The Analyst
FULL PAPER

Preparation of single-stranded PCR products for electrospray ionization mass spectrometry using the DNA repair enzyme lambda exonuclease

Allison P. Null, James C. Hannis and David C. Muddiman*

Department of Chemistry, Virginia Commonwealth University, 1001 West Main Street, Richmond, Virginia 23284, USA. Email: dcmuddim@saturn.vcu.edu

Received 5th October 1999, Accepted 4th January 2000
Published on the Web 24th February 2000

Electrospray ionization mass spectrometry (ESI-MS) has been utilized to obtain accurate mass measurements of intact PCR products; however, single-stranded PCR products are necessary to detect sequence modifications such as base substitutions, additions or deletions. The locations of these modifications can subsequently be determined using additional stages of mass spectrometry. The recombinant enzyme lambda exonuclease selectively digests one strand of a DNA duplex from a 5′ phosphorylated end leaving the complementary strand intact. Using this rapid enzymatic step, we were able to produce single-stranded PCR products by digestion of an intact PCR product derived from the Human Tyrosine Hydroxylase (HUMTHO1) gene, which contains a tetrameric repeating motif. The non-template directed 3′ adenylation common when using *Taq* polymerase resulted in three distinct species (blunt-ended, mono-adenylated and di-adenylated), which added complexity to the spectrum of the double-stranded product. The data from the single-stranded products shows that one strand is preferentially adenylated over the other, which cannot be determined from the mass spectrum of the double-stranded PCR product alone. The ESI-FTICR (Fourier transform ion cyclotron resonance) mass spectra of the lambda exonuclease treated PCR products exhibited less than expected signal-to-noise (S/N) ratios. This is attributed to inaccurate concentration calculations due to remaining double-stranded PCR product amplified with unphosphorylated primers, and to matrix effects contributed by the lambda exonuclease reaction buffer. To further test this hypothesis, we investigated and determined the limit of detection to be 0.27 μM using standard curve statistics for single acquisitions of a synthetic 75-mer. The concentrations of the noncoding and coding strands produced by lambda exonuclease digestion were calculated to be 0.29 and 0.37 μM, respectively, taking into account the presence of double-stranded product. The products were electrosprayed from concentrations at the limit of detection requiring the averaging of 5–10 acquisitions to produce a sufficient S/N ratio, indicating that product concentration, base composition and matrix effects play a combined, significant role in detection of lambda exonuclease treated PCR products. Although additional work will be required to further exploit this strategy, lambda exonuclease clearly provides mass spectrometrists with a method to generate single-stranded PCR products.

Introduction

The accurate definition of short genetic sequences derived from genomic DNA using the polymerase chain reaction (PCR)[1] is critical to geneticists for gene mapping and genetic disease diagnosis and to forensic scientists for human identification. Detection of PCR products is currently accomplished using gel electrophoresis; however, gel-based methods, which use fluorescent tags or radioactive isotopes, are laborious, time consuming and often inaccurate. The inherent mass accuracy and short analysis time of a mass spectrometric approach provide the basis for development of electrospray ionization mass spectrometry (ESI-MS)[2] for the characterization of nucleic acids.[3–6]

ESI-MS has been utilized to obtain accurate mass measurements of intact PCR products subsequently used to differentiate bacterial strains,[7] to detect single nucleotide polymorphims,[8] to detect deletions of portions of a gene[9] and to accurately define the number of tandem repeats.[10] However, to identify a base deletion, addition or substitution in a PCR product, detection of the single-stranded oligonucleotide is demanded. For example, a nucleotide substitution of an adenosine for a thymidine in one strand will consequently result in a thymidine being substituted for an adenosine in the complementary strand resulting in a zero mass difference. The mass of either single-strand clearly reflects the exact *nature* of the polymorphism, 9 Da in this particular example, indicative of an A–T substitution. The implementation of tandem or multi-stage mass spectrometry potentially allows for a means to determine the exact *location* of the polymorphism. McLafferty and coworkers have reported dissociation of single-stranded and double-stranded oligonucleotides that produced significant sequence information and demonstrated that the complete dissociation of single-stranded oligonucleotides at the 50-mer level is achievable.[6,11] However, detection of single-stranded PCR products is essential for the following reasons: (1) when collisional activation studies are invoked, product ions are derived from one strand thus reducing the complexity of the interpretation; (2) since each strand has roughly the same molecular weight, detection of the single-stranded species reduces the overall mass by about a factor of two which is more amenable to dissociation due to fewer internal modes of vibration as taught by the Rice–Ramsperger–Kassel–Marcus/Quasi-equilibrium theory;[12,13] (3) it increases the likelihood of achieving isotopic resolution for both the intact species and product-ion spectra which allows for more accurate mass measurements and direct-charge state determination;[14] (4) when carrying out multiplex PCR, a method commonly used in genetics and forensic science to amplify multiple loci in a single PCR, mass analysis of the multiple species can be accomplished more readily if only a single-stranded species from each locus is present since it decreases the complexity of the mass spectrum by a factor of two. It should be noted that all the arguments

stated above, except the last one, are valid regardless of whether both strands are present in the mass spectrum. There have been numerous reports of the detection of double-stranded PCR products by ESI-MS[7,10,15–19] with the largest extending beyond 500 base pairs in size.[20] Previous attempts at electrospraying PCR products that were heat denatured resulted in diminished intensity of the single-stranded molecular ion signal with a concomitant increase in the double-stranded species with time.[10] Thus, it appears that it will be necessary to remove one strand prior to analysis to prevent reannealing of the complementary strands. Khramer *et al.*[21] have recently described a method for PCR clean-up and preparation of single-stranded PCR products utilizing biotin–streptavidin chemistry, which should be compatible with ESI-MS. They report the use of a biotin-labeled primer in the PCR phase that allows one strand of a PCR product to bind to streptavidin-coated magnetic beads while the other is removed by heat denaturation. However, due to significant sample loss during removal of the unbiotinylated strand and mass error, the authors were unsuccessful in obtaining mass spectra of the isolated strand.[21] We describe an alternative method for isolation of single-stranded PCR products using the recombinant enzyme, lambda exonuclease.

Exonucleases enable DNA polymerases to repair misincorporations that exist in double-stranded DNA by excising non-complementary nucleotides in either the 5′-3′ or 3′-5′ direction allowing the DNA polymerase to insert the correct nucleotide.[22–24] Exonuclease activity commonly plays a proofreading role in DNA polymerases that are used in PCR and DNA sequencing, including *Taq*,[25] *Pfu*,[26] DNA polymerase I[27] and Sequenase.[28] Lambda exonuclease is a recombinant enzyme that selectively digests a single strand of a DNA duplex in the 5′–3′ direction from a 5′ phosphorylated end generating single-stranded DNA for analysis.[29] This enzyme has recently been utilized to prepare single-stranded templates for DNA sequencing from PCR products by gel electrophoresis.[30,31]

To obtain accurate sequence information from single-stranded DNA by ESI-MS (and MS/MS) for characterization of PCR products, a rapid, reliable strategy that would be compatible with the buffers commonly used in ESI of large oligonucleotides is essential. Herein, we illustrate the potential for using a 5′ phosphorylated primer in the PCR phase with subsequent digestion of the PCR product with lambda exonuclease prior to analysis using ESI Fourier transform ion cyclotron resonance[32,33] (FTICR) mass spectrometry to efficiently and reproducibly detect single-stranded PCR products.

Experimental

PCR amplification

The protocol for PCR amplification was optimized in our laboratory on a 24-well Perkin Elmer GeneAmp 2400 thermal cycler with final PCR amplification accomplished on a 96-well MJ Research PTC-225 Peltier Thermal Cycler (Watertown, MA, USA) at Commonwealth Biotechnologies Inc. (Richmond, VA, USA). Primers were synthesized and purified by anion exchange at the Midland Certified Reagent Co. (Midland, TX, USA) with one primer from each pair modified with a 5′ phosphorylation. Reagents for each 50 μL reaction included: 2.5 units of Ampli-Taq Gold polymerase (specific for hot-start PCR), 1× GeneAmp PCR buffer (Perkin-Elmer, Branchburg, NJ, USA), 12.5 pmol of each primer, 0.2 mM dNTPs (Promega, Madison, WI, USA) and 100 ng of K562 template DNA (Promega). Amplification was accomplished utilizing hot-start/Touchdown PCR to eliminate non-specific primer annealing using an initial hold temperature of 94 °C for 12 min followed by six cycles of 94 °C for 15 s (denaturation), 63 °C for 15 s (primer annealing), and 72 °C for 15 s (elongation). Subsequent cycles involved reducing the annealing temperature by 3 °C every six cycles until reaching the touchdown temperature of 53 °C. Final elongation was accomplished at 72 °C for 5 min.

Ethanol precipitation

PCR salts, dNTPs, excess primers and enzyme were removed from the PCR product by performing an ethanol precipitation as outlined by Sambrook *et al.*[34] and optimized by Crouse and Amorese,[35] which has previously been reported for use with ESI-MS.[36,37] Four 50 μL PCR reactions were pooled into a 1.5 mL microcentrifuge tube and 100 μL of ammonium acetate (Sigma, St. Louis, MO, USA) was added resulting in a final concentration of 2.5 M. 750 μL of cold ethanol (Sigma) was added, the tube was inverted several times to mix and then precipitated at 4 °C overnight to ensure good recovery. The PCR product was collected by centrifugation at 16 000*g* for 30 min followed by a rinse with 200 μL cold ethanol and subsequent lyophilization. The PCR product was reconstituted in 20 μL of 10 mM ammonium acetate.

Digestion of PCR products with lambda exonuclease

Reactions were performed in a final volume of 10 μL in a 200 μL PCR tube that included 20 pmol of the 82 bp PCR product derived from the Human Tyrosine Hydroxylase (HUMTHO1) gene[38] equivalent to *ca.* 2 reactions, 1 μL of lambda exonuclease 10 × buffer and 1 μL (5 units) of lambda exonuclease (Boehringer Mannheim, Indianapolis, IN, USA). Reactions were carried out for 1 h at 37 °C using the GeneAmp PCR system 2400 (Perkin-Elmer) followed by an enzyme deactivation step at 75 °C for 10 min. Following digestion, reaction contents were removed by centrifugation for 1 min. at 16 000*g* and the single-stranded PCR product transferred to a fresh tube. The product was purified with an additional ethanol precipitation step as described above.

Microdialysis

Single-stranded and double-stranded PCR products and the synthetic 75-mer oligonucleotide were further desalted using a microdialysis system previously described[19] which was based on the initial design reported by Smith and coworkers.[39] Briefly, the PCR products were infused at a flow rate of 2 μL min^{-1} against the counter-current buffer of 10 mM ammonium acetate with a gravity induced flow-rate of *ca.* 3 mL min^{-1}. Concentrations of the PCR products were determined using Beer's law and on-line UV-vis measurements at the exit of the microdialysis unit.[19]

Preparation of synthetic 75-mer

The 75-mer, 5′-$(CTG)_{25}$-3′, was synthesized by the Midland Certified Reagent Co. (Midland, TX, USA), resuspended in 10 mM ammonium acetate, microdialyzed and quantified using UV-vis absorbance measurements. Serial dilutions were made ranging from 5 μM to 0.05 μM in electrospray buffer (52% acetonitrile, 8% propan-2-ol, 40% aqueous with a final concentration of 2 mM ammonium acetate and 20 mM piperidine and imidazole).

Mass spectrometry

The PCR products and oligonucleotide were electrosprayed from 50 μm fused-silica capillary pulled to a fine tip and

remotely coupled to a potential of *ca.* −2100 V with a 3.3 nL s^{-1} flow rate.[40] The electrospray tip was positioned directly in front of the Analytica of Branford (Branford, CT, USA) source modified to accept a heated-metal capillary[41] that was held at 200 °C for all work described here. The mass spectrometer was an IonSpec (Irvine, CA, USA) 4.7 Tesla Fourier transform ion cyclotron resonance mass spectrometer as previously described elsewhere[40] with the exception of a 12-bit analog-to-digital converter and a 300 mL ballast volume to maintain the foreline pressure. Ions were injected into the trapped ion cell using a shutter pulse width of 200 ms for the spectra of the double-stranded product and 1 s for the spectra of the products of lambda exonuclease digestion. Trapped ions were excited using a 4 ms Chirp waveform with $V_{p\text{-}p}$ = 30 V from m/z = 400 to 3500 using a preamplifier gain of 1 ×, without a window function, acquiring 32 k of data at a 500 kHz ADC rate, except when attempts were made to achieve isotopic resolution. All electrospray reagents were purchased from Sigma, at the highest purity available and used as received.

Results and discussion

Fig. 1 shows a portion of the DNA sequence encoding the human tyrosine hydroxylase (HUMTHO1) gene including primer regions, the nonconsensus $(CATT)_{coding}/(AATG)_{noncoding}$ repeating region and the primer pairs utilized for the two reactions involved in these investigations. The repeat region of the Human Tyrosine Hydroxylase (HUMTHO1) gene is one of thirteen forensic loci included in the Federal Bureau of Investigation's COmbined DNA Index System (CODIS) and is a well characterized system currently used in our laboratory to study short tandem repeats (STR).[10] The primers utilized in these studies directly flank the repeating sequence while maintaining a 3′-GC pair to ensure effective amplification. As shown in Fig. 1, one primer in each reaction set is modified with a 5′ phosphorylation, which will serve as a marker for targeted degradation by lambda exonuclease. The resulting 82 bp phosphorylated PCR product is derived from the HUMTHO1 gene using K562 DNA as a template. K562 DNA is purified from a subculture of the human myelogenous leukemia cell line and is homozygotic for the HUMTHO1 9.3 allele (nine pure repeats with one incomplete repeat). The theoretical average masses of the double-stranded PCR products and the lambda exonuclease digested products are listed in Table 1. Reaction 1 results in a PCR product that has been phosphorylated on the 5′ end of the coding strand whereas reaction 2 results in a PCR product that has been phosphorylated on the 5′ end of the noncoding strand (see Fig. 1). Mass measurements of both single-stranded and double-stranded PCR products derived from the HUMTHO1 9.3 allele, with a mass accuracy of better than 100 ppm, have shown the significant information that one can obtain using an ESI-FTICR-MS approach for STR genotyping.[10]

Immediately after the PCR, products were ethanol precipitated to remove PCR contents and salts. The double-stranded products were prepared for mass analysis using a rapid microdialysis step and quantified using UV-vis absorbance measurements. To prepare single-stranded DNA, PCR products were digested with lambda exonuclease for one hour at 37 °C followed by an additional ethanol precipitation. Since the makeup of the lambda exonuclease buffer included with the enzyme is proprietary, we performed three rapid dialysis steps to ensure removal of any buffer components that may interfere with electrospray ionization followed by quantification (*vide infra*). All desalted PCR products were lyophilized and electrosprayed from a buffer containing 52% acetonitrile, 8% propan-2-ol, 40% aqueous with a final concentration of 2 mM ammonium acetate, 20 mM piperidine and 20 mM imidazole.[10,42,43]

Fig. 2 shows the ESI-FTICR mass spectra of the double-stranded PCR product and the PCR products resulting from lambda exonuclease digestion. Fig. 2A shows the single-acquisition mass spectrum of the double-stranded 82 bp PCR product obtained from reaction 1 primers. Inspection of Fig. 2A clearly reveals a triplet at each charge-state with experimental masses corresponding to species **2**, **4** and **5** listed in Table 1. This is consistent with the non-template directed 3′ deoxyribonucleotide addition, preferentially adenosine, characteristic

Coding strand

Reverse primer

5′-*ATTGG*CCTGTTCCTCCCTTATTTCCC *T* **(CATT)$_3$CAT(CATT)$_6$**CACCATGGAGTCTGTGTTCCC*TGTGA*-3′

3′-*TAACC*GGACAAGGAGGGAATAAAGGG *A* **(GTAA)$_3$GTA(GTAA)$_6$**GTGGTACCTCAGACACAAGGG*ACACT*-5′

Forward primer

Noncoding strand

Primer	Sequence (5'-3')	Genomic Position
Reaction 1		
P-THO1-*for*	Ⓟ-CCTGTTCCTCCCTTATTTCCC	1149
THO1-*rev*	GGGAACACAGACTCCATGGTG	1227
Reaction 2		
THO1-*for*	CCTGTTCCTCCCTTATTTCCC	1149
P-THO1-*rev*	Ⓟ-GGGAACACAGACTCCATGGTG	1227

Fig. 1 Amplified region of the HUMTHO1 locus and primer sequences. The region shown represents the 82 base-pair PCR product that contains a nonconsensus tetrameric repeating motif (shown in bold) and the following five nucleotides in the genomic sequence (shown in italics). The primers were designed to flank the repeating region as closely as possible while maintaining a 3′ G–C pair to maximize yield, hence the base flanked by asterisks was not included in the primer region. One primer in each reaction is phosphorylated on the 5′ end to target that synthesized strand for enzymatic digestion by lambda exonuclease.

of *Taq* polymerase as reported by Clark.[44] The inset of Fig. 2A shows an expansion plot of the 24⁻ charge-state representing blunt-ended, mono-adenylated and di-adenylated PCR products. Notably, the mono-adenylated product is the most abundant which suggests that one strand may be preferentially adenylated; however, this data does not allow one to identify which strand is preferred (*vide infra*). The double stranded product generated by PCR carried out with reaction 2 primers (Fig. 1) resulted in an identical spectrum (data not shown).

Table 1 Average theoretical masses for PCR products derived from HUMTHO1 with corresponding lambda exonuclease digestion products

Double-stranded PCR product—		
1	Blunt-ended	50 536 Da
2	Blunt-ended + phosphorylation	50 616 Da
3	Blunt-ended + adenosine	50 849 Da
4	Blunt-ended + phosphorylation + adenosine	50 929 Da
5	Blunt-ended + phosphorylation + 2 adenosines	51 242 Da
Single-stranded product from lambda exonuclease digestion—		
6	Noncoding strand	25 783 Da
7	Noncoding strand + adenosine	26 096 Da
8	Coding strand	24 753 Da
9	Coding strand + adenosine	25 066 Da

Fig. 2B shows an ESI-FTICR mass spectrum obtained after lambda exonuclease digestion of a double-stranded PCR product phosphorylated on the coding strand. The inset of Fig. 2B shows an expansion plot of all species present at the 22⁻ charge-state. Interestingly, the major species is the blunt-ended single-stranded PCR product with a small amount of mono-adenylated present. The masses of both are in agreement with those listed in Table 1 for **6** and **7**. Additional peaks at lower *m/z* represent loss of both guanosine nucleotides (dpG) and neutral guanine (GH) from the blunt-ended single strand. Inspection of the noncoding strand sequence (see Fig. 1) shows that guanosines are present at the 5′ and 3′ ends which is in agreement with the nucleotide loss seen in this spectrum. Fig. 2C shows an ESI-FTICR mass spectrum after lambda exonuclease digestion in which the phosphorylated noncoding strand should be completely digested. The inset of Fig. 2C shows an expansion plot of the 23⁻ charge-state where the major species is the adenylated PCR product with a small amount of blunt-ended present, a clear indication that the coding strand is preferentially adenylated. These masses correspond to those of **9** and **8**, respectively (Table 1). There is no evidence that nucleotide loss has occurred with the coding strand, which has a cytidine on both termini. Considering that the spectra of the single strands were acquired under identical conditions (*i.e.*,

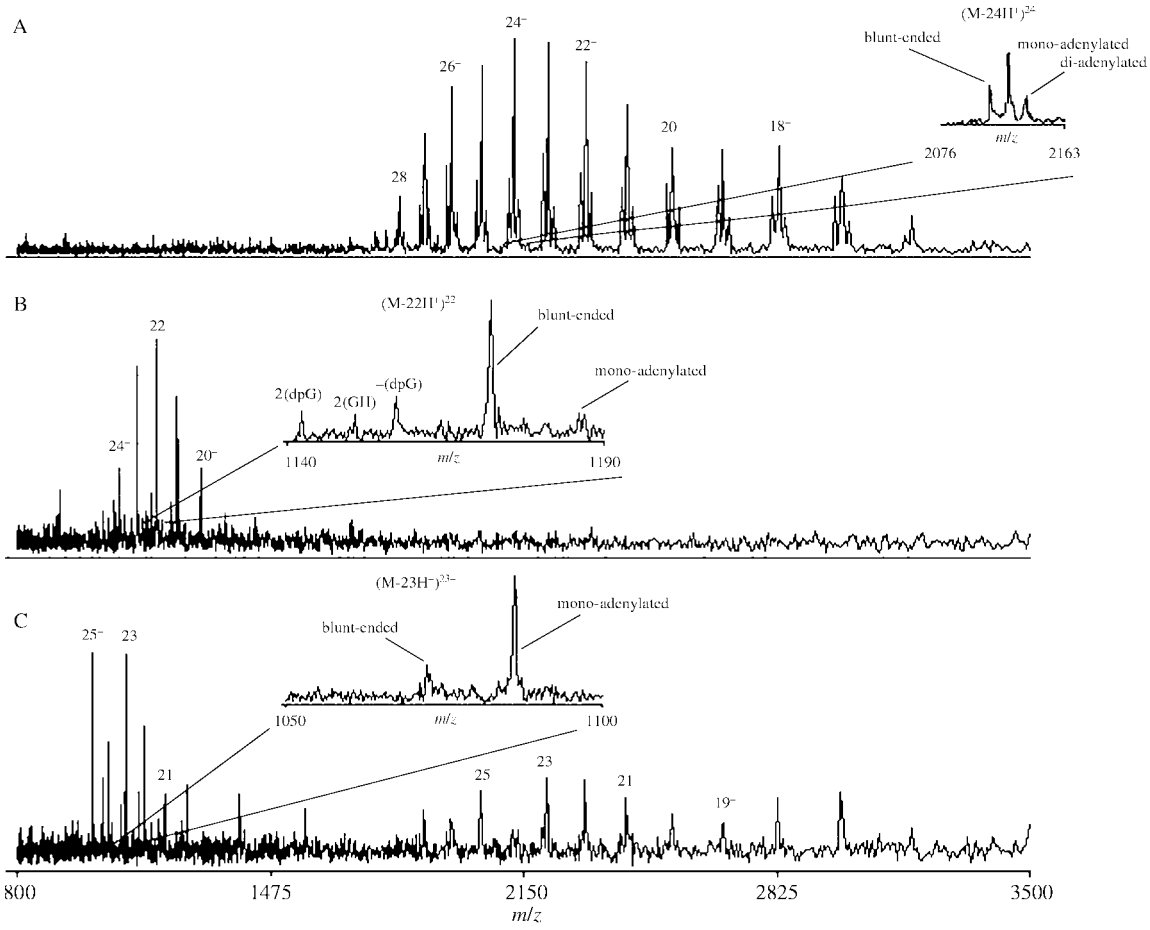

Fig. 2 ESI-FTICR mass spectra of the double-stranded PCR product derived from the HUMTHO1 locus and subsequent digestion using lambda exonuclease. (A) Single-acquisition mass spectrum of the 82 base-pair PCR product containing a 5′ phosphorylation on the coding strand. An expansion of the 24⁻ charge state shows the three species produced in the PCR with masses corresponding to the phosphorylated blunt-ended (**2**), mono-adenylated (**4**) and di-adenylated (**5**) products (see Table 1). The total concentration of all three species was 2 μM and 13 fmol of total material was injected. (B) The mass spectrum of the PCR product phosphorylated on the coding strand after digestion with lambda exonuclease represents the average of ten spectra infused at a concentration of 0.29 μM with a total of 9 fmol material injected. Expansion of the 22⁻ charge-state shows that the majority of the noncoding strand is blunt-ended (**6**) and that only a small amount of adenylated (**7**) is present. Loss of guanosine nucleotides (dpG) and neutral guanine (GH) are indicated at lower *m*/z. (C) The mass spectrum of the PCR product phosphorylated on the noncoding strand after digestion with lambda exonuclease represents the average of 5 spectra infused at 0.37 μM with a total of 6 fmol injected. The charge-states of the double-stranded (undigested) PCR product, clearly present at low signal-to-noise, have been labeled. Expansion of the 23⁻ charge-state of the single-stranded product shows that the major peak is the preferentially adenylated coding strand (**9**) with a small amount of blunt-ended present (**8**). Nucleotide and base loss was not detected.

identical ionization/detection potentials, gas densities), a propensity for guanosine loss is indicated and may be attributed to interface conditions of the mass spectrometer.

Further inspection of Fig. 2C in the *m/z* range 1600–3300 shows the presence of the intact double-stranded PCR product with a mass that corresponds to that of the mono-adenylated, non-phosphorylated PCR product (species **3** of Table 1). The presence of double-stranded PCR product after the exonuclease digestion along with the fact that the double-stranded PCR product routinely produced a very intense signal (Fig. 2A) while the single-stranded PCR product (*i.e.*, the digested product) required the averaging of 5–10 spectra, led us to consider the possibility that the phosphorylated primers utilized in the PCR phase were not pure. Fig. 3 shows the ESI-FTICR mass spectra of the 5′-phosphorylated primers utilized in the PCR. The mass spectrum of the 5′-phosphorylated primer (P-THO1-*for*) is shown in Fig. 3A and the relative intensities of the two species clearly show that *ca.* 50% of the primer is unphosphorylated. Additional peaks at higher *m/z* are attributed to sodium and potassium adducts. Fig. 3B shows the 5′-phosphorylated primer (P-THO1-*rev*) utilized in reaction 2 where *ca.* 50% of the primer is unphosphorylated which, like the forward primer, would render the PCR product untouchable by lambda exonuclease. Thus, highlighting another important (although obvious) contribution of mass spectrometry, namely, to characterize PCR primers.

The single-stranded PCR products shown in Figs. 2B and 2C undoubtedly show a preference for adenylation of the coding strand (Fig. 2C inset). The greater intensity of the mono-adenylated peak in the spectrum of the double-stranded PCR product suggests that adenylation of one strand is preferred, but provides no further information regarding the preferred strand. Furthermore, inspection of the K562 DNA sequence beyond the primer regions (Fig. 1) shows that there is no thymidine present that may act as a template for the 3′ adenosine addition. Assuming all else equal, knowing which strand was preferentially adenylated would be advantageous so that it could be targeted for degradation providing a less complex mass spectrum.

The poor signal-to-noise ratio in the spectra of the single-stranded PCR products was initially a concern when these measurements were made considering that peptide and protein studies result in limits of detection of 4.16 nM for cyclosporin A[45] and 45 nM for equine heart cytochrome *c*.[46] The digested PCR products in Fig. 2 were expected to achieve a better signal-to-noise ratio than shown since they were electrosprayed at concentrations in the low μM range. These results led us to investigate the limits of detection achievable when analyzing single-stranded PCR products using ESI-FTICR. A single-stranded oligonucleotide (75-mer), 5′-$(CTG)_{25}$-3′, was quantified and serially diluted from 5 μM down to 0.05 μM in electrospray buffer. For those concentrations in which a signal was obtained (1–5 μM), peak intensities were derived by averaging the five most abundant charge states from five single-acquisition spectra using 32k data points per spectrum. Fig. 4A shows a representative single-acquisition ESI-FTICR mass spectrum of the 75-mer at a concentration of 1.3 μM, which is at approximately the same concentration as the double-stranded PCR product shown in Fig. 2A. Thus, the signal-to-noise in ESI mass spectra of single-stranded *versus* double-stranded oligonucleotides is nearly identical when independently electrosprayed at similar concentrations. The 19^- charge-state has been expanded to demonstrate the achievement of isotopic resolution, obtained by acquiring 512k data points. Fig. 4B shows the plot of absolute peak intensity *versus* concentration of the 75-mer. Using linear least-squares regression analysis, the upper 95% confidence limit of the *y*-intercept ($t_{\text{one-sided}}$ = 1.771) was determined to be 0.00131 which corresponds to a limit of detection of 0.27 μM. Although the limit of detection is 0.27 μM, the typical concentration of an oligonucleotide electrosprayed in our laboratory is between 1–2 μM to achieve a high quality mass spectrum. However, it should be noted that these concentrations are more than two orders of magnitude greater than what is required for peptides and proteins.[45,46]

The concentrations of digested PCR product (Fig. 2) were determined using absorbance measurements and Beer′s Law, but could not be based on the single-stranded PCR product alone as initially assumed. As shown in Figs. 3A and 3B, approximately half of the ‘phosphorylated’ primers were in fact unphosphorylated so that half of the PCR products generated from each set of primers were untouchable by the lambda exonuclease leaving double-stranded product in the digest. As a

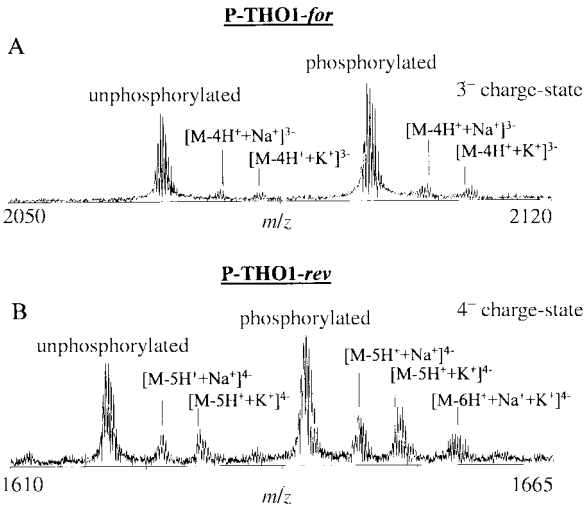

Fig. 3 ESI-FTICR mass spectra of the 5′-phosphorylated primers. (A) A single-acquisition mass spectrum of the 3⁻ charge state of the phosphorylated forward primer (P-THO1-*for*) shows that about half of the primer is unphosphorylated. Sodium and potassium adducts are labeled. (B) A single-acquisition mass spectrum of the 4⁻ charge state of the phosphorylated reverse primer (P-THO1-*rev*) reveals that about half of the primer is unphosphorylated. Sodium and potassium adducts are also indicated.

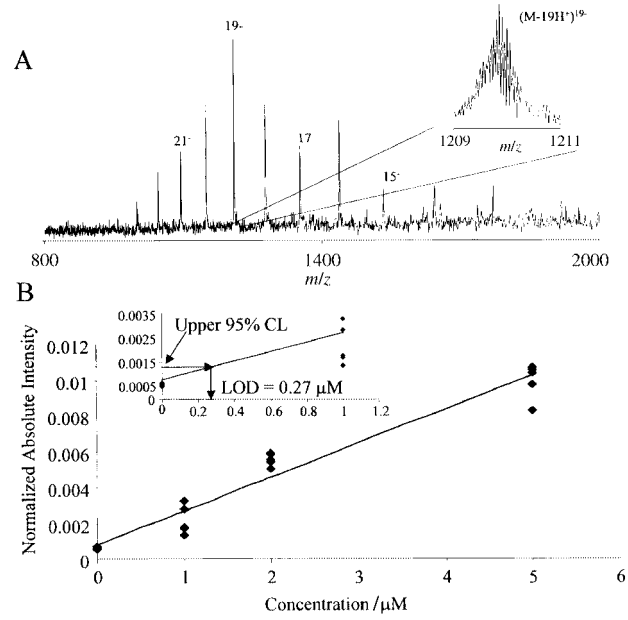

Fig. 4 ESI-FTICR mass analysis of a 75-mer for detection limit determination. (A) The single-acquisition mass spectrum of a 75-mer, 5′-$(CTG)_{25}$-3′, was electrosprayed at 1.3 μM and acquired using a 512k data point set to achieve isotopic resolution as shown in the expansion of the 19^- charge state. (B) The normalized absolute intensities were obtained from five replicate spectra for several concentrations of a 75-mer oligonucleotide acquiring a 32k data point set. Least-squares regression analysis was used to determine the upper 95% confidence limit of the *y*-intercept ($t_{\text{one-sided}}$ 1.771) corresponding to a detection limit of 0.27 μM for a single acquisition.

result, the digested PCR products were quantified assuming that 50% of the lambda exonuclease digested product remained double-stranded resulting in concentrations of 0.29 and 0.37 μM for the single-stranded species. Following this argument, we argue that single-stranded PCR products ionize more efficiently than double-stranded since a greater signal-to-noise ratio is produced for the single-stranded species when both species are present in the same solution at nearly identical concentrations.

The concentrations of the single-stranded PCR products are at the limit of detection, therefore, the spectra of the single-stranded PCR products in Fig. 2 required the averaging of 5–10 acquisitions, increasing the signal-to-noise by $N^{1/2}$ where N is the number of averaged spectra.[47] Cumulatively, the data explains the poor signal-to-noise ratio exhibited in the spectra of the single-stranded PCR product digested with lambda exonuclease compared to that of the 75-mer since: (1) Half of the primers were unphosphorylated so the concentration of the PCR product after enzymatic digestion was calculated *assuming* that 50% was single-stranded and 50% was double-stranded. In addition, these calculations were made *assuming* 100% efficiency of lambda exonuclease. Consequently, the concentrations may actually be lower than what we calculated; (2) matrix effects will play a large role in the ability to detect PCR products by mass spectrometry. The 75-mer was resuspended in 10 mM ammonium acetate, essentially matrix-free, whereas the digested sample was in a buffer of unknown composition possibly introducing interference into the analysis. The matrices present in the lambda exonuclease-treated product may also compete for charge in the electrosprayed droplet reducing the signal-to-noise ratio. Using lambda exonuclease, we have demonstrated a technique in which single-stranded PCR products can be selectively produced from a double-stranded PCR product for analysis by ESI-FTICR mass spectrometry with future work directed towards generating pure, relatively matrix-free, single-stranded products with unit efficiency.

Conclusion

It is clear that production of single-stranded PCR products is critical for accurate characterization of nucleic acids; however, it is unclear what method will be the most compatible with electrospray ionization mass spectrometry. Lambda exonuclease is an efficient method in preparing single-stranded DNA from the double-stranded PCR product, provided the phosphorylated primers are pure and acceptable efficiency of the lambda exonuclease reaction can be achieved. We have shown that selective digestion using the DNA repair enzyme lambda exonuclease offers the ability to obtain mass spectra of single-stranded PCR products using a single enzymatic step. Further investigation revealed that the limit of detection of a 75-mer is 0.27 μM indicating the capabilities of ESI-MS for analyzing low concentrations of PCR product. Subsequent research will be targeted toward matrix reduction, decreasing the time requirement for PCR product purification and isolation of phosphorylated primers prior to PCR; however, it is clear that lambda exonuclease offers a broadly applicable strategy to generate single-stranded PCR products.

Acknowledgements

We would like to gratefully acknowledge the financial support of the Merck Genome Research Institute (Grant #31), American Society for Mass Spectrometry Research Award sponsored by PE Biosystems (D.C.M), ACS Division of Analytical Chemistry Graduate Student Fellowship (J.C.H.) and the Mary E. Kapp Foundation of the Department of Chemistry, Virginia Commonwealth University.

References

1 K. Mullis and F. Faloona, *Methods Enzymol.*, 1987, **155**, 335.
2 J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong and C. M. Whitehouse, *Science*, 1989, **246**, 64.
3 D. P. Little, R. A. Chorush, J. P. Speir, M. W. Senko, N. L. Kelleher and F. W. McLafferty, *J. Am. Chem. Soc.*, 1994, **116**, 4893.
4 D. P. Little and F. W. McLafferty, *J. Am. Chem. Soc.*, 1995, **117**, 6783.
5 D. P. Little, T. W. Thannhauser and F. W. McLafferty, *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 2318.
6 D. P. Little, D. J. Aaserud, G. A. Valaskovic and F. W. McLafferty, *J. Am. Chem. Soc.*, 1996, **118**, 9352.
7 D. C. Muddiman, D. S. Wunschel, C. L. Liu, L. Pasatolic, K. F. Fox, A. Fox, G. A. Anderson and R. D. Smith, *Anal. Chem.*, 1996, **68**, 3705.
8 T. Tsuneyoshi, K. Ishikawa, Y. Koga, Y. Naito, S. Baba, H. Terunuma, R. Arakawa and D. J. Prockop, *Rapid Commun. Mass Spectrom.*, 1997, **11**, 719.
9 Y. Naito, K. Ishikawa, Y. Koga, T. Tsuneyoshi, H. Terunuma and R. J. Arakawa, *J. Am. Soc. Mass Spectrom.*, 1997, **8**, 737.
10 J. C. Hannis and D. C. Muddiman, *Rapid Commun. Mass Spectrom.*, 1999, **13**, 954.
11 F. W. McLafferty, D. J. Aaserud, Z. Guan, D. P. Little and N. L. Kelleher, *Int. J. Mass Spectrom. Ion Processes*, 1997, **165/166**, 457.
12 R. A. Marcus, *J. Chem. Phys.*, 1952, **20**, 359.
13 H. M. Rosenstock, M. B. Wallenstein and A. L. Wahrhaftig, *Proc. Natl. Acad. Sci. USA*, 1952, **38**, 667.
14 K. D. Henry and F. W. McLafferty, *Org. Mass Spectrom.*, 1990, **25**, 490.
15 Y. Naito, K. Ishikawa, Y. Koga, T. Tsuneyoshi and H. Terunuma, *Rapid Commun. Mass Spectrom.*, 1995, **9**, 1484.
16 D. S. Wunschel, K. F. Fox, A. Fox, J. E. Bruce, D. C. Muddiman and R. D. Smith, *Rapid Commun. Mass Spectrom.*, 1996, **10**, 29.
17 D. S. Wunschel, D. C. Muddiman, K. Fox, A. Fox and R. D. Smith, *Anal. Chem.*, 1997, **70**, 1203.
18 D. C. Muddiman and R. D. Smith, *Rev. Anal. Chem.*, 1998, **17**, 1.
19 J. C. Hannis and D. C. Muddiman, *Rapid Commun. Mass Spectrom.*, 1999, **13**, 323.
20 D. C. Muddiman, A. P. Null and J. C. Hannis, *Rapid Commun. Mass Spectrom.*, 1999, **13**, 1201.
21 M. T. Krahmer, Y. A. Johnson, J. J. Walters, K. F. Fox, A. Fox and M. Nagpal, *Anal. Chem.*, 1999, **71**, 2893.
22 H. Echols and M. F. Goodman, *Annu. Rev. Biochem.*, 1991, **60**, 477.
23 A. R. Fersht, *Trends Biol. Sci.*, 1980, **5**, 262.
24 M. F. Goodman, S. Creighton, L. B. Bloom and J. Petruska, *Crit. Rev. Biochem. Mol. Biol.*, 1993, **28**, 83.
25 M. J. Longley, S. E. Bennett and D. W. Mosbaugh, *Nucleic Acids Res.*, 1990, **18**, 7317.
26 K. S. Lundberg, D. D. Shoemaker, M. W. W. Adams, J. M. Short, J. A. Sorge and E. J. Mathur, *Gene*, 1991, **108**, 1.
27 P. K. Cooper and P. C. Hanawalt, *Proc. Natl. Acad. Sci. USA*, 1972, **69**, 1156.
28 S. Tabor and C. C. Richardson, *Proc. Natl. Acad. Sci. USA*, 1987, **84**, 4767.
29 J. W. Little, *J. Biol. Chem.*, 1967, **242**, 679.
30 R. G. Higuchi and H. Ochman, *Nucleic Acids Res.*, 1989, **17**, 5865.
31 S. Tagaki, M. Kimura and M. Katsuki, *Biotechniques*, 1993, **14**, 218.
32 M. B. Comisarow and A. G. Marshall, *Chem. Phys. Lett.*, 1974, **25**, 282.
33 K. D. Henry, E. R. Williams, B. H. Wang, F. W. McLafferty, J. Shabanowitz and D. F. Hunt, *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 9075.
34 J. Sambrook, E. F. Fritsch and T. Maniatis, *Molecular Cloning*, Cold Spring Harbor Laboratory, Plainview, NY, 2nd edn., 1989, vol. 3.
35 J. Crouse and D. Amorese, *Focus*, 1987, **9**, 3.
36 J. T. Stults and J. C. Marsters, *Rapid Commun. Mass Spectrom.*, 1991, **5**, 359.
37 P. A. Limbach, P. F. Crain and J. A. McCloskey, *J. Am. Soc. Mass Spectrom.*, 1995, **6**, 27.
38 A. Edwards, A. Civitello, H. A. Hammond and T. C. Caskey, *Am. J. Hum. Genet.*, 1991, **49**, 746.

39 C. Liu, D. C. Muddiman and R. D. Smith, *J. Mass Spectrom.*, 1997, **32**, 425.
40 J. C. Hannis and D. C. Muddiman, *Rapid Commun. Mass Spectrom.*, 1998, **12**, 443.
41 S. K. Chowdhury, V. Katta and B. T. Chait, *Rapid. Commun. Mass Spectrom.*, 1990, **4**, 81.
42 M. Greig and R. H. Griffey, *Rapid Commun. Mass Spectrom.*, 1995, **9**, 97.
43 D. C. Muddiman, X. H. Cheng, H. R. Udseth and R. D. Smith, *J. Am. Soc. Mass Spectrom.*, 1996, **7**, 697.
44 J. M. Clark, *Nucleic Acids Res.*, 1988, **16**, 9677.
45 E. F. Gordon and D. C. Muddiman, *Rapid Commun. Mass Spectrom.*, 1999, **13**, 164.
46 E. F. Gordon, B. A. Mansoori, C. F. Carroll and D. C. Muddiman, *J. Mass Spectrom.*, 1999, **34**, 1055.
47 D. A. Skoog and F. J. Holler, *Principles of Intrumentation Analysis*, Harcourt Brace and Company, Orlando, 5th edn., 1998.

Paper a908022h