Rapid mass spectrometric identification of human genomic polymorphisms using multiplexed photocleavable mass-tagged probes and solid phase capture

Naomi Hammond,*[a] Peter Koumi,[b] G. John Langley,[a] Alex Lowe[b] and Tom Brown[a]

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A mass spectrometric approach for rapid and simultaneous detection of several single nucleotide polymorphisms (SNPs) is reported. Oligonucleotide single base extension (SBE) primers, labelled at the 5′-end with photocleavable, quaternised and brominated peptidic mass tags, are extended by a mixture of the four dideoxynucleotides of which one is biotinylated. The 3′-biotinylated extension products are captured by streptavidin-coated solid phase magnetic beads, whilst non-biotinylated extension products and unreacted primers are washed away. Quaternised and brominated mass tags, cleaved from captured extension products during analysis by matrix-assisted laser desorption/ionisation-time-of-flight (MALDI-TOF) MS, are detected at pmol levels. This method is applied to the analysis of mitochondrial DNA polymorphisms for the purpose of human identification.

Introduction

Genotyping of single nucleotide polymorphisms (SNPs), the most common human genetic variation, is widely used for identifying disease genes, in forensic identification of individuals, and genetic mapping.[1] The search for a rapid and inexpensive technique for determining SNP variation is ongoing.[2–4]

Single base extension (SBE) is commonly used for genotyping SNPs.[4,5] In this method a primer is annealed to the DNA template and extended by a single dideoxynucleotide that is complementary to the nucleotide at the variable site. Fluorescently-labelled dideoxynucleotides and solid-phase capturable SBE primers, with slab gel electrophoretic detection, have been employed for multiplexed SNP genotyping.[2] The multiplexing capabilities of this approach are limited as spectral differentiation between large numbers of fluorophores is not possible. In contrast, matrix-assisted laser desorption/ionisation-time-of-flight (MALDI-TOF) mass spectrometry (MS) can be used to simultaneously analyse multiple SBE products.[6–9] Direct analysis of DNA is difficult due to its negatively charged sugar–phosphate backbone which forms adducts with alkali metal ions such as sodium and potassium.[10] Stringent purification is therefore essential to obtain unambiguous results.

The GOOD assay resolves some of the incompatibilities between DNA and MALDI-TOF MS by chemical modifications of the SBE primers. Primers are partially modified with up to three phosphorothioates, followed by extension with quaternised ddNTPs and the unmodified segment is digested by phosphodiesterase II. The negative charges on the remaining phosphorothioate segment are neutralised by an alkylation reaction, leaving a single positive charge from the quaternary ammonium ion for MALDI-TOF MS detection.[11]

Alternatively, SBE products can be indirectly detected by MALDI-TOF MS by labelling them with a photocleavable mass marker, which is detected instead of the DNA products. Photocleavable peptide–DNA conjugates have also been used as hybridisation probes.[12] The peptides are released during photocleavage induced by the nitrogen laser pulse (337 nm), greatly simplifying detection. This provides a wide range of available mass tags that are not restricted by the masses of unmodified DNA strands.

In this novel approach (Fig. 1), SBE primers are labelled at the 5′-end with photocleavable, quaternised, and brominated peptidic mass tags. These are extended by a mixture of the four dideoxynucleotides of which one is biotinylated.[13] The 3′-biotinylated extension products are captured by streptavidin-coated solid phase magnetic beads, whilst non-biotinylated extension products and unreacted primers are washed away. Quaternised, brominated mass tags are photocleaved from the captured extension products during MALDI-TOF MS analysis for detection. Detection of mass tags is automatable if cluster pattern recognition software is used for analysis of the brominated isotope patterns.

This procedure differs from the existing SBE assays using MALDI-TOF MS, predominantly in that the photo-released peptide mass tags are used and not DNA based ones. Peptides are more readily detected by MALDI-TOF MS than DNA. A wide range of commercially available standard and isotopically labelled amino acids can be used, eliminating the need for expensive and time consuming chemical modification of the SBE primers. As the ddNTPs are biotinylated and not the SBE primers, all unextended primers are removed and no background signals, except from matrix ions result.

Results and discussion

The *o*-nitrobenzyl based photocleavable linker, similar to that of Holmes,[14] was synthesised according to Scheme 1. It was coupled to the 5′-end of three SBE primers using standard automated solid phase DNA synthesis methods. Mass tags were manually

[a]*School of Chemistry, University of Southampton, Southampton, UK SO17 1BJ. E-mail: tb2@soton.ac.uk; Fax: +44 (0)2380 592991; Tel: +44 (0)23 8059 2974*
[b]*The Forensic Science Service, Trident Court, 2920 Solihull Parkway, Birmingham Business Park, Birmingham, UK B37 7YN*

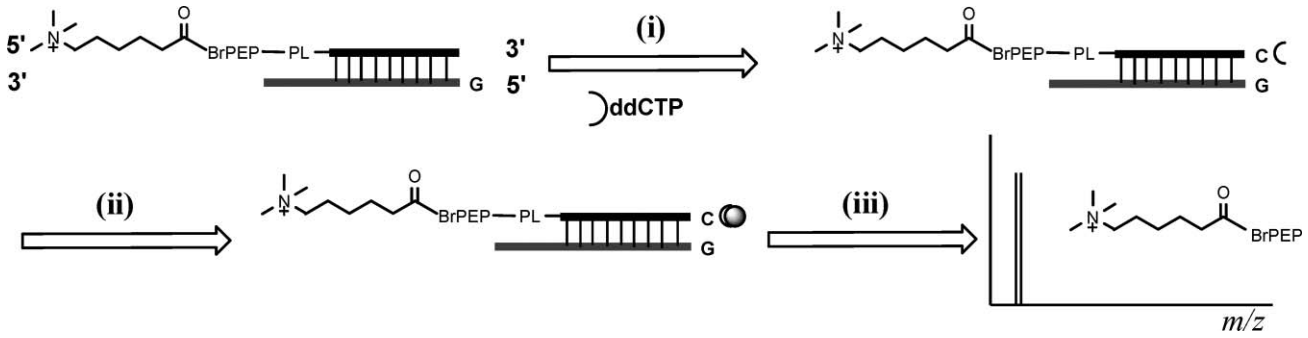
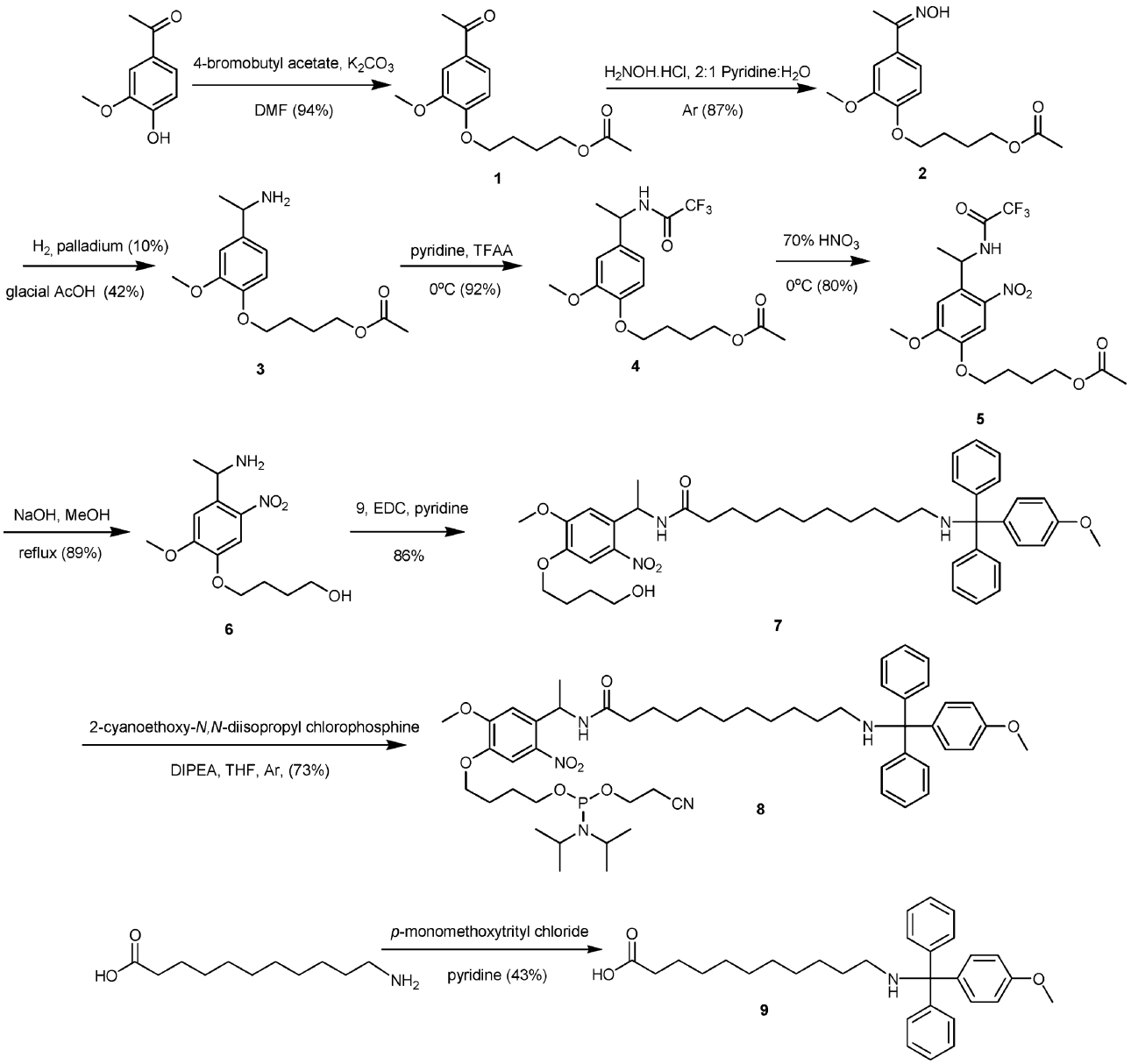


Fig. 1 SBE assay. (i) The derivatised mass tagged primer anneals one base upstream of the SNP site and is extended by a ddNTP. The reaction is carried out four times, each time replacing one of the four ddNTPs with a biotinylated version. (ii) Biotinylated extension products are captured on streptavidin-coated beads whilst non-biotinylated extension products are washed away. (iii) Captured extension products are directly cleaved and the peptide moiety analysed by MALDI-TOF MS. The genotype is determined by the molecular ion of the brominated mass marker. PL denotes photocleavable linker, BrPEP denotes brominated peptide.



Scheme 1 Synthesis of the photocleavable *o*-nitrobenzyl photocleavable linker.

Table 1 Single base extension primer sequences and characterisation by mass spectrometry and UV melting analysis

Location of 3′ insertion	Sequence: 5′–3′	Mass[c] *m/z* (calcd/found)	T_m /°C[d] with/without mass tag
H00073	Quat[a]BrPheGlyGly–PL[b]–CCAGCGTCTCGCAATGCTATCGCGTGCA	9551/9550	74.6/75.1
H00247	Quat[a]BrPhePheGly–PL[b]–CTGTGTGGAAAGTGGCTGTGCAGACATT	9815/9812	73.1/72.2
H16129	Quat[a]BrPheGly–PL[b]–GTACTACAGGTGGTCAAGTATTTATGGTAC	10244/10241	67.5/67.1

[a] Quat denotes derivatised with a quaternary ammonium. [b] PL denotes photocleavable linker. [c] Molecular weights are reconstructed from the multiply charged peaks. [d] Melting studies were performed with exact complements.

conjugated to the photocleavable linker-oligonucleotide, each primer having its own unique mass tag (Table 1).[15]

The mass tag was designed to have well resolved peaks (~60 Da apart) and to contain a single bromine atom to allow for subsequent automated spectral analysis. The amino terminus of the peptide was derivatised with a quaternary ammonium salt,[16] to allow mass tag detection at the pmol level.

Each allele of a particular SNP requires a separate reaction, *i.e.* to detect a G insertion, biotinylated ddCTP is added along with non-biotinylated ddATP, ddGTP and ddTTP. All ddNTPs are required to eliminate any possibility of false extension. The method has been evaluated with just one biotinylated ddNTP (biotin-11-ddCTP) and this adequately demonstrates its efficiency and selectivity.

The method described has been successfully validated by probing three substitution polymorphisms from two human mitochondrial DNA samples, and extension with biotin-11-ddCTP. The base compositions of each SNP investigated for both samples are shown in Table 2. SBE primer-mass tag conjugate sequences[2] and masses are shown in Table 1 along with T_m data. The presence of the 5′-photocleavable linker mass tags does not appreciably effect the melting temperature of the primers. Photocleavage product masses are shown in Table 3.

When the assay was tested with DNA source 1 (Table 2), three mass tags were detected, as all SBE primers were extended with biotin-11-ddCTP. With DNA source 2, just one mass tag (corresponding to H00073) was detected (Fig. 2). Any primers not extended by the biotinylated dideoxynucleotide are washed away during solid phase capture providing unambiguous detection of all C-extended nucleotides.

Single nucleotide deletions can be detected (Table 2, H00247) by designing the 3′-end of a primer to be complementary to the base at which the deletion is positioned. If the target sequence has a deletion at this point, the primer does not fully hybridise

Table 3 Cleaved mass tag

Location of 3′ insertion	Mass tag *m/z* (calcd/found)
H00073	695.3/695.3
H00247	785.4/785.3
H16129	638.3/638.3

Table 2 SNP genotype of two DNA samples analysed at three different sites

	Target sequence of template: 5′–3′	
Location of 3′ insertion	Source 1	Source 2
H00073	**G**TGCACGCGATAGCATTGCGAGACGCTGG	**G**TGCACGCGATAGCATTGCGAGACGCTGG
H00247	**G**AATGTCTGCACAGCCACTTTCCACACAG	**GD**[a]ATGTCTGCACAGCCACTTTCCACACAG
H16129	**G**GTACCATAAATACTTGACCACCTGTAGTAC	**A**GTACCATAAATACTTGACCACCTGTAGTAC

[a] D denotes a deletion. As the primer is mismatched at the 3′-end, extension does not occur. The SNP genotype is highlighted in bold.

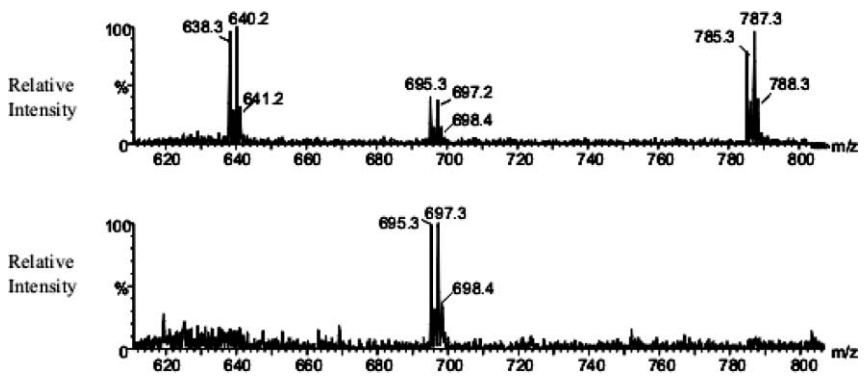


Fig. 2 Reflectron MALDI-TOF MS of photocleaved mass tags after SBE, demonstrating selectivity of assay. Top spectrum from DNA source 1: expected bromine isotopic mass $(M)^+$ 638.3, 695.3 and 785.4 *m/z* units. Lower spectrum from DNA source 2: expected bromine isotopic mass $(M)^+$ 695.3 *m/z* units.

and extension does not occur. If the deletion is part of a homo-oligomer repeat region (*i.e.* GGGGG), the 3′-end of the primer can be designed to be complementary to the last base in the region. A deletion would stop full hybridisation and extension (Fig. 3).

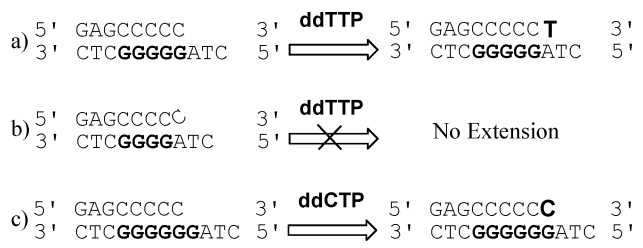


Fig. 3 Application of the assay to the detection of deletions and insertions in a homo-oligomer repeat region (bold). The 3′-end of the primer is designed to anneal to last base of the repeat region, assuming no deletions or insertions. a) Primers which anneal to a template containing no deletions or insertions are extended by a ddNTP complementary to the next base after the repeat region. b) As primers do not fully hybridise to templates containing a deletion, extension does not occur. c) Templates containing an insertion are extended by a ddNTP complementary to the repeat region.

Single nucleotide insertions in a homo-oligomer repeat region can be genotyped in a similar manner, by designing the 3′-end of a primer to anneal to the last base in the region (assuming no insertions). If the target sequence has an insertion, the biotinylated base added will be complementary to that of the repeat region, but different if no insertions are present (Fig. 3).[2]

Typing deletions or insertions of homo-oligomer repeat regions could prove difficult, although this has not been experimentally determined. It may be possible for either the primer or template to loop out and form a stable hybridised duplex for SBE, giving false typing results.

To quantify the assay for application in forensic science, the percentages of total DNA present in one tube from sources 1 and 2 were varied pre-PCR. Mass tag peak height ratios from SNPs H00073 and H00247 (Table 3), of 695 and 785 *m/z* units respectively, were calculated from each set of spectra obtained (Table 4). The mass tags generated from SBE of H00073 are present in both sample types, whilst the tags from H00247 are only present in sample 1. Low background levels of the H00247 mass tags are detected during analysis of 100% source 2. When total DNA consists of ≤0.5% source 1, peaks from the tag are reduced to the background level. As the percentage of source 2 increases, the peak ratios increase almost proportionally (see theoretical peak height values, Table 4). Therefore, the proportion of two different DNA sources in a single sample can be factored into the interpretation of a forensic result by analysing two SNPs, if the two alternative allele types are present for one of the SNPs.

Mass tag mass spectral peaks were well resolved, supporting the capability to extend the multiplex much further, by utilising suitable amino acids or non-natural amino acid derivatives.

Experimental

Chemical synthesis

Solvents and reagents were purchased from Aldrich or Fisher. Derivatised peptides were purchased from Activotec. All reactions were carried out under an argon atmosphere in oven-dried glassware.

Table 4 Quantification of the assay

Source 1(%)[a]	Source 2(%)[a]	H00073 : H00247 peak height ratios[b]	Theoretical peak height ratios[c]
100	0	1 : 1.82	n/a
0	100	1 : 0.06	1 : 0.00
25	75	1 : 0.67	1 : 0.46
2.4	97.6	1 : 0.14	1 : 0.04
0.5	99.5	1 : 0.06	1 : 0.01

[a] % of total DNA present in PCR sample. [b] Both peak heights from each data set are obtained from the same spectra. [c] Theoretical values are calculated from the 100% source 1 H00247 peak height.

4-(4-Acetyl-2-methoxyphenoxy)butyl acetate (1). A slurry of acetovanillone (15.36 g, 92.4 mmol), 4-bromobutyl acetate (18.84 g, 96.6 mmol), and K_2CO_3 (20.00 g, 144.9 mmol) in DMF (100 mL) was stirred at room temperature for 17 hours. Water (50 mL) was added to the reaction mixture, dissolving the majority of K_2CO_3. The mixture was poured into saturated NaCl (100 mL) and the aqueous phase extracted with EtOAc (3 × 100 mL). The combined organic phase was washed with H_2O (2 × 50 mL) and brine (100 mL), dried (Na_2SO_4), filtered and the solvent removed *in vacuo* to afford the keto-ester **1** (25.35 g, 94%) as a pale yellow crystalline solid; mp: 60–61 °C (ethyl acetate) (Found: C, 64.2; H, 7.2. $C_{15}H_{20}O_5$ requires C, 64.3; H, 7.2%); ν_{max}/cm^{-1} 2961m (CH_2, CH_3), 1732s, 1721s, 1667s (C=O), 1359s (CO-CH_3) and 1255s (C–O); δ_H (300 MHz, $CDCl_3$) 2.00–1.80 (4 H, m, 2 × CH_2), 2.04 (3 H, s, CH_3CO_2), 2.56 (3 H, s, CH_3COC), 3.92 (3 H, s, OCH_3), 4.11 (2 H, t, *J* 5.9, CH_2), 4.15 (2 H, t, *J* 6.2, CH_2), 6.88 (1 H, d, *J* 8.2, CH_{ar}), 7.55–7.52 (2 H, m, CH_{ar}); δ_C (75 MHz, $CDCl_3$) 20.92 (CH_3CO_2), 25.28 (CH_2), 25.65 (CH_2), 26.17 ($COCH_3$), 56.02 ($C_{ar}OCH_3$), 63.96 ($CH_3CO_2CH_2$), 68.36 ($COCH_2$), 110.54 (CH), 111.19 (CH), 123.16 (CH), 130.50 (C), 149.29 (C), 152.74 (C), 171.06 (CH_3CO_2), 196.75 (CH_3COC); LRMS (ES^+) *m/z* 303.2 $(M + Na)^+$ (100%), HRMS (ES^+) *m/z* 281.1387 $(M + H)^+$, $C_{15}H_{21}O_5$ requires 281.1384.

4-[4-(1-Hydroxyiminoethyl)-2-methoxyphenoxy]butyl acetate (2). Keto-ester **1** (25.74 g, 91.9 mmol) and $H_2NOH{\cdot}HCl$ (9.57 g, 137.7 mmol) were dissolved in a 2 : 1 v/v pyridine : H_2O mixture (120 mL) forming a yellow solution which was stirred at room temperature for 18 hours. The work-up procedure was the same as described for compound **1**. Final traces of pyridine were removed by co-evaporating with toluene (4 × 30 mL) to afford oxime **2** (23.68 g, 87%) as a pale yellow solid (Found: C, 60.95; H, 7.2; N, 4.6. $C_{15}H_{21}NO_5$ requires C, 61.0; H, 7.2; N, 4.7%); ν_{max}/cm^{-1} 2961m (CH_2, CH_3), 1732s, 1721s, 1667s (C=O), 1360s (CO–CH_3) and 1254s (C–O); δ_H (300 MHz, $CDCl_3$) 1.71–1.91 (4 H, m, 2 × CH_2), 1.97 (3 H, s, CH_3CO_2), 2.20 (3 H, s, CH_3CNOH), 3.82 (3 H, s, OCH_3), 3.99 (2 H, t, *J* 6.2, CH_2), 4.07 (2 H, t, *J* 6.3, CH_2), 6.78 (1 H, d, *J* 8.4, CH_{ar}), 7.06 (1 H, dd, *J* 8.4 and 2.1, CH_{ar}), 7.19 (1 H, d, *J* 2.2, CH_{ar}); δ_C (75 MHz, $CDCl_3$) 11.98 (CH_3CNOH), 20.95 (CH_3CO_2), 25.32 (CH_2), 25.77 (CH_2), 55.99 (OCH_3), 64.11 ($CH_3CO_2CH_2$), 68.39 ($COCH_2$), 109.14 (CH), 112.37 (CH), 119.17 (CH), 129.42 (C), 149.34 (C), 149.57 (C), 155.67 (CH_3CNOH), 171.18 (CH_3CO_2); LRMS (ES^+) *m/z* 318.2 $(M + Na)^+$ (100%), HRMS (ES^+) *m/z* 296.1496 $(M + H)^+$, $C_{15}H_{21}NO_5$ requires 296.1493.

4-[4-(1-Aminoethyl)-2-methoxyphenoxy]butyl acetate (3). A suspension of oxime **2** (20.58 g, 69.7 mmol) and palladium (10%) on activated carbon (2.0 g) was made in glacial acetic acid (200 mL). The mixture was degassed by placing under reduced pressure, refilled with hydrogen and placed under a pressure of 1.1 atm *via* a balloon. The mixture was stirred vigorously at room temperature, refilling the balloon with further hydrogen as required. An additional 1 g of catalyst was added after 48 hours. After 12 hours the mixture was filtered through Celite and the solvent reduced *in vacuo* to ~10 mL. Water (100 mL) was added and the solution acidified to pH 1 with 6 M HCl. The aqueous phase was washed with Et_2O (2 × 50 mL), basified to ~pH 12 with Na_2CO_3 and extracted with EtOAc (3 × 100 mL). The combined organic phases were washed with H_2O (50 mL) and saturated NaCl (50 mL). The organic phase was dried (Na_2SO_4), filtered and evaporated *in vacuo*, and purified by column chromatography on silica gel using an eluent system of EtOAc + 8% MeOH + 1% Et_3N to yield the amine **3** (8.15 g, 42%) as an orange–brown oil; ν_{max}/cm^{-1} 2956m, 2871m (CH_2, CH_3), 2361w, 1732s (C=O), 1678w (C=O), 1365m (CO–CH_3), 1235s (C–O); δ_H (300 MHz, $CDCl_3$) 1.36 (3 H, d, *J* 6.6, CH_3CH), 1.61 (2 H, s, NH_2), 1.71–1.92 (4 H, m), 2.02 (3 H, s, CH_3CO_2), 3.86 (3 H, s, OCH_3), 3.99–4.15 (5 H, m), 6.79–6.86 (2 H, m, CH_{ar}), 6.91 (1 H, d, *J* 1.5, CH_{ar}); δ_C (75 MHz, $CDCl_3$) 20.94 (*C*H_3CO_2), 25.34 (CH_2), 25.76 (*C*H_3CHNH_2), 25.87 (CH_2), 51.02 (CHNH_2), 56.00 (CO*C*H_3), 64.14 (CH_3CO_2*C*H_2), 68.61 (CH_2), 109.65 (CH), 113.30 (CH), 117.66 (CH), 140.78 (*C*CHNH_2), 147.22 (*C*OCH_2), 149.57 (*C*OCH_3), 171.09 (CH_3*C*O_2); LRMS (ES^+) *m/z* 265.2 (M − NH_3 + H)$^+$ (100%), 304.2 (M + Na)$^+$ (14), HRMS (ES^+) *m/z* 304.1517 (M + Na)$^+$, $C_{15}H_{23}NaNO_4$ requires 304.1519.

4-(2-Methoxy-4-[1-(2,2,2-trifluoroacetamido)ethyl]phenoxy)butyl acetate (4). Amine **3** (8.15 g, 29.0 mmol) was dissolved in pyridine (80 mL), cooled to 0 °C. TFAA (12.3 mL, 56.9 mmol) was added forming a light orange solution. The mixture was stirred at 0 °C for 1.5 hours after which time the dark orange solution was poured into saturated NaCl (200 mL, cooled to 0 °C) whilst stirring and the organic phase extracted with EtOAc (3 × 100 mL). The combined organic phases were washed with H_2O (50 mL) and saturated NaCl (50 mL), dried (Na_2SO_4), filtered and evaporated *in vacuo*. The product was purified by column chromatography on silica gel using an eluent system of EtOAc : hexane 2 : 1 affording amide **4** (10.02 g, 92%) as a light yellow–off-white powder (Found: C, 54.2; H, 5.9; N, 3.7. $C_{17}H_{22}F_3NO_5$ requires C, 54.1; H, 5.9; N, 3.7%); ν_{max}/cm^{-1} 3296m (N–H), 2956w, 2878w (CH_2, CH_3), 2361w, 1736m (C=O), 1693s (C=O), 1366m (CO–CH_3), 1232s (C–O) 1148s (C–F); δ_H (300 MHz, $CDCl_3$) 1.57 (3 H, d, *J* 7.0, CH_3CH), 1.77–1.94 (4 H, m, 2 × CH_2), 2.03 (3 H, s, CH_3CO_2), 3.86 (3 H, s, OCH_3), 4.02 (2 H, t, *J* 6.1, CH_2), 4.13 (2 H, t, *J* 6.2, CH_2), 5.09 (1 H, quin, *J* 7.2, C*H*CH_3), 6.83–6.85 (3 H, m, CH_{ar}), 6.49 (1 H, s, NH); δ_C (75 MHz, $CDCl_3$) 20.93 (CH_3), 21.07 (CH_3), 25.46 (CH_2), 25.92 (CH_2), 49.67 (*C*HCH_3), 56.21 (CO*C*H_3), 64.22 (CH_2), 68.69 (CH_2), 110.61 (CH), 113.44 (CH), 115.56 (q, *J* 277.8, CF_3), 118.37 (CH), 133.82 (C), 148.48 (*C*OCH_3), 149.93 (NH*C*O), 156.26 (q, *J* 38.9, *C*OCF_3), 171.29 (CH_3*C*O_2); δ_F (282 MHz, $CDCl_3$) 76.13; LRMS (ES^+) *m/z* 400.1 (M + Na)$^+$ (100%), 777.4 (2M + Na)$^+$ (13), HRMS (ES^+) *m/z* 400.1345 (M + Na)$^+$, $C_{17}H_{22}NaF_3NO_5$ requires 400.1342.

4-(2-Methoxy-5-nitro-4-[1-(2,2,2-trifluoroacetamido)ethyl]-phenoxy)butyl acetate (5). Benzyl amide **4** (7.37 g, 19.5 mmol) was slowly added to 70% HNO_3 (150 mL), cooled to 0 °C, forming a light yellow solution. The solution gradually turned orange in colour and after 2 hours the reaction was quenched by pouring into iced water (1500 mL). The resultant slurry was chilled at 4 °C overnight, and filtered whilst cold, resulting in a pale yellow solid. The solid was dissolved in EtOAc and the residual aqueous phase removed. The organic phase was dried (Na_2SO_4), filtered and evaporated *in vacuo* to afford the nitrated amide **5** (6.57 g, 80%) as a pale yellow crystalline solid; mp: 134–135 °C (ethyl acetate) (Found: C, 48.45; H, 5.1; N, 6.5. $C_{17}H_{21}F_3N_2O_7$ requires C, 48.3; H, 5.0; N, 6.6%); ν_{max}/cm^{-1} 3341m (N–H), 2951w, 2890w (CH_2, CH_3), 1730m (C=O), 1698s (C=O), 1525s, 1512s (NO_2), 1177s (C–F); δ_H (300 MHz, $CDCl_3$) 1.62 (3 H, d, *J* 7.4, CH_3CH), 1.78–1.98 (4 H, m), 2.05 (3 H, s, CH_3CO_2), 3.94 (3 H, s, OCH_3), 4.08 (2 H, t, *J* 6.2, CH_2), 4.14 (2 H, t, *J* 6.3, CH_2), 5.52 (1 H, quin, *J* 7.2, C*H*NH), 6.87 (1 H, s, CH_{ar}), 7.39 (1 H, d, *J* 7.3, NH), 7.59 (1 H, s, CH_{ar}); δ_C (75 MHz, $CDCl_3$) 20.24 (*C*H_3CO_2), 21.07 (*C*H_3CH), 25.41 (CH_2), 25.68 (CH_2), 48.69 (CH_3*C*H), 56.58 (*C*H_3CO), 64.10 (CH_2), 69.10 (CH_2), 110.36 (CH), 111.34 (CH), 115.56 (q, *J* 276.7, CF_3), 130.95 (C), 140.74 (C), 147.93 (C), 154.20 (C), 156.31 (q, *J* 38.8, *C*OCF_3), 171.33 (CH_3*C*O_2); δ_F (282 MHz, $CDCl_3$) 76.21; LRMS (ES^+) *m/z* 445.2 (M + Na)$^+$ (100%), HRMS (ES^+) *m/z* 445.1199 (M + Na)$^+$, $C_{17}H_{21}NaF_3NO_7$ requires 445.1193.

4-[4-(1-Aminoethyl)-2-methoxy-5-nitrophenoxy]-1-butanol (6). Nitro-amide **5** (6.57 g, 15.6 mmol) was dissolved in MeOH (200 mL) and 1 M NaOH (60 mL, 60 mmol) added, forming an orange solution which was refluxed for 6 hours. Upon cooling to room temperature, the resultant dark red solution was concentrated to ~50 mL, water (200 mL) added and acidified to pH 1 with 2 M HCl. The solution was washed with Et_2O (4 × 40 mL) and the aqueous phase basified to pH 10.5 with saturated Na_2CO_3. Saturated NaCl (20 mL) was added to the aqueous phase and extracted with DCM (3 × 60 mL, 9 × 30 mL). The combined organic phases were dried (Na_2SO_4), filtered and evaporated *in vacuo* to afford the nitro-amine **6** (3.96 g, 89%) as an orange oil; ν_{max}/cm^{-1} 3359w, 3288w (O–H), 2922w, 2862w (CH_2, CH_3), 1503s (NO_2), 1263s (O–H), 1045s (C–OH); δ_H (400 MHz, $CDCl_3$) 1.43 (3 H, d, *J* 6.4, CH_3CH), 1.72–1.81 (5 H, m, 2 × CH_2 overlapping OH), 1.92–1.97 (2 H, m), 3.72 (2 H, t, *J* 6.2, CH_2OH), 3.97 (3 H, s, OCH_3), 4.10 (2 H, t, *J* 6.2, CH_2OC), 4.80 (1 H, q, *J* 6.4, C*H*NH_2), 7.31 (1 H, s, CH_{ar}), 7.47 (1 H, s, CH_{ar}); δ_C (100 MHz, $CDCl_3$) 24.81 (CH_3), 25.69 (CH_2), 29.59 (CH_2), 46.02 (CHNH_2), 56.43 (*C*H_3OC), 62.36 (CH_2OH), 69.38 (*C*H_2OC), 108.99 (2 × CH_{ar}), 137.48 (C), 140.91 (C), 146.78 (C), 153.78 (C); LRMS (ES^+) *m/z* 268.1 (M − NH_3 + H)$^+$ (100%), 285.2 (M + H)$^+$ (51), 307.1 (M + Na)$^+$ (23), HRMS (ES^+) *m/z* 285.1439 (M + H)$^+$, $C_{13}H_{21}N_2O_5$ requires 285.1445.

11-(4-Methoxytritylamino)undecanoic acid (9). To a solution of 11-aminoundecanoic acid (7.15 g, 35.5 mmol) in pyridine (100 mL), *p*-monomethoxytrityl chloride (10.00 g, 32.3 mmol) was added and stirred at room temperature for 3 hours. The solution was concentrated to ~20 mL *in vacuo*, DCM (600 mL) added and subsequently washed with saturated KCl (5 × 75 mL) and H_2O (1 × 100 mL). The organic phase was dried (Na_2SO_4), filtered and evaporated *in vacuo* to afford a pale yellow oil. The product was purified by column chromatography on silica using

an eluent system of DCM + 8% MeOH + 1% Et_3N to afford acid **9** (7.25 g, 43%) as a pale yellow oil; ν_{max}/cm^{-1} 3348br (O–H), 2922m, 2852m (CH_2, CH_3), 1737w (C=O), 1508s (NH), 1268s (C–O); δ_H (400 MHz, $CDCl_3$) 1.16–1.29 (13 H, m, 6 × CH_2, NH), 1.40–1.49 (2 H, m, CH_2), 1.57–1.64 (2 H, m, CH_2), 2.12 (2 H, t, *J* 7.0, CH_2NH), 2.23 (2 H, t, *J* 7.6, CH_2COOH), 3.79 (3 H, s, OCH_3), 6.79–6.84 (2 H, m, CH_{ar}), 7.25–7.30 (6 H, m, CH_{ar}), 7.36–7.41 (2 H, m, CH_{ar}), 7.47–7.48 (4 H, m, CH_{ar}); δ_C (100 MHz, $CDCl_3$) 26.17 (CH_2), 27.52 (CH_2), 29.63 (CH_2), 29.67 (CH_2), 29.72 (2 × CH_2), 29.78 (CH_2), 31.02 (CH_2), 36.88 (COOH*C*H_2), 43.71 (NCH_2), 55.28 (OCH_3), 70.48 (NHC), 113.12 (2 × CH), 126.49 (2 × CH), 128.00 (4 × CH), 128.70 (4 × CH), 129.92 (2 × CH), 138.73 (C), 146.78 (2 × C), 157.87 (*C*OCH_3), 179.78 (COOH); LRMS (ES^+) *m*/*z* 496.3 (M + Na)$^+$ (5%); HRMS (ES^+) *m*/*z* 474.3012 (M + H)$^+$, $C_{31}H_{40}NO_3$ requires 474.3003.

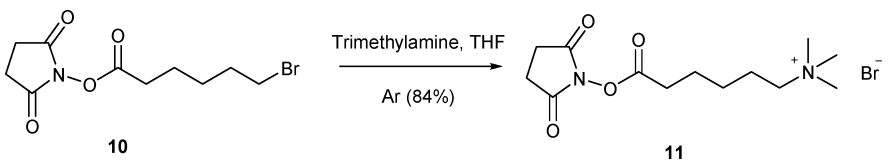
11-(4-Methoxytritylamino)-*N*-{1-[4-(4-hydroxybutoxy)-3-methoxy-6-nitrophenyl]ethyl}undecanamide (7). A solution of acid **9** (4.47 g, 9.4 mmol), nitroamine **6** (2.24 g, 7.9 mmol) and EDC·HCl (1.81 g, 9.4 mmol) in pyridine (80 mL) was stirred at room temperature for 3 hours. Pyridine was removed *in vacuo* and final traces coevaporated with toluene. The product was purified by column chromatography on silica using an eluent system of EtOAc + 1% Et_3N to afford the coupled amide **7** (5.00 g, 86%) as a yellow crystalline solid; mp: 64–65 °C (ethyl acetate) (Found: C, 71.32; H, 7.75; N, 5.6. $C_{44}H_{57}N_3O_7$ requires C, 71.4; H, 7.8; N, 5.7%); ν_{max}/cm^{-1} 2925s (CH_2, CH_3), 2851w (CH_2, CH_3), 1713w (C=O), 1508s (NO_2), 1247s (O-H), 1033s (C–OH); δ_H (400 MHz, $CDCl_3$) 1.21–1.28 (13 H, m, 6 × CH_2, CH_2N*H*), 1.53 (3 H, d, *J* 7.0, CH_3CHNH), 1.45–1.60 (4 H, m, 2 × CH_2), 1.76 (2 H, app quin, *J* 6.7, CH_2), 1.96 (2 H, app quin, *J* 6.7, CH_2), 2.12–2.17 (5 H, m, 2 × CH_2, OH), 3.72 (2 H, t, *J* 6.2, CH_2OH), 3.78 (3 H, s, CH_3O), 3.93 (3 H, s, CH_3O), 4.09 (2 H, t, *J* 6.2, CH_2OC_{ar}), 5.49 (1 H, quin, *J* 7.1, C*H*NH), 6.32 (1 H, d, *J* 7.0, CHN*H*), 6.81 (2 H, d, *J* 8.8, 2 × CH_{ar}), 6.90 (1 H, s, CH_{ar}), 7.17 (2 H, t, *J* 7.3, 2 × CH_{ar}), 7.27 (4 H, t, *J* 7.7, 4 × CH_{ar}), 7.38 (2 H, d, *J* 8.8, 2 × CH_{ar}), 7.47 (4 H, d, *J* 7.8, 4 × CH_{ar}), 7.55 (1 H, s, CH_{ar}); δ_C (100 MHz, $CDCl_3$) 20.91 (*C*H_3CHNH), 25.52 (CH_2), 25.62 (CH_2), 7.35 (CH_2), 29.25 (CH_2), 29.30, (CH_2), 29.39 (CH_2), 29.45 (CH_2), 29.51 (CH_2), 29.58 (CH_2), 30.86 (CH_2), 36.68 (CH_2), 43.60 (CH_2), 47.67 (CHNH), 55.17 (OCH_3), 56.33 (OCH_3), 60.37 (CH_2), 62.25 (CH_2OH), 69.29 (*C*H_2OC), 70.41 (C), 109.85 (CH), 111.06 (CH), 113.02 (2 × CH), 126.07 (2 × CH), 127.69 (4 × CH), 128.59 (4 × CH), 129.82 (2 × CH), 133.71 (C), 140.65 (2 × C), 146.05 (C), 147.05 (C), 153.65 (C), 157.78 (C), 171.12 (C); LRMS (ES^+) 740.5 (M + H)$^+$ (100%), HRMS (ES^+) *m*/*z* 740.4261 (M + H)$^+$, $C_{44}H_{58}N_3O_7$ requires 740.4270.

11-(4-Methoxytritylamino)-*N*-(1-{4-[4-*O*-(2-cyanoethoxy-diisopropylaminophosphinoxy)butoxy]-3-methoxy-6-nitrophenyl}ethyl)-undecanamide (8). Amide **7** (1.00 g, 1.4 mmol), 2-cyanoethoxy-*N*,*N*-diisopropyl chlorophosphine (0.34 mL, 1.5 mmol) and DIPEA (0.59 mL, 3.4 mmol) were dissolved in freshly distilled THF (10 mL) and stirred at room temperature for 2 hours under an argon atmosphere. Degassed DCM (30 mL) and degassed saturated KCl (8 mL) was added and the organic phase extracted, dried ($MgSO_4$), filtered and evaporated *in vacuo* to afford a yellow oil. The product was purified by column chromatography on silica using an eluent system of 7 : 3 EtOAc : toluene + 1% Et_3N to afford phosphoramidite **8** (0.96 g, 73%) as a yellow oil; δ_H (400 MHz, DMSO) 1.16–1.22 (25 H, m, 6 × CH_2, 4 × NCH_3, 1 × CHN*H*), 1.44–1.48 (7 H, m, 2 × CH_2, CH_3CHNH), 1.75 (2 H, app quin, *J* 6.7, CH_2), 1.85 (2 H, app quin, *J* 6.6, CH_2), 2.00–2.02 (2 H, m, CH_2), 2.13 (2 H, t, *J* 6.9, CH_2), 2.79 (2 H, t, *J* 5.9, CH_2), 3.77 (3 H, s, CH_3O), 3.56–3.82 (6 H, m, 2 × CH_2, 2 × CHNP), 3.93 (3 H, s, CH_3O), 4.10 (2 H, t, *J* 5.5, CH_2OC_{ar}), 5.43 (1 H, quin, *J* 7.0, C*H*NH), 6.89 (2 H, d, 2 × CH_{ar}), 7.20–7.24 (4 H, m, 4 × CH_{ar}), 7.29–7.35 (5 H, m, 5 × CH_{ar}), 7.44 (4 H, d, *J* 8.0, 4 × CH_{ar}), 7.52 (1 H, s, CH_{ar}), 8.49 (1 H, d, *J* 7.8, CHN*H*); δ_P (121 MHz, DMSO) 147.76; LRMS (ES^+) 940.7 (M + H)$^+$ (100%), HRMS (ES^+) *m*/*z* 940.5362 (M + H)$^+$, $C_{53}H_{75}N_5O_8P$ requires 940.5354.

6-[(*N*-Succinimidyl)oxy]-6-oxohexyl(trimethyl)ammonium bromide (11)[16]. Following the procedure of Bartlet-Jones,[16] trimethylamine (1.45 mL, 15.4 mmol) in anhydrous tetrahydrofuran (THF) (3 mL) was added to a solution of (*N*-succinimidyl)oxy-6-bromohexanoate **10** (1.48 g, 5.1 mmol) in anhydrous THF (3 mL). The reaction mixture was stirred for 12 hours under an atmosphere of argon (Scheme 2). The resulting white solid was filtered and recrystallised in hot ethanol to yield ester **11** (1.14 g, 84%) as a white solid; mp: 185–186 °C; ν_{max}/cm^{-1} 2929br (CH_2, CH_3), 1810m, 1720m (C=O), 1717s (C=O), 1480m (C=O), 1420w (C=O), 1369m (C=O), 1209s, 1050s, 1003w; δ_H (300 MHz, CD_3OD) 1.52–1.64 (2 H, m, CH_2), 1.80–1.97 (4 H, m, 2 × CH_2), 2.78 (2 H, t, *J* 7.0, $COOCH_2$), 2.91 (4 H, s, $COCH_2CH_2CO$), 3.21 (9 H, s, 3 × $N(CH_3)$), 3.41–3.45 (2 H, m, CH_2); δ_C (75 MHz, CD_3OD) 23.43 (CH_2), 25.04 (CH_2), 26.39 (CH_2), 26.54 ($COCH_2CH_2CO$), 31.24 ($COOCH_2$), 53.60 (3 × $N(CH_3)$), 67.63 (CH_2N), 170.19 (COO), 171.92 (2 × NCO); LRMS (ES^+) 270.9 (M)$^+$ (100%), HRMS (ES^+) *m*/*z* 271.1649 (M)$^+$, $C_{13}H_{23}N_2O_4$ requires 271.1652.

Synthesis of oligonucleotides

Standard DNA phosphoramidites, solid supports and additional reagents were purchased from Link Technologies or Applied Biosystems Ltd. All oligonucleotides were synthesised on an Applied Biosystems 394 automated DNA/RNA synthesiser using a 0.2 or 1.0 μmole phosphoramidite cycle of acid-catalysed detritylation, coupling, capping and iodine oxidation. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior



Scheme 2 Synthesis of derivatising reagent.

to use. Monomer **8** was coupled for 300 seconds rather than the standard 25 sec. Stepwise coupling efficiencies and overall yields were determined by the automated trityl cation conductivity monitoring facility and in all cases were >98.0%. Cleavage of oligonucleotides from the solid support was achieved by exposure to concentrated ammonia solution (30 min at room temp) followed by heating in conc. aqueous ammonia, 55 °C for 5 hours in a sealed tube. SBE primers were manually functionalised with the appropriate peptides prior to cleavage from the solid support (see below).

All oligonucleotides were purified by reversed phase HPLC on a Gilson system controlled by Gilson 7.12 software using an ABI Aquapore column (C8), 8 mm × 250 mm, pore size 300 Å. The following protocol was used: Run time 30 min, flow rate 4 mL min^{-1}, binary system, gradient: time in min (% buffer B) 0 (0); 3(0); 5(20); 21 (100); 25(100); 27 (0); 30(0). Elution buffer A: 0.1 M ammonium acetate, pH 7.0, buffer B: 0.1 M ammonium acetate with 35% acetonitrile pH 7.0. Elution of oligonucleotides was monitored by ultraviolet absorption at 295 nm. After HPLC purification oligonucleotides were desalted using disposable NAP 10 Sephadex columns (Pharmacia), aliquoted into Eppendorf tubes and stored at −20 °C in the dark.

Synthesis of derivatised mass tagged SBE primers

Oligonucleotides with phosphoramidite **8** at the 5′-end were prepared on a 1 μmol scale by standard solid-phase phosphoramidite methods. The columns were removed from the synthesiser and manually washed according to the method of Zaramella *et al.*[15] with morpholine (1 mL, 2% in MeCN) for 1 minute, followed by MeCN (8 × 1 mL) and DMF (3 × 1 mL) prior to the addition of the photocleavable amino linker. Solid phase coupling of derivatised peptides to the amino-modified oligonucleotide was achieved with peptide–HBTU–*N*-methylmorpholine (25 : 25 : 108) in DMF (300 μL) overnight. The resultant yellow coupling solution was removed and the column washed with DMF (4 × 1 mL), ether (2 × 1 mL) and dried. The oligonucleotides were cleaved from the solid support, deprotected and purified by HPLC as described above.[15]

The molecular masses of the conjugates were verified by ESMS in negative mode as solutions diluted 5-fold in MeCN–H_2O–TPA (100 : 100 : 1). Molecular weights were reconstructed from the *m*/*z* values of the multiply deprotonated molecules using the mass deconvolution programme MassEnt (Maximum Entropy).

UV-melting studies

The UV absorbance *vs.* temperature profiles were obtained simultaneously at 260 nm using an intra-cuvette temperature probe on a ChemCary 400 UV Visible spectrometer in Hellma® SUPRASIL synthetic quartz, 10 mm pathlength cuvettes. The SBE primers, with or without conjugated mass tags, and their exact complements were each at a concentration of 1 μM in 52 mM Tris, 13 mM $MgCl_2$ pH 9.5 in a volume of 1.5 mL. The samples were filtered with Kinesis regenerated cellulose 13 mm, 0.45 μM syringe filters and rapidly heated from 20 °C to 80 °C at 10 °C min^{-1} then cooled to 20 °C prior to analysis. The UV melting curves were recorded for three consecutive heat and cool cycles. The temperature was increased in increments of 0.5 °C min^{-1} and the T_m values determined from the maximum of the first order derivative of the average of three cycles.

PCR amplification

DNA (4 ng) containing the polymorphic sites H00073, H00247 and H16129 were amplified from mitochondrial DNA in a total volume of 20 μL, containing 10 pmol each primer (L16048, 5′-TCATGGGGAAGCAGATTTGG-3′; H00326, 5′-CAGAGATGTGTTTAAGTGCTGT-3′), 200 μM dNTPs (Promega, Madison, WI), 1.5 mM $MgCl_2$ (Promega, Madison, WI), 1.5 U *Taq* DNA polymerase in storage buffer B (Promega, Madison, WI) and 1X PCR buffer B (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton®X-100, pH 9.0). Using an Eppendorf Mastercycler gradient PCR system, the reactions were initiated with denaturation at 95 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s and subsequently held at 72 °C for 3 min. Excess dNTPs and primers were degraded by adding 2 U *Escherichia coli* exonuclease I (USB, Cleveland, Ohio) and 2 U shrimp alkaline phosphatase (Roche Diagnostics, Indianapolis, IN) in 1 × shrimp alkaline phosphatase buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 8.5). The samples were incubated at 37 °C for 45 min followed by enzyme denaturation at 95 °C for 15 min.

Single base extension

The sequence and masses of the mass tagged SBE primers used for detecting the three SNPs are shown in Table 1. Optimised SBE reactions contained 3 μL Exo I and SAP treated PCR product, 80 pmol each primer, 100 pmol biotin-11-ddCTP (PerkinElmer, Boston, MA), 100 pmol each ddATP, ddGTP, ddTTP (all Roche Diagnostics, Indianapolis, IN), 4 μL Thermo Sequenase reaction buffer (260 mM Tris-HCl, 65 mM $MgCl_2$, pH 9.5), 1.92 U Thermo Sequenase (Amersham Biosciences) in a total volume of 20 μL. Reactions were cycled 35 times at 94 °C for 30 s and 52 °C for 30 s.

Solid phase purification

20 μL of Dynabeads M-270 streptavidin (Invitrogen) were prewashed with binding and washing (B–W) buffer (0.5 mM Tris-HCl, 2 M NH_4Cl, 1 mM EDTA, pH 7.0), and resuspended in 20 μL B–W buffer. 20 μL SBE products were added to the beads and bound by incubating at 48 °C for 45 min. Captured products were washed with B–W buffer (×3), with distilled water (×3) and resuspended in 2 μL deionised water.

MALDI-TOF MS analysis

1 μL of the suspended purified captured extension products were directly pipetted onto a MALDI sample platen followed by 1 μL matrix (DHB in MeCN : H_2O 1 : 1, 0.1% TFA) and air dried. Samples were analysed using a Micromass TofSpec2E MALDI-TOF MS spectrometer. Positive ion reflectron data was acquired at an accelerating voltage of 20 kV, a 3000 V pulse voltage, and a 39 ns delay time. Approximately 100 laser shots were averaged to produce the final mass spectrum in each case.

Conclusions

A novel, indirect method to genotype SNPs, employing MALDI-TOF MS detection, is described. The use of peptidic mass tags avoids problems present with direct detection of DNA by MS, whilst extension with biotinylated ddNTPs allows solid phase capture and removal of unextended mass tag-SBE primers. The multiplexed assay can be easily extended to genotype many more SNPs simultaneously.

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