter syringe. Please note that using a wash solution helps to reduce impurities from previous samples and reduces the amount of sample expended during the time the mass spectrometer requires to equilibrate.

12| Operate the Q TRAP system in the negative-ion mode because of the negatively charged phosphate backbone of oligonucleotides. Set the needle voltage to −4.5 kV, the source temperature to 150 °C and the data obtained to an *m/z* ratio between 500 and 1,200 AMU. Collect data at a scan rate of 1,000 AMU s^{-1} and set the declustering potential to −25 to −50 V, the entrance potential to −6 to −8 V, the collision cell entrance potential to −25 V and the collision energy to −10 V. Set the collisionally activated dissociation to 'high', the curtain gas to 30 psi and the ion source gas 1 (GS1) and gas 2 (GS2) to 30 and 10 psi, respectively. Please note that the parameters mentioned here work well for most oligonucleotides, but some of the parameters can be further optimized for each different sequence by using the quantitative optimization tool in Analyst software.

13| To analyze the sample, use either the enhanced MS or the enhanced multiply charged scan (EMC) mode. Typically, the EMC mode is better suited to oligonucleotides, as it removes the singly charged ions in the ion trap before detection, leading to a higher signal-to-noise ratio. However, using the EMC mode can lead to a reduction in the total signal intensity, and switching on the Q0 trapping when using the EMC mode gives the maximal increase in signal-to-noise ratio.

14| Introduce the sample using the turbo ion spray at a flow rate of 7 µl min^{-1} after having run the wash solution for 10–12 min and after having thus confirmed that there is not a significant residual signal from previous samples. Acquire data in the enhanced MS or EMC mode under the same conditions used for running the wash solution.
? TROUBLESHOOTING

15| Use the Bayesian Protein Reconstruct tool in the BioAnalyst software or a similar mass reconstruction software to reconstruct the final mass spectrum from the raw data. Set the step mass to 0.1–1, the sound-to-noise threshold to 20–25 (range is from 1 to 200) and the minimum intensity, which filters out data points below that level before reconstruction, to 0–2%. Please note, however, that varying these parameters does not significantly alter the final mass reconstruction. Select the start and stop mass for the output of the reconstruction depending on the expected molecular weight of the oligonucleotides being analyzed, to allow the calculation of masses within that range.

● **TIMING**
Step 1: 1 h; Steps 2 and 3: 4–7 h; Steps 4 and 5: 45 min; Steps 6–8: 45 min; Step 9, 30 min; Step 10, 10 min; Steps 11–14, 30 min; Step 15, 5 min

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
4	Pellets are not formed	Short incubation time	Incubate at −80 °C for another 4–5 h
		Low centrifugation speed	Increase the speed of centrifugation
			Add higher amounts of glycogen. Do not exceed 3 μl for every 30 μl of the starting siRNA sample
14	Prominent peaks representing Na^+ adducts	Desalting not successful	Repeat the desalting with ammonium acetate solution procedure two more times before washing the pellet and using it for mass spectrometric analysis
	Very low signal-to-noise ratio	Contamination from previous samples	Run the wash solution mentioned above for a longer period of time
		Low oligonucleotide concentration	Use a higher concentration of the oligonucleotide. The propensity of samples to ionize depends on the nucleotide content and on the presence of chemical modifications. A higher oligonucleotide concentration would improve signal-to-noise ratio for samples that do not ionize easily
			Use EMC mode with the Q0 trapping switched on, if not already being used
	No signal obtained	Sample not reaching ionization chamber	Confirm that the sample is reaching the ionization chamber by testing a standard sample that typically runs well
		Instrument requires tuning	Tuning of the instrument can help to adjust the voltage of the ion source and ensure that adequate ions are produced and directed toward the mass filter and that the sensitivity of the detector is satisfactory
		Low oligonucleotide concentration	Use a larger amount of oligonucleotide

ANTICIPATED RESULTS

A good mass spectrum obtained using this method contains very little contamination from sodium adducts and excellent signal-to-noise ratio. As an

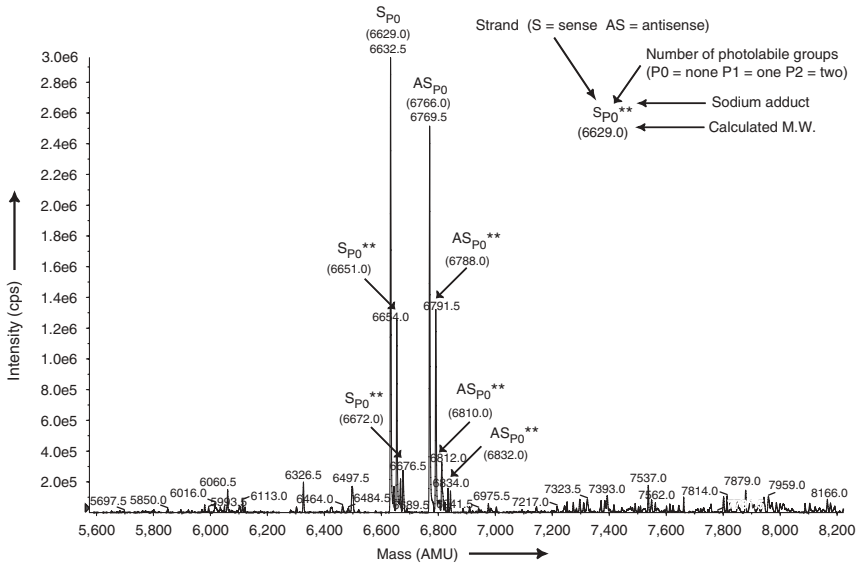

Figure 2 | Reconstructed ESI mass spectrum of a GFP-targeting siRNA (sense strand, 5′-GCA GCA CGA CUU CUU CAA GdTdT-3′; antisense strand, 5′-CUU GAA GAA GUC GUG CUG CdTdT-3′) containing a 5′-phosphate on the antisense strand. Peaks are annotated to indicate the strand (AS, antisense; S, sense) and the number of photolabile modifications (P0, none; P1, one, etc.). Below the annotation is the actual molecular weight of species, which is positioned above the actual observed molecular weight. The double asterisk sign refers to the formation of Na^+ adducts. A significant amount of Na^+ adduct formation, as observed here, indicates inefficient desalting and a requirement for further desalting.

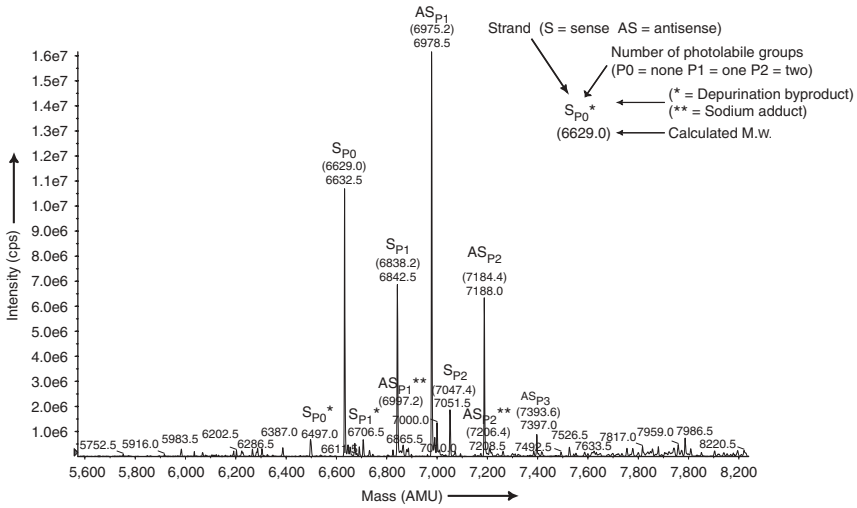

Figure 3 | Reconstructed ESI mass spectrum of a GFP-targeting siRNA (sense strand, 5′-GCA GCA CGA CUU CUU CAA GdTdT-3′; antisense strand, 5′-CUU GAA GAA GUC GUG CUG CdTdT-3′) containing a 5′-antisense phosphate after modification with a photolabile DMNPE group, as shown in **Figure 1a**. Peaks are annotated to indicate the strand (AS, antisense; S, sense) and the number of photolabile modifications (P0, none; P1, one, etc.). Below the annotation is the molecular weight expected for the species, which is positioned above the observed molecular weight. This represents a typical mass spectrum obtained using this protocol, and it contains very little Na^+ adduct contamination (marked by a double asterisk) and some deadenylation byproducts (marked with a single asterisk).

example of a procedure that brought about poor results, **Figure 2** shows the mass spectrum of a GFP-targeting siRNA containing a phosphate on the 5′-antisense strand. The peaks labeled with a double asterisk sign indicate sodium adducts. We observe Na^+ adducts at such high intensity in 2–3% of the samples and it indicates inefficient desalting.

Figure 3, which shows the mass spectrum of a GFP-targeting siRNA containing a 5′-antisense phosphate, after modification with the DMNPE caging group, represents a typical mass spectrum obtained using the protocol. The expected and observed molecular weights for the modified sense and antisense strands are shown in **Figure 3**. We observe very little Na^+-adduct contamination in this spectrum. However, some depurination (deadenylation, in this case) products, marked with a single asterisk, are observed, possibly due to heat-induced degradation during the ionization process. Using this method, we observe that the two strands of the siRNA are separated, likely due to heating during the ionization process. The separation of the two strands can be useful for characterizing individual modifications on the sense and antisense strand when random modification of the siRNA is conducted.

Typically the observed molecular weight using this method is within 5 mass units of the calculated molecular weight. An internal standard of a known molecular weight can be used for applications of this method where a higher accuracy is desired. **Figure 4** shows an example of the use of an internal standard to obtain more accurate molecular weight information using this method. A 21 mer oligonucleotide containing a C6 amino linker on the 5′-end was analyzed using a 21 mer oligonucleotide of a known molecular weight (MW 6766.0) as an internal standard. The uncorrected molecular weight for the oligonucleotide containing a C6 amino linker obtained using this method (observed MW 6868.9) differs from the expected molecular weight (calculated MW 6865.2) by 3.7 AMU. However, the difference between the calculated and observed molecular weight for the internal standard was almost identical (3.8 AMU). Correcting for the difference in the known molecular weight of the internal standard (MW 6766.0) to that observed by mass spectrometry (observed MW 6769.8) results in a mass determination within 0.1 mass units (corrected MW 6865.1) in this case and typically between 0 and 1 AMU.

In addition to using the reconstructed mass spectrum for analysis, we can also

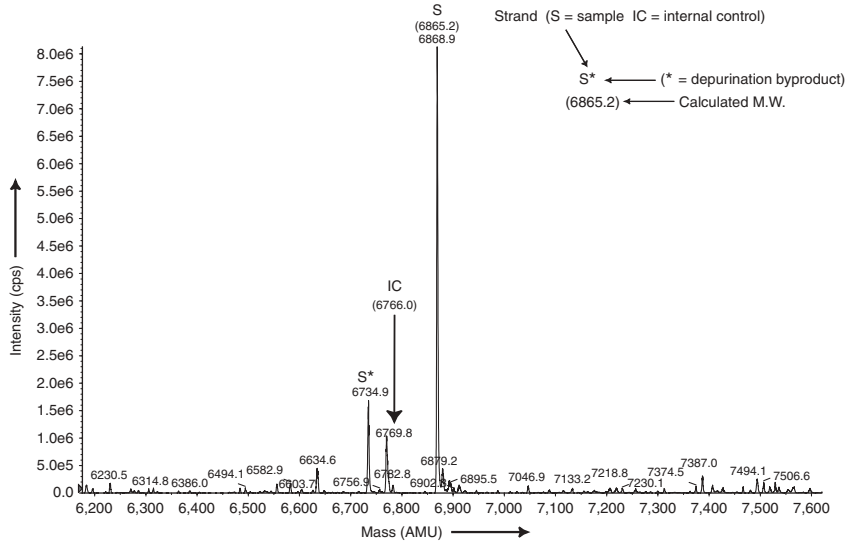

Figure 4 | Reconstructed ESI mass spectrum of a 21 mer oligonucleotide (sequence, 5′-CUU GAA GAA GUC GUG CUG CdTdT-3′) containing a C6 amino linker on the 5′-end in the presence of the same 21 mer oligonucleotide containing only a phosphate modification on the 5′-end of a known molecular weight as an internal standard. Peaks are annotated to indicate the strand (S, sample, IC, control). Below the annotation is the molecular weight expected for the species, which is positioned above the observed molecular weight. A deadenylation byproduct is observed and marked with a single asterisk.

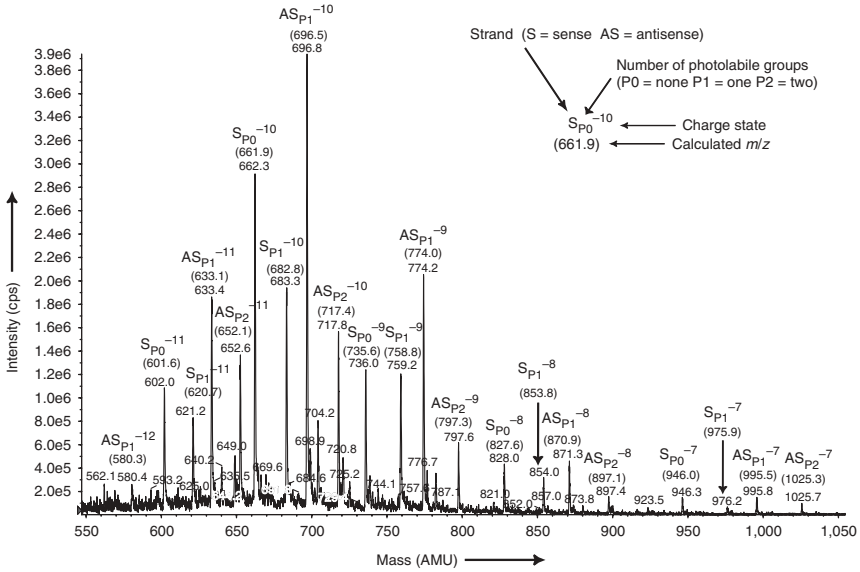

Figure 5 | Raw ESI mass spectrum (before mass reconstruction) of a GFP-targeting siRNA (sense strand, 5′-GCA GCA CGA CUU CUU CAA GdTdT-3′; antisense strand, 5′-CUU GAA GAA GUC GUG CUG CdTdT-3′) containing a 5′-antisense phosphate after modification with a photolabile DMNPE group, as shown in **Figure 1a**. Peaks are annotated to indicate the strand (AS, antisense; S, sense), the charge state (e.g., −10. −9) and the number of photolabile modifications (P0, none; P1, one, etc.). Below the annotation is the actual calculate *m/z* for each species, which is positioned above the actual observed *m/z*.

identify the peaks directly from the raw data trace. Using the ESI-MS method, we observe an *m/z* ratio resulting in several different species with multiple charges corresponding to a single final molecular weight. *m/z* values corresponding to a particular siRNA strand are determined by calculating the molecular weight for each different charge state and then dividing by the number of charges for each state. **Figure 5** shows the raw mass spectrum of the DMNPE-modified GFP-targeting siRNA containing a 5′-antisense phosphate. When possible, it is helpful to use a mass reconstruction software, as it facilitates rapid analysis of the data and allows identification of peaks that can be neglected when analyzing the raw spectrum directly.

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