行政院國家科學委員會專題研究計畫　期中進度報告

合併運用 DNA 突變分析儀與質譜儀於快速基因分析—新技術之開發與臨床應用(1/3)

計畫類別： 個別型計畫
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執行單位： 國立臺灣大學醫學院婦產科

計畫主持人： 謝豐舟
共同主持人： 李建南

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附件一

行政院國家科學委員會補助專題研究計畫 □成果報告 ■期中進度報告

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計畫參與人員： 蘇怡寧

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢

□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：台大醫學院婦產科

中　華　民　國　93 年 6 月 8 日

一、中文摘要

關鍵詞：質譜儀，乙型海洋性貧血，基因分析

愈來愈多之單一核酸變異已被確認，並且開啟了一道提供我們發現更多致病基因，藥物動力學，及了解人類起源之署光。利用高速，經濟而有效率之基因診斷工具，在單一核酸變異分析中仍變得極為重要。目前，最敏感之基因診斷工具仍是直接序列分析，但是，序列分析昂貴耗時即期較高之技術依賴性仍其缺點。所以，如何結合新的檢驗工具來更有效率地分析核酸變異，仍變得極為急迫。於本計畫中，我們運用核酸質譜分析之新技術於基因診斷中。

我們利用 MALD1-TOF 此一核酸分析之新技術，將突變點依突變點核苷酸質量之不同予以確認。本方法以 PCR 之產物進一步進行第二次之微序列反應，進而以質譜儀來執行此一微序列差異之分析。以此一新技術，我們可一次在短時間內得到多重微序列分析之結果。而其快速，低成本之優點，可望成為新一代取代直接序列分析之工具。

醫學上結合 MALD1-TOF 之技術來從事基因診斷，其特點為 MALD1-TOF 可以微序列之方式來直接確認診斷。和傳統之突變分析方法比較，臨床上以 MALD1-TOF 之技術，將可使基因診斷變得更有效率敏感且更符合經濟規模效益。

二、英文摘要

Keywords：Multiplex genotyping, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, β-globin gene

Single nucleotide polymorphisms (SNPs) are currently being identified and mapped at a remarkable pace, providing a rich genetic resource with vast potential for disease gene discovery, pharmacogenetics, and understanding the origins of modern humans. High-throughput, cost effective genotyping methods are essential in order to make the most advantageous and immediate use of these SNP data. Currently, the most sensitive screening technique for genes that predispose patients for particular diseases is direct sequencing. However, sequencing is technically demanding, costly and time-consuming. To find fast, sensitive, and cost effective methods for detecting alterations of DNA in the target gene is warrant. In this study, we will develop a rapid and highly specific mutation screening method for the genetic diagnosis based on the technique of matrix-assisted laser desorption/ionization time-of- flight mass spectrometry (MALDI-TOF).

we incorporate the use of matrix-assisted laser desorption/ionization time-of- flight mass spectrometry (MALDI-TOF) in our laboratory as a tool for further differentiating genotypes based on the mass of the variant DNA sequence which had been identified by DHPLC which we had previously developed. We will combine a PCR amplification reaction with a secondary mini-sequencing reaction containing oligonucleotide primers that anneal immediately upstream of the polymorphic site, dideoxynucleotides, and a thermostable polymerase used to extend the PCR product by a single base pair. Mass spectrometry (MS) analysis of mini-sequencing reactions was performed using a MALDI-TOF instrument. We plan to perform either single or multiplex mini-sequencing reactions, and genotyped up to seven different variant sites in one time. The advantages of direct targeting the polymorphic site and the potential of multiple reaction in one time make MALDI-TOF a potential economic, efficient alternative technique than direct sequencing in confirming the diagnosis.

By combining DHPLC and MALDI-TOF techniques in genetic diagnosis, the advantages including that DHPLC got the powerful ability to identify random mutations and MALDI-TOF could identify the mutation site by direct targeting with highly efficient performance. Compared to classic approaches of mutation screening, DHPLC in combination with MALDI-TOF could take place of the direct sequencing in a more rapid, economic and highly sensitive way in genetic diagnosis.

三、研究方法

DNA Sample Preparation

All β-globin affected cases come from the individuals who come to National Taiwan University Hospital to be tested for the national screening program of thalassemia. All samples were extracted from peripheral whole blood, chorionic villus sampling, amniocentesis or cord blood sampling by using Puregene DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. DNA samples represent common genotypes of β-globin gene in Taiwanese population. The diagnosis of β-thalassemia were confirmed previously by Polymerase chain reaction- (PCR) based techniques including analyses of amplification created restriction sites (ACRS) and sequence analysis.

Primers Design and PCR Condition

PCR Primers used in this study were: 5'-TACGGCTGTCATCACTTAGACCTCA-3' and 5'-TGCAGCTTGTCACAGTGCAGCTCACT-3' were used to amplify from exon 1 to 5' portion of exon 2, 5'-GTGTACACATATTGACCAAA-3' and 5'-AGCACACAGACCAGCACGT-3' were used to amplify exon 3 and its flanking regions. The PCR primers were identical to those used previously for screening program, which could detect over 98% mutations in the Taiwanese population.

PCR for the fragments was performed in a total volume of 25 μL containing 100ng of genomic DNA, 0.12μM of each primer, 100μM dNTPs, 10mM Tris- HCl (pH = 8.3), 50mM KCl, 2mM $MgCl_2$, and 0.5 units of AmpliTaq Gold^{TM} enzyme (PE Applied Biosystems, Foster City, CA, USA). Amplification was performed in a mutliblock system (MBS) thermocycler (ThermoHybaid, Ashford, UK). PCR amplification was performed with an initial denaturation step 95℃ for 10 min, followed by 35 cycles consisting of denaturation at 94℃ for 30 s, annealing at 56℃ for 1 min, and extension at 72℃ for 30 s, and then an extension step of 72°C for 10 min.

Preparation of PCR Products for Primer Extension (Mini-sequencing) Reaction

To eliminate the dNTPs and primers from PCR products, PCR products were treated to remove unincorporated primers and dNTPs by GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ), then the purified PCR products was subsequently used for the primer extension reactions. The amount of PCR products were quantified by using spectrophotometer and samples were diluted to a final concentration of 10 ng/μL.

The mini-sequencing extension primers for VSET and PinPoint assay used in this study to detect eight most common genotypes of β-globin gene mutation including

c.-78A>G, c.2T>G, c.52A>T, c.84_85insC, c.130G>T, c.125_128delTCTT, c.216_217insA, and c.316-197C>T were described in Table 1. Primer extension reactions were carried out in 20 μL containing 50 ng input PCR product and 1 μL of 10 pmol mini-sequencing primer, 0.5 μL of 1mM of each ddNTPs and dNTPs (Amersham Pharmacia Biotech, Piscataway, NJ), 0.5 units of Thermo Sequenase DNA polymerase (Amersham Biosciences, Piscataway, NJ), and 2 μL of 10x reaction buffer provided by the manufacturer. For PinPoint assay, all four unlabeled ddNTPs were included (Fei, et al., 1998; Haff and Smirnov, 1997b; Li, et al., 1999). The very short extension (VSET) assay used 0.5 μL of 1 mM of only one dNTP and 0.5 μL of 1mM of other three ddNTPs (Sun, et al., 2000). The reaction was carried out in a mutliblocksystem (MBS) thermocycler (ThermoHybaid, Ashford, UK) with an initial denaturation step at 96°C for 1 min followed by 50 cycles of 96°C for 15 s, 43°C for 15 s and 60°C for 100 s, then 96°C for 30 s. The nucleotide compositions and product lengths for the primer extension reactions including for VSET and PinPoint assay are also listed in Table 1.

Sample Purification

Three different DNA desalting methods were tested for 10μM single strand DNA primer (5'-AACTCATCCACGTTCACCT-3') in 5mM $MgCl_2$. For ethanol precipitation, 3M ammonium acetate solution of one-fifth to one-third sample volume was added to DNA and incubated in 65℃ for 60min and then 37℃ for 2 hours. After incubation, 10 volumes of 100% ethanol were added, followed by incubating the tube in a freezer at -20℃ for overnight. Then, the tube was centrifuged, followed by removing the supernatant solution. Finally, 5μl of water was added to re-dissolve the precipitated DNA. For cationic exchange resin desalting, the 5WX8-200 Dowex cation exchange resin (Supelco Park, Bellefonate, PA) was selected as according to published protocol of Harksen. A. *et al*. (Harksen, et al., 1999) The cationic exchange beads were first exchanged with 1M NH_4OAc and placed on a piece of parafilm. 5 to 10μL of DNA sample and an equal amount of matrix (50mg/ml 3-hydroxypicolinic acid, 3HPA) were added to 0.1mg beads, followed by mixing up and down in the pipette tip several times. The supernatant were loaded onto the sample plate for MALDI analysis. The third method for DNA purification test was using Poros 50-R2 (PerSeptive Biosystems, Framingham, MA) reversed-phase chromatography media (Haff and Smirnov, 1997b). Pipette tips were filled with adequate Poros and then DNA sample was bound to it in the presence of 100mM triethylammonium acetate (TEAA, pH=6.5). Solvent was removed by applying compressed air to the tip (Guo, 1999), followed by to rinse with 10mM TEAA (pH=6.5), and elution with 20% acetonitrile.

For desalting strategy which combined the Poros chromatography and cationic exchange resin, vacuum-dried sample following Poros purification protocol was resolved in 3 to 5μL water and desalted following the cationic exchange bead procedure described as above. Finally, sample and matrix co-crystallized onto MALDI sample plate.

Mass Spectrometry Analysis

Prior to MALDI-TOF MS analysis, the sample was mixed with matrix solution (50mg/ml 3-hydropicolinic acid in a 4:5:1 mixture of water, acetonitrile and 50mg/ml diammonium citrate) and spotted on the 96x2 well teflon sample plate (PerSeptive Biosystems, Framingham, MA, USA).

MALDI-TOF mass spectra were acquired by a reflectron time-of-flight mass spectrometer (Voyager-DE PRO, Applied Biosystems, Foster City, CA, USA). The instrument was equipped with a 337 nm nitrogen laser source and delivered pulses at 3-20 Hz. Measurements were taken in linear, negative ion mode at a 20kV acceleration voltage and 380ns delayed ion extraction. The excess unextended oligonucleotide primers were used as internal standards for mass calibration. 100 laser shots were averaged for a typical spectrum, and following data acquisition, noise reduction was performed by smoothing.

Sequence Analysis

Amplicons were purified by solid-phase extraction and bidirectionally sequenced with the PE Biosystems Taq DyeDeoxy terminator cycle sequencing kit (PE Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing reactions were separated on a PE Biosystems 373A/3100 sequencer.

四、結果

Improved sample preparation for MALDI-TOF MS

Metal cation adduction is a common problem for nucleic acid detection by mass spectrometry and various desalting techniques have been extensively discussed (Nordhoff, et al., 1996). Considerable ion suppression caused by excess metal salt will reduce the sensitivity of mass spectrometric measurement. In addition, partial substitution of protons by metal cations will result in a distribution of adduct peaks and may interfere with interpretation of primer extension products. Therefore, efficient desalting is crucial for SNP genotyping in which significant amount of metal salts and buffer are required to optimize primer extension reactions.

To address the issue, several sample purification methods were evaluated in this

study including ethanol precipitation, cationic exchange resin, and reverse-phase resin (POROS). In addition to the un-removed Na^+ and K^+ in the solution, 5mM $MgCl_2$ was added to mimic the salt content in the primer extension reaction. Figure 1 shows the comparison of the mass spectra obtained from an unpurified primer containing 5mM $MgCl_2$ and those after three different sample purifications. The results show various degree of desalting in the following descending order, POROS desalting, cationic exchange resin, and ethanol precipitation. As shown in Figure 1(a), the mass spectrum obtained from the un-purified primer displays a number of peaks representing a series of adducts formed with the Mg^{2+}, Na^+ and K^+. Compared to un-desalted primer, the average intensity and signal-to-noise ratio were improved 12 and 28 times, respectively, for POROS desalting method (Figure 1(d)). Furthermore, we combined POROS and cationic exchange resin desalting for optimal desalting effect. The MALDI-TOF MS spectrum in Figure 1(e) showed almost exclusively molecular ions of the 20-mer primer and complete disappearance of salt adduct. Dramatic desalting effect was demonstrated on the 120 times superior signal-to-noise ratio. It is noted that no recovery loss arise from the sequential desalting. The following multiplex SNP genotyping would use this improved sample preparation procedure to ensure free salt adduct that may interfere spectra interpretation.

Comparison of VSET and PinPoint assay for healthy heterozygous subjects analysis

For subsequent optimal multiplex SNP analysis of hereditary β-globin mutation, we first evaluated uni-plex reaction by VSET and PinPoint assays for genotyping the eight most common SNPs of β-globin Gene in Taiwan (as confirmed by direct sequencing). For PinPoint assay (figure 2), the primer extension products were produced by mini-sequencing primer plus the extended ddNTP. The primer extension products of wild types and mutant analogues have same length of bases and are closely spaced with mass difference of different nucleotides at the polymorphistic site (Table. 1). It was seen from Figure 2(c) and (g) that the mutation product of c.216_217insA and c.52A>T were poorly resolved due to the small mass difference of 9 Da (the difference between A and T). In this case, higher mass spectra resolution is required to well separate the A/T heterozygotes. Although this renders the PinPoint assay less clear to differentiate the heterozygous product, our result still unambiguously genotype the A/T heterozygous alleles attributing to the free interference of salt adduct from our modified sample preparation procedure as presented above.

On the other hand, the difficulty of resolving A/T heterozygotes can be overcome by the VSET assay. Figure 3(a)-(h) show the results of typing eight heterozygous

individuals using VSET assay. Clearly, in addition to the first peak corresponding to the un-extended mini-sequencing primer, the pattern of two extended peaks can be identified for heterozygous carriers while only one single extended peak was observed for wild-type homozygous counterparts. By the characteristic patterns and comparison of the expected extension products, all eight biallelic SNP marker in HBB mutant can be more easily identified by VSET assay without any ambiguity due to unresolved peaks. Moreover, lower level of desalting is needed even for the difficult A/T heterozygotes.

Rational design for HBB multiplex genotyping assay using mass tuning strategy

To establish cost-down and time-effective screening of eight common disease-causing mutations in β-globin gene (HBB), multiplex genotyping is particularly advantageous. Based on the comparison between VSET and PinPoint assay, the later primer extension method was preferred for HBB eight-plex SNPs assay, in which the primer and its extension product are in narrow mass range to facilitate the display and interpretation of eight SNPs. To resolve eight pairs of primer/extended product in single mass spectrum without ambiguity, overlapping of unextended primer and its product has to be avoided. This can be achieved by rational design of primer using "mass tuning" strategy, in which the primers vary with successively increasing length. For optimal MALDI-TOF MS detection sensitivity, the masses of the eight pairs of primer/extended products ideally should be designed below 40 bases, to give both adequate resolution between possible components and ionization efficiency (Ross, et al., 1998). To compromise with the primer extension specificity where longer primers are preferred, the lengths of primer are designed with at least one base apart, ranging from 18 to 27 bases (Table 1b). They were constructed such that their masses are sufficiently different to be spectrally separated from each other within a mass spectrum. Therefore, the number of typed loci can be increased by mass tuning to avoid overlaps between primers and its extended products.

Figure 4 shows the mass spectra of the eightfold multiplex genotyping for the most common HBB mutation in Taiwan. As shown in Figure 4(b) and 4(c), the eight pairs of primer/extended products for either wild type or heterozygous carrier are well separated in one-base increment, demonstrating that the mass tuning strategy can easily resolve high degree of multi-plexing for unambiguous data interpretation. In Figure 4(d) where eight heterozygous mutations were measured, the two extended peaks in all eight SNPs can be spectrally resolved. At the longest length of 27 bases, the extended peak with masses of 8651.3 and 8666.2 can be identified for a heterozygous carrier on c.316-197C>T without ambiguity. The result showed that the PinPoint assay in combination with mass tuning strategy is sufficiently accurate for

the eightfold multiplex genotyping diagnosis.

Diagnosis of hereditary β-globin Gene (HBB) mutations in a core family by the eight-plex genotyping assay

Finally, the multiplex genotyping by PinPoint assay and MALDI-TOF MS was applied to the identification of inherited β-globin alleles within a nuclear family composed of parents and three children. As shown in Figure 5, all eight polymorphic sites show the expected *m/z* of primer and single-base extension products, enabling the assignment of each mutant parental allele feasible. The spectra of parents display characteristic heterozygous peaks, in excellent agreement with the diagnosis that they are heterozygous carriers for the mutation c.316-197C>T and c.125_128 delTCTT, respectively. The peak doublet of mass difference 15 Da shown in Figure 5 diagnosed that child III inherited the c.316-197C>T allele from the father. However, the other two affected children (IV and V) in this core family were identified as compound heterozygous patients for both parental mutations, c.316-197C>T and c.125_128 delTCTT. The multiplex genotyping assay confirmed that their offspring imperatively have to carry half of the alleles from the mother and half of the alleles from the father as the permanent rule of heritage. Our analysis is further confirmed by direct sequencing analysis of the family (data not shown).

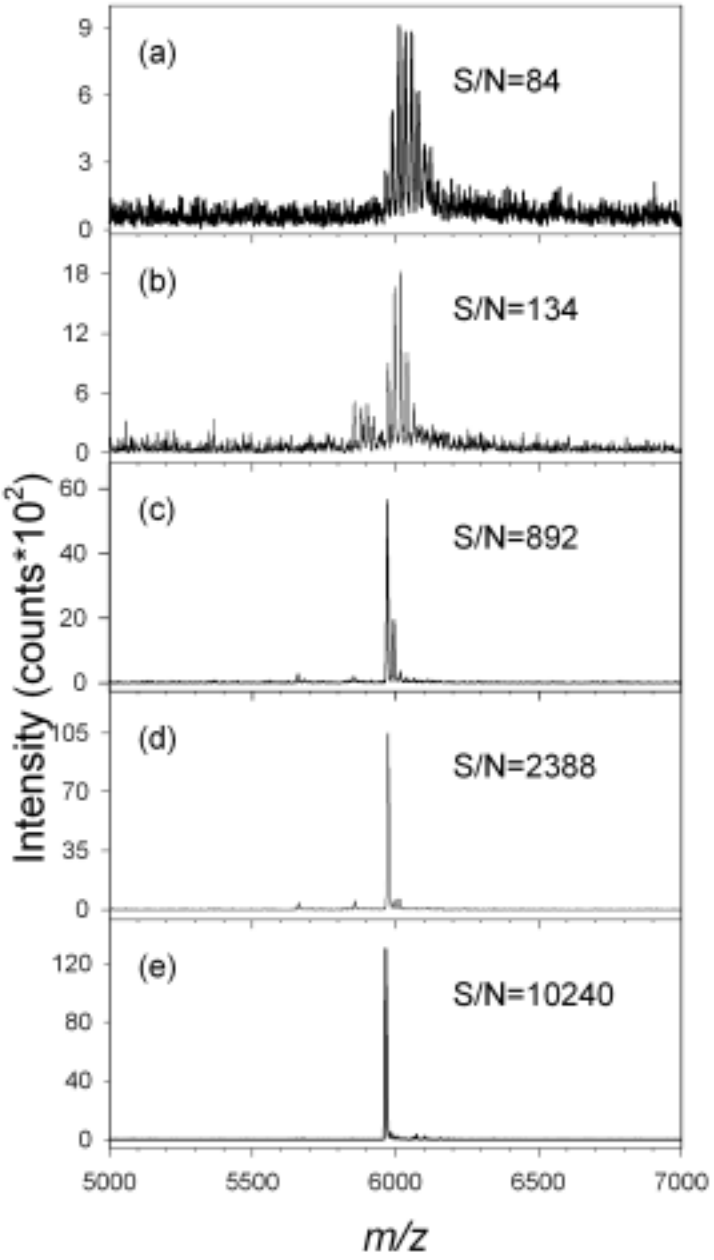

Figure 1. Evaluations of several different DNA desalting methods for 10 μM DNA primer (5'-AACTTCATCCACGTTCACCT-3') containing 5mM $MgCl_2$. (a) Direct detection without any desalting method; (b) ethanol precipitation; (c) cationic exchange resin desalting; (d) POROS R2 desalting; and (e)

combination of POROS R2 and cationic exchange resin desalting.

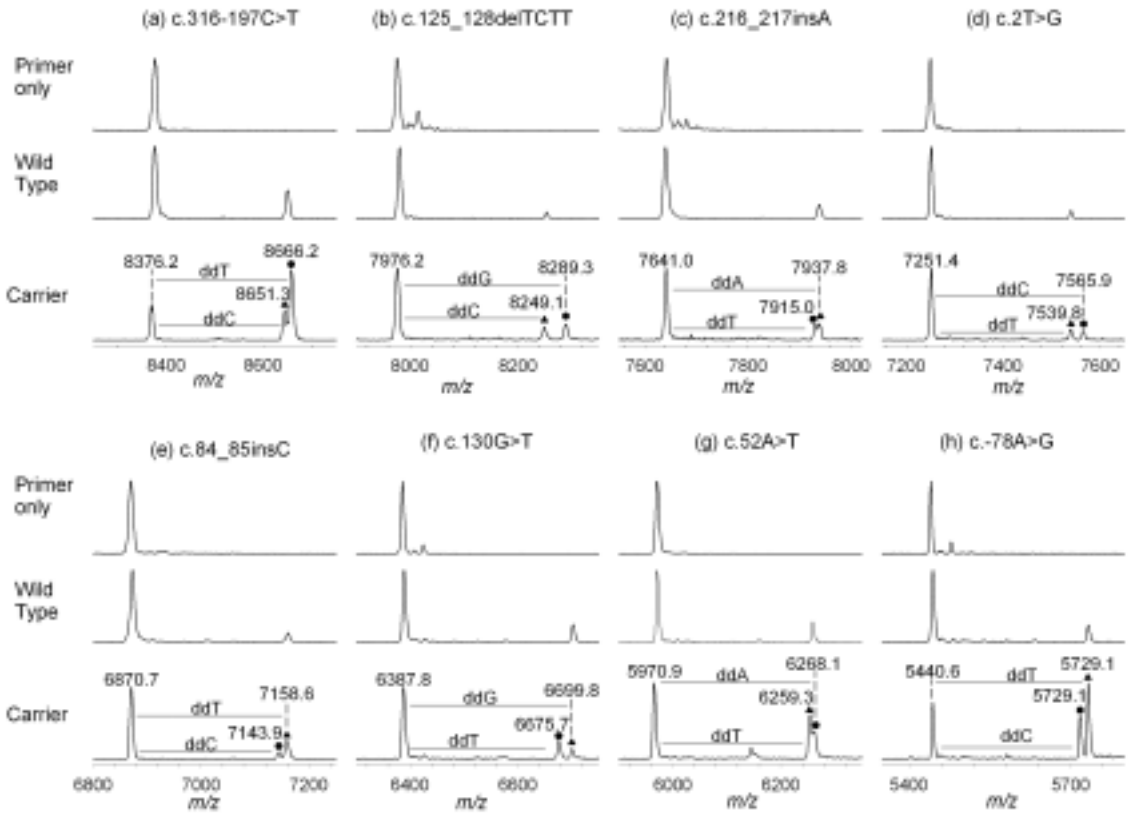

Figure 2. Mass spectra using PinPoint assay for genotyping heterozygous carriers and wild-type subjects detected to identify eight common mutations of the HBB gene for the Taiwanese population. The primers for PinPoint assay are designed to detect: (a) c.316-197C>T; (b) c.125_128delTCTT; (c) c.216_217insA.; (d) c.2T>G; (e) c.84_85insC; (f) c.130G>T; (g) c.52A>T; and (h) c.-78A>G. The peaks labeled with filled triangles and circles are the primer-extension products from the wild-type and mutant subjects, respectively.

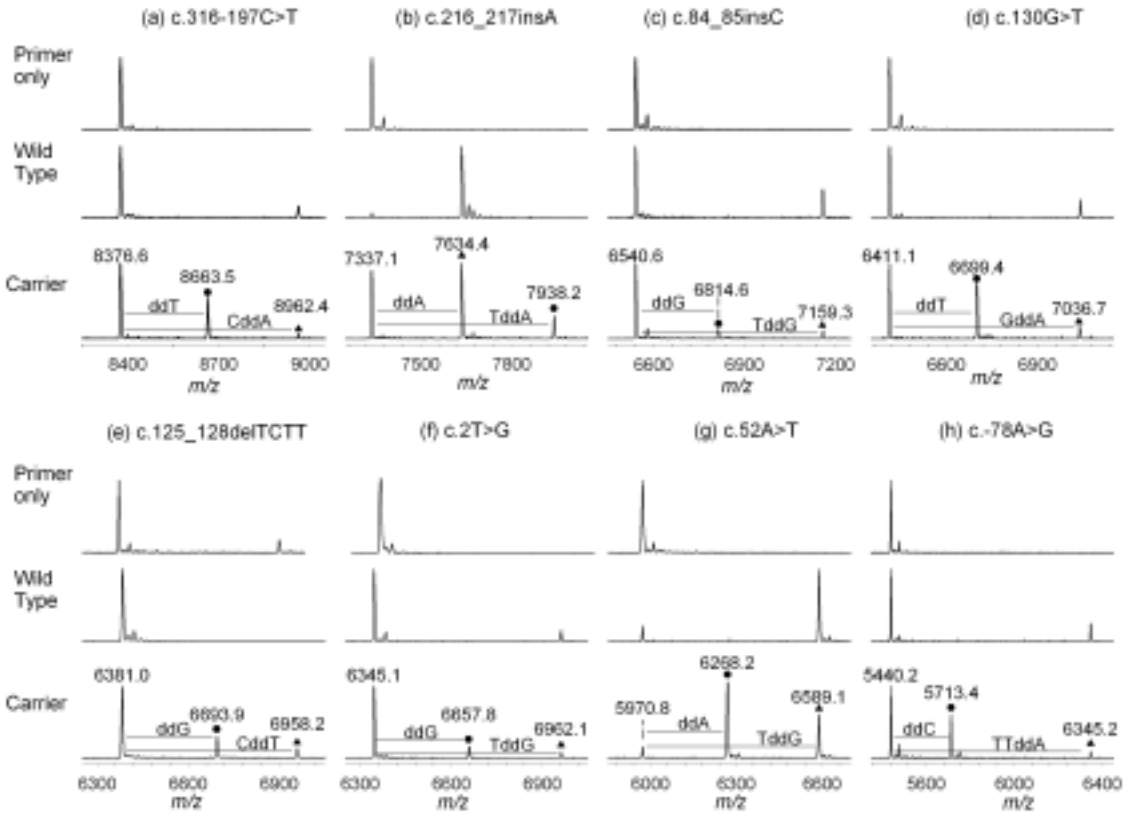

Figure 3. Mass spectra using very short extension (VSET) assay for genotyping heterozygous carriers and wild-type subjects to identify eight common mutations of the HBB gene for the Taiwanese population. The primers for VSET assay are designed to detect: (a) c.316-197C>T; (b) c.216_217insA; (c) c.84_85insC.; (d) c.130G>T; (e) c.125_128delTCTT; (f) c.2T>G; (g) c.52A>T; and (h) c.-78A>G. The peaks labeled with filled triangles and circles are the primer-extension products from the wild-type and mutant subjects, respectively.

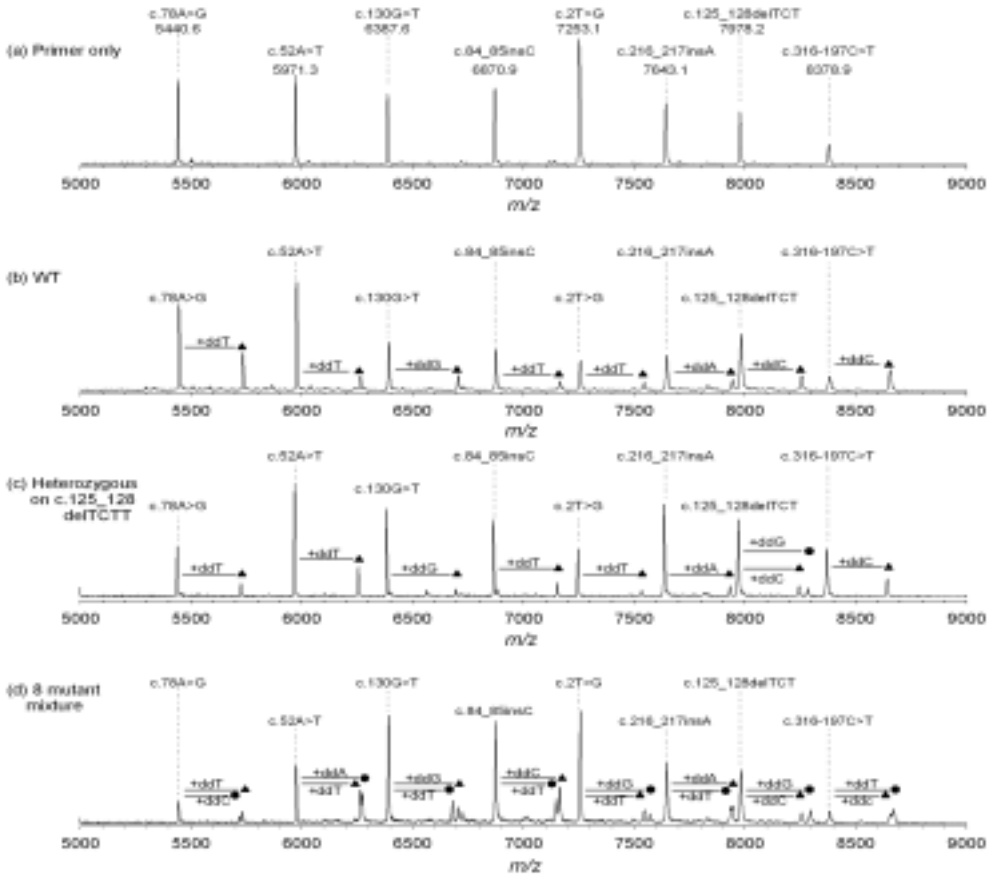

Figure 4. Representative MALDI-TOF mass spectra from multiplex PinPoint assay of eight polymorphic sites in the HBB gene. (a) Mass spectrum of mixture from control primers for the single base extensions. (b) Mass spectrum of the 8-fold multiplex assay of the homozygous wild-type subjects. (c) Mass spectrum of the 8–fold multiplex assay from a carrier exhibiting a c.125_128delTCTT mutation. (d) Mass spectrum of the 8-fold multiplex assay from eight heterozygous carrier mixtures. The peaks labeled with filled triangles and circles are the primer-extension products of the wild-type and mutant subjects, respectively.

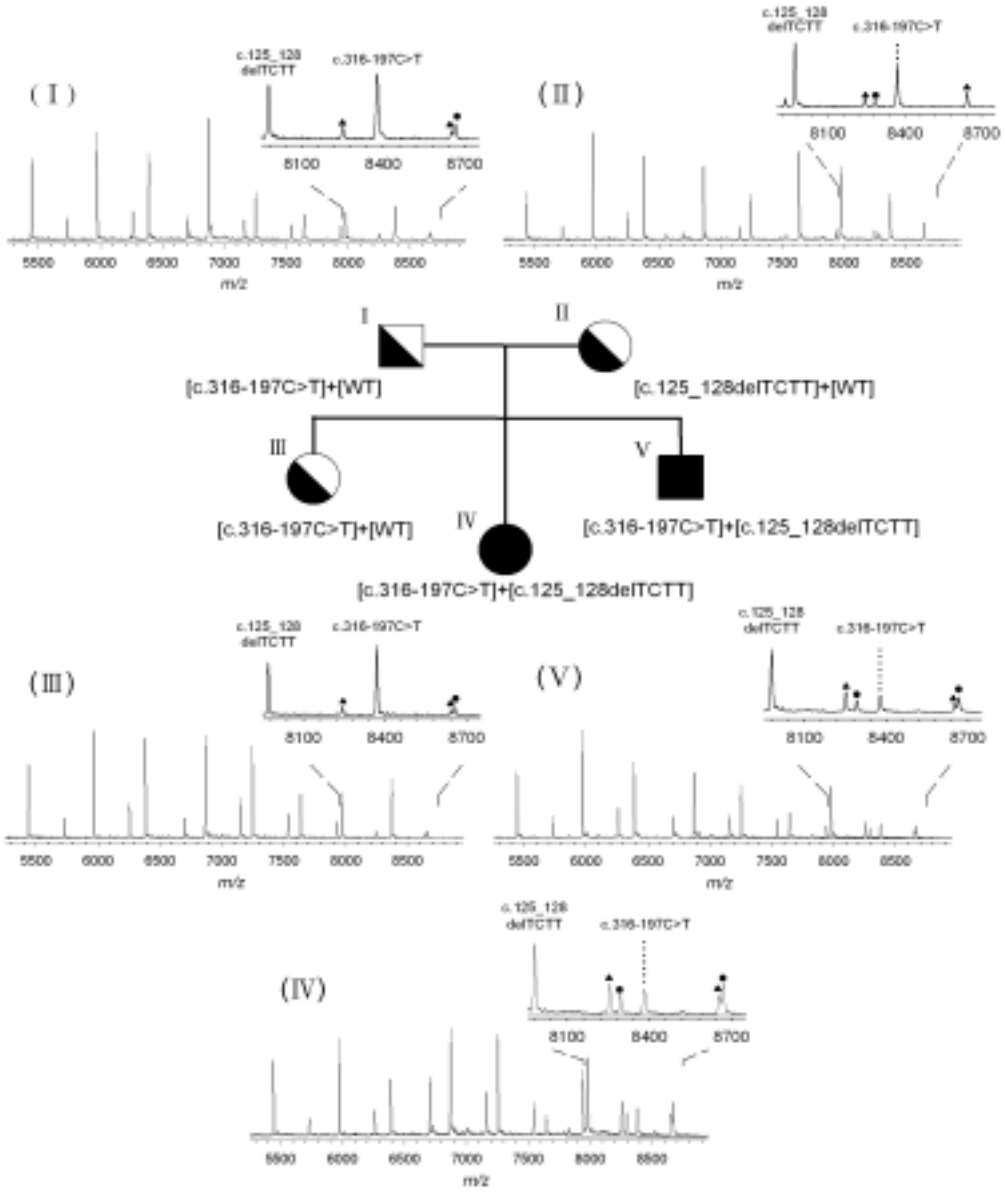

Figure 5. Eight-plex analysis of a core HBB mutation family with heterozygous carrier. The heterozygous single- and double mutations in the HBB gene are correctly genotyped for each family member. The results show that the five family individuals I-V are a heterozygous carrier on c.316_317C>T (I), a heterozygous carrier on c.125_128delTCTT (II), a heterozygous carrier on c.316_317C>T (III), and two individuals of double heterozygous mutation on c.316_317C>T and c.125_128delTCTT (IV and V), respectively. The peaks labeled with filled triangles and circles are the primer-extension products from the wild-type and mutant subjects, respectively.