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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(2/3)

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中文摘要

乙型海洋性貧血是常見的單一基因遺傳疾病，起因於人類乙型血球蛋白基因突變。此研究建立了一套可靠、正確的分子診斷方法，合併多重引子延長法與基質輔助雷射脫附游離/飛行時間質譜儀(MALDI-TOF MS)，可對乙型血球蛋白基因常見的 8 個突變點位進行快速突變分析，佔了台灣地區乙型海洋性貧血基因型的 98 % 左右。MALDI-TOF MS 利用”質譜儀調校”來提高對於乙型血球蛋白基因之單一核苷酸檢測的敏感度與專一性。利用 POROS 有效的去鹽與陽離子交換層析儀使 MALDI-TOF MS 有效的用於多重基因定型分析。此單一核苷酸檢測方法擁有高靈敏度，可以有效的分辨常見的 8 個突變點位之同型合子與異型合子。結果顯示，此方法可以準確的篩檢乙型海洋性貧血之基因突變型，擁有高效率、高靈敏度、高準確率之優點。如此一來，MALDI-TOF MS 可大量分析樣品之優點，可以應用於臨床醫學上，做為乙型海洋性貧血基因分析之第一線篩檢診斷工具。

Abstract

β-thalassemia is a common monogenic disease caused by mutations in the human β-globin gene (HBB), many of which are differentially represented in human subpopulations stratified by ethnicity. This study describes an efficient and highly accurate method to screen for the eight most-common disease-causing mutations, covering more than 98% of HBB alleles in the Taiwanese population, using parallel minisequencing and multiplex assay by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The MALDI-TOF MS was optimized for sensitivity and resolution by ‘‘mass tuning’’ the PinPoint assay for eight HBB SNPs. Efficient sequential desalting using POROS and cationic exchange chromatography allowed for an unambiguous multiplex genotyping by MALDI-TOF MS. The embellishing SNP assay allowed for highly accurate identification of the eight most common β-thalassemia mutations in homozygous normal control, carrier, and eight heterozygous carrier mixtures, as well as the diagnosis of a high-risk family. The results demonstrated a flexible strategy for rapid identification of clustering SNPs in HBB with a high degree of accuracy and specificity. It can be adapted easily for high-throughput diagnosis of various hereditary diseases or to establish family heritage databases for clinical applications.

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

一、前言

The introduction of matrix-assisted laser desorption/ionization (MALDI) time-of-fight (TOF) mass spectrometry (MS) has resulted in the direct and rapid identification of intact biomolecules (Marvin et al. 2003). For the analysis of complex minisequencing products in multiplex genotyping, MS offers greater accuracy than electrophoresis-based or hybridization-array-based methods, which are often susceptible to complications from nucleic acid secondary structure (Li et al. 1993; Southern et al. 1994). Therefore, MALDI-TOF MS in combination with a minisequencing strategy has become one of the most promising mutation analysis tools to

analyze polymorphisms in disease-causing genes, including the genes encoding cystic fibrosis (Braun et al. 1997), coronary artery diseases (Nakai et al. 2002), cardiovascular disease, and even the disease-causing mutations on human Y chromosome (Paracchini et al. 2002; Wise et al. 2003). Compared with other DNA analysis methods, the advantages of MALDI-TOF MS include the direct and absolute mass readout of DNA (Tost and Gut 2002) and high-throughput analysis (Van Ausdall and Marshall 1998) that facilitates simultaneous multiplex genotyping in a single experiment.

Although multiplex assay is generally a cost-effective way for high-throughput SNP genotyping, two factors have limited the practicality of using multiplex minisequencing and MALDI-TOF MS for screening HBB mutations. The limitations are based on the following factors: (1) difficult and time-consuming optimization of multiplex primer extension (Tost and Gut 2002), and (2) spectral interference from contaminating salt adducts (Guo 1999; Tost and Gut 2002). The former issue presents a specific challenge because highly multiplex genotyping parameters may be difficult to optimize. For HBB, as shown in Fig.1, two sets of clustering polymorphic sites, CD27/28 +C versus CD17 A>T, and CD43 G>T versus CD41/42 –TCTT, are in close proximity to each other and almost overlap. The genotyping of these closely associated and clustered mutations within the β-thalassemia gene poses practical difficulty for multiplex minisequencing in a single tube (Wang et al. 2003). The second limiting factor for multiplex HBB genotyping using MALDI-TOF MS is that contaminating salt, detergent, or glycerol can degrade spectral quality and reduce the accuracy of mass assignment (Bleicher and Bayer 1994; Guo 1999; Kim et al. 2002; Tost and Gut 2002). In a highly multiplex assay, this increase in interference is greater than the linear scale of the multiplex factor. Thus, sample purification is a crucial step in DNA sample analysis by MALDI-TOF MS.

二. 研究目的

To address the complications of primer extension arising from the close proximity and clustering of β-thalassemia mutations, we developed an alternative multiplex genotyping method utilizing eight parallel minisequencing reactions followed by multiplex MALDI-TOF analysis. Minsequencing reaction products are pooled for sequential desalting and MALDI-TOF MS assay, thus maintaining the advantages and efficiency of multiplex processing and analysis. Using this approach, the eight common HBB alleles in the Taiwanese population can be analyzed with high accuracy in a single assay. The method is equally successful with heterozygous carriers or homozygous-affected and normal individuals. Our results demonstrate the feasibility of rapid, accurate, and flexible multiplex genotyping for high throughput, unconstrained diagnostics of β-thalassemia.

三、文獻探討

Several assays have been developed to genotype common β-thalassemia mutations, including amplification refractory mutation analysis (Fortina et al. 1992), DNA-probe assays (Ugozzoli et al. 1998), fluorescent multiplex PCR (Sherlock et al. 1998), real-time PCR (Vrettou et al. 2004), and microelectronic chip-based assays (Foglieni et al. 2004). Each of these methods has

advantages and limitations, and a highly accurate, high-throughput method to simultaneously genotype common HBB alleles has yet to be developed and widely implemented.

四、研究方法

Mass spectrometry analysis Prior to MALDI-TOF MS analysis, the sample was mixed with matrix solution (50 mg/ml 3-hydropicolinic acid in a 4:5:1 mixture of water, acetonitrile and 50 mg/ml diammonium citrate) and spotted on the 96·2-well teflon sample plate (PerSeptive Biosystems). MALDI-TOF mass spectra were acquired by a reflectron TOF MS (Voyager-DE PRO, Applied Biosystems). The instrument was equipped with a 337 nm nitrogen laser source at 3–20 Hz. Measurements were taken in linear, negative ion mode at 20 kV acceleration voltage and 380 ns delayed ion extraction. The excess unextended oligonucleotide primers were used as internal standards for mass calibration. A typical mass spectrum was obtained by the average of 100 laser shots followed by noise reduction and Gaussian smoothing using Data Explorer software (Applied Biosystems).

五、結果與討論

Parallel minisequencing reactions using PinPoint assays were evaluated for genotyping the eight common HBB alleles in the Taiwanese population. The typical mass spectrum of minisequencing primers plus the extended ddNTPs for wild types and carriers are shown in Fig.3. Primer extension products templated on the wild-type or mutant allele have the same length, and their mass difference is due to sequence difference at the polymorphic site (Table 1). Figure 3c and g show analysis of CD71/72 +A and CD17 A >T containing samples; although the mutant products partially overlap with one another because the mass difference is only 9 Da (the difference between A and T), the samples migrate at m/z=7929.1 and 6268.1, respectively, providing sufficient resolution for accurate genotyping. For maximal benefit and clinical application, this method should differentiate homozygotes and heterozygotes carrying all eight common HBB mutations. Thus, we next performed the one-spectrum analysis on subjects in different genetic states, including a homozygous normal control, a carrier with a SNP (CD41/42 –TCTT), and mixture containing eight heterozygous carriers, as shown in Fig. 4. Because of the proximity of the two pairs of mutation sites (CD41/42 –TCTT and CD43 G > T, CD 27/28 +C and CD17 A > T), the eight minisequencing reactions were performed in parallel followed by the multiplex MALDI-TOF MS assay to overcome the obstruction caused by partially overlapping primers. The extension products from the eight parallel minisequencings were pooled for subsequent sequential desalting to reduce sample loss during purification as well as to speed up the procedure. As shown in Fig.4b and c, the eight pairs of primer/extended products for either wild-type or heterozygous carrier are well separated in one-base increments, which facilitate unambiguous data interpretation. Figure 4d shows assay results with the most complex input material, a mixture of eight heterozygous samples. In this assay, peaks for mutant and wild-type product were resolved for all eight SNPs, demonstrating that heterozygous carriers can be accurately genotyped by this method. This result demonstrated that parallel minisequencing followed by multiplex MALDI-TOF assay in combination with the mass tuning

strategy is sufficiently accurate for the eight-fold multiplex genotyping diagnosis in β-globin gene.

In this study, a total of 80 subjects were analyzed, including 37 wild-type controls and 43 heterozygous cases. All of the mutations can be identified without ambiguity, consistent with the characterization by direct sequencing. The highly accurate detection rate demonstrated the reproducibility of the approach for clinical diagnostics. Thus, the current approach is a reliable technique for mutation screening of β-thalassemia.

Ultimately, it would be useful to apply multiplex HBB genotyping for prenatal diagnosis. Here, we evaluated the potential of our assay for this application by analyzing HBB status in a nuclear family at risk for β-thalassemia. Figure 5a shows the MS analysis of all eight polymorphic sites. Seven normal allelles were detected, and the products of wild-type and mutant alleles corresponding to IVS2+654 C > T and CD41/42 –TCTT polymorphisms were detected. The mass spectra shown in Fig.5b are enlarged to facilitate analysis of the spectral region relevant to the genotype assignment. The spectra of parents (I and II) display characteristic heterozygous peaks consistent with the fact that they are heterozygous carriers for the mutation IVS2+654 C >T and CD41/42 –TCTT, respectively. For child III, the spectrum reveals a doublet peak at m/z=8648.7 and 8664.2 of mass difference 15 Da (Fig.5b), indicating that the IVS2+654 C > T allele was inherited from the father. Spectra for the other two children (IV and V) were identified as compound heterozygotes; they inherited both mutant alleles IVS2+654 C > T and CD41/42 –TCTT, and thus they are affected individuals and not carriers. These two mutations are the most commonly observed SNPs in the Taiwanese population (Su et al. 2003). In following with the Mendelian rules of inheritance, the multiplex genotyping assay confirmed that the offspring analyzed carried half of their alleles from their mother and half from their father. These data demonstrate that our multiplex genotyping strategy for common HBB alleles in the Taiwanese population is capable of genotyping heterozygote carriers and homozygote normal and affected individuals.

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七、計畫成果自評

In summary, a cost-effective, efficient, accurate, high-throughput, multiplex genotyping strategy was developed for eight common HBB alleles in the Taiwanese population. This method uses parallel minisequencing, multiplex sequential desalting, and MALDI-TOF MS, and it accurately identifies all relevant genotypes, including a mixture of eight heterozygous carriers. Our result accurately displayed the hereditary map of parents and their offspring at the molecular level. The strategy described here can be adapted for genotyping other disease-causing mutations; as such, it may be widely applicable in the clinical setting for diagnostic genotyping.

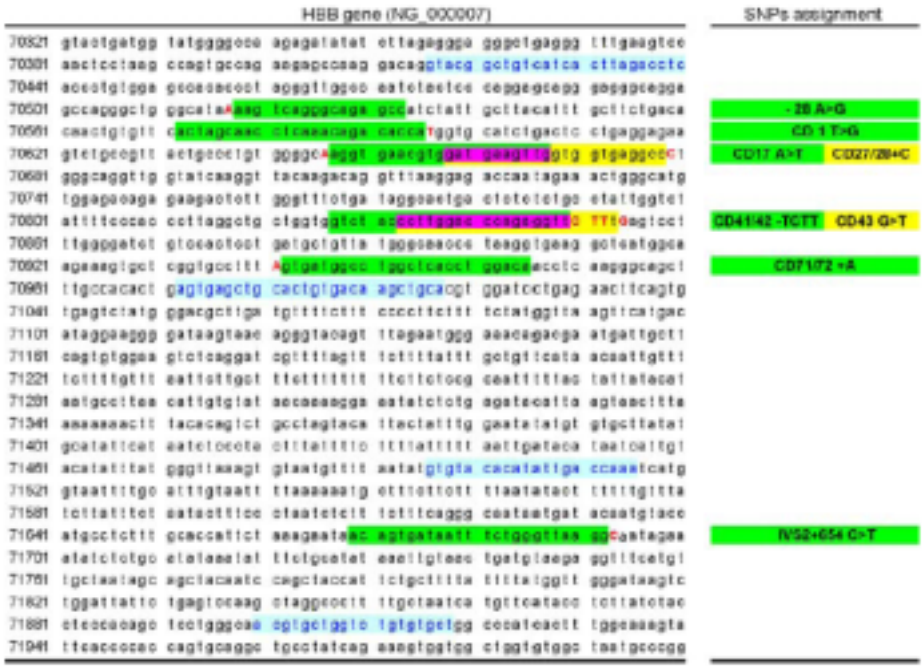

Fig. 1 Human b-globin gene sequence and primer locations. The mutation sites are marked in capital letters and red font. The shaded DNA sequences indicate primers. Assignments are shown in the right column and shaded in the same color as the relevant primer. Overlapping sequences are shaded in pink. The sequences for PCR amplification are shaded in cyan

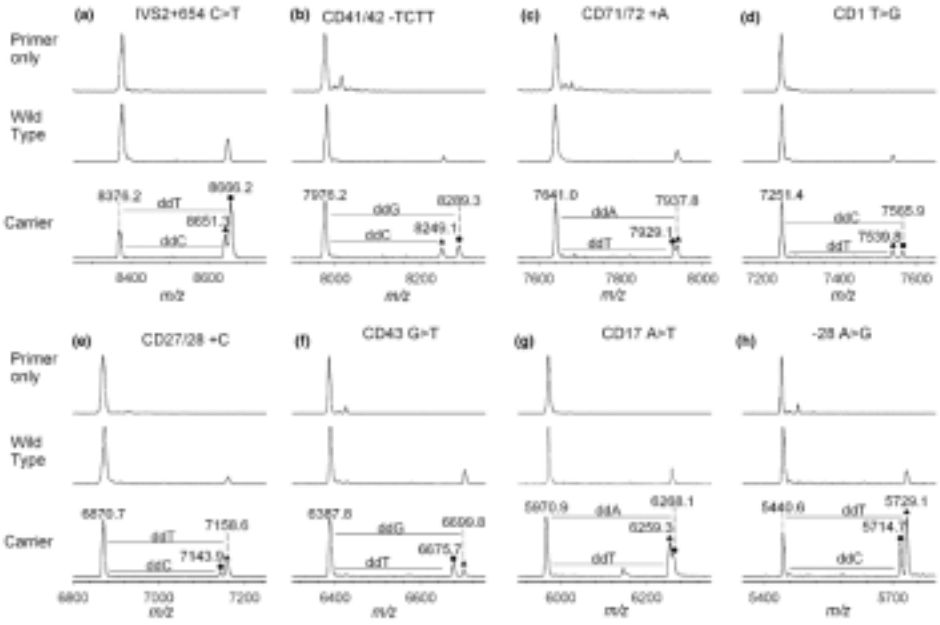

Fig. 2 MALDI-TOF MS analysis of eight human b-globin (HBB) gene alleles. For each SNP, the top, middle, and bottom panels show the mass spectra of primer, extension products of heterozygous carrier, and wild-type subject, respectively. a IVS2+654 C > T, b CD41/42 –TCTT, c CD71/72 +A, d CD1 T > G, e CD27/28 +C, f CD43 G>T, g CD17 A>T, and h _28 A>G. All panels: filled triangles and circles indicate primer-extension products from the wild-type and mutant subjects, respectively

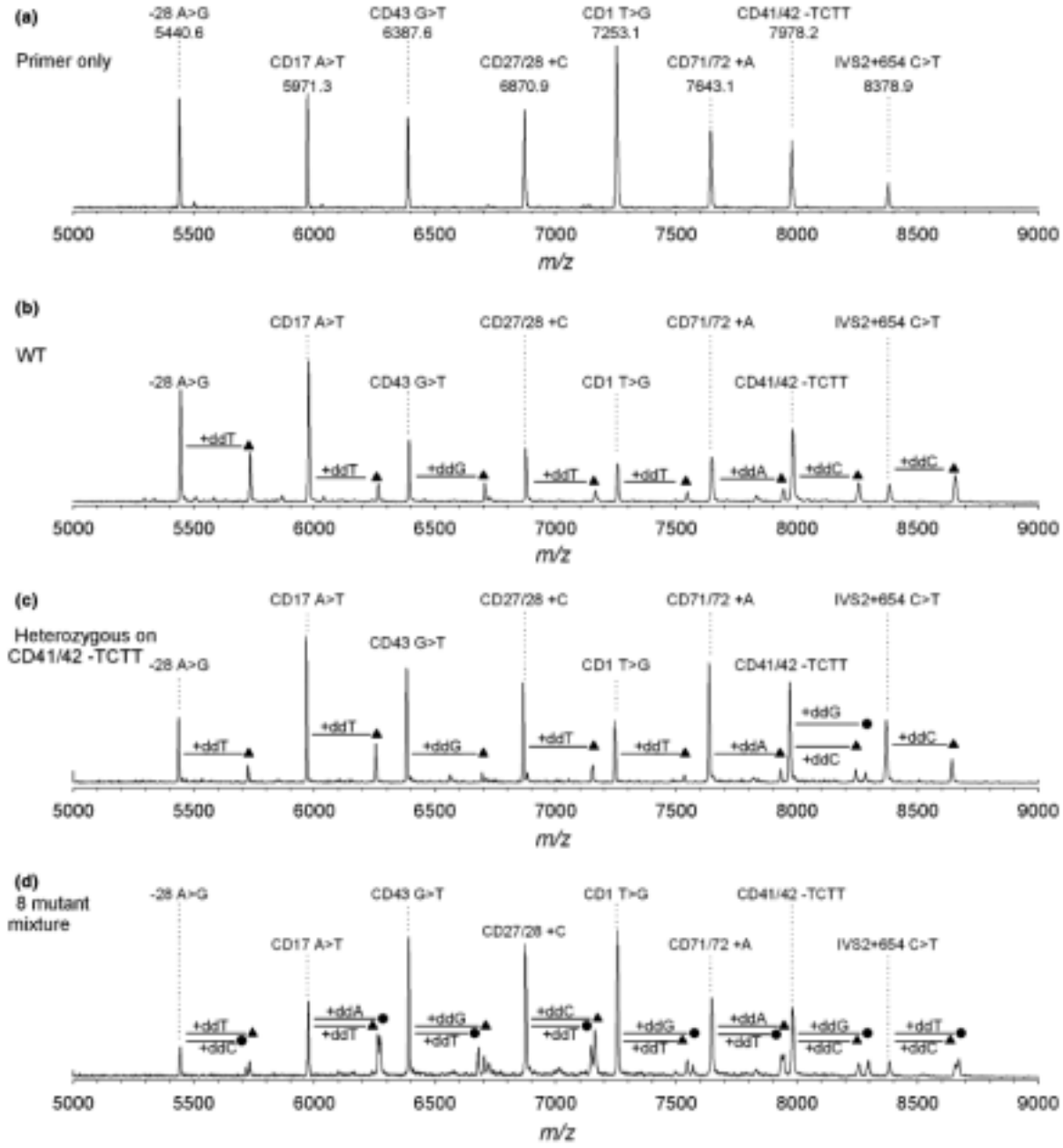

Fig. 3 Representative mass spectra from eight parallel PinPoint minisequencing followed by multiplex MALDI-TOF MS assay in the human b-globin (HBB) gene. a Control primers from minisequencing reactions. b Homozygous wild-type subjects. C Heterozygote with CD41/42 –TCTT mutation. d Mixure of eight heterozygous carriers. a–c: filled triangles and circles indicate primer-extension products from the wild-type and mutant subjects, respectively

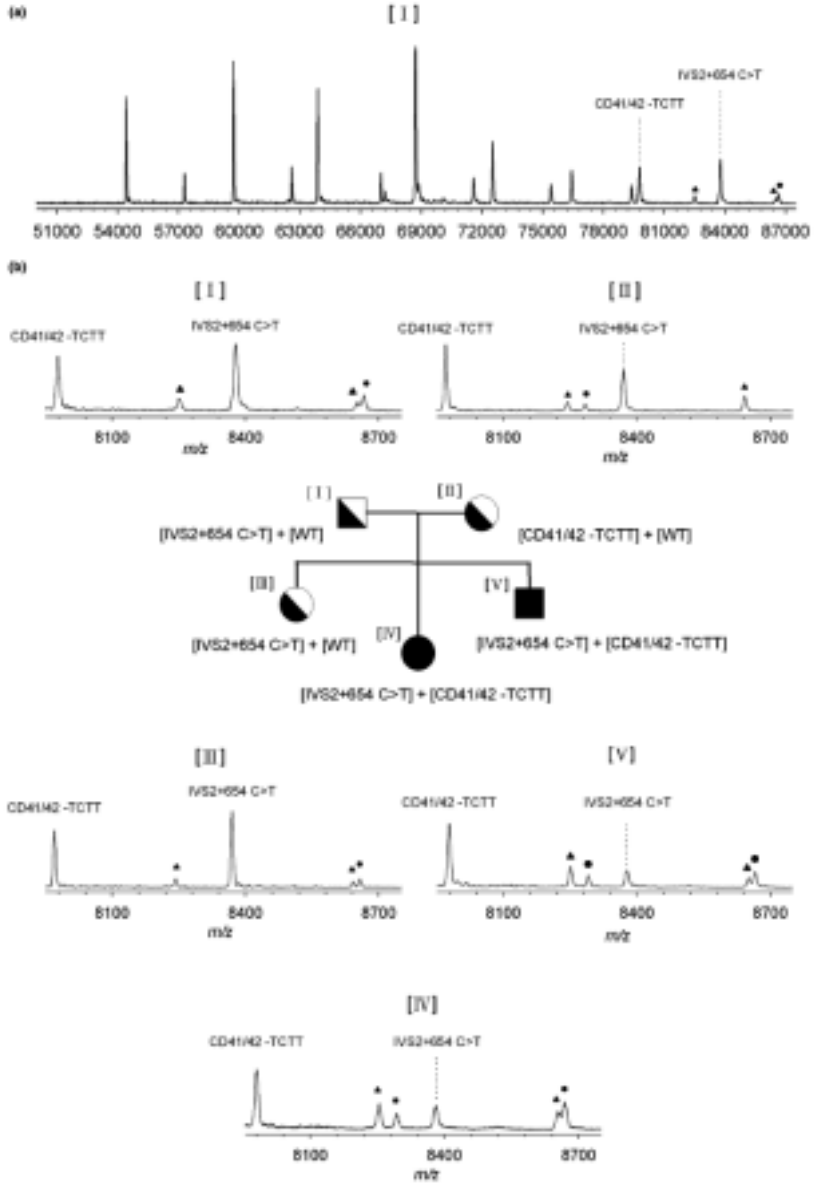

Fig. 4 Genotyping of hereditary b-globin (HBB) gene mutations in a core family. a A representative mass spectra showing all eight polymorphic sites (father I). b Expanded mass spectra showing IVS2+654 C>T and CD41/42 –TCTT. Filled triangles and circles indicate primer-extension products from the wild-type and mutant subjects, respectively

PinPoint assay				
SNP site	Primer sequence (5′-3′)	Primer mass[a] (Da)	Normal product mass[a] (Da)	Mutant product mass[a] (Da)
IVS2 + 654 C > T	ATAACAGTGATAATTTCTGGGTTAAGG	8376.2	8649.5(+ddC)	8664.5(+ddT)
CD41/42 –TCTT	GTGGTCTACCCTTGGACCCAGAGGTT	7977.2	8250.2(+ddC)	8290.2(+ddG)
CD71/72 + A	TGTCCAGGTGAGCCAGGCCATCACT	7642.0	7939.0(+ddA)	7930.0(+ddT)
CD1 T > G	ACTAGCAACCTCAAACAGACACCA	7251.0	7539.0(+ddT)	7564.0(+ddG)
CD27/28 + C	GATGAAGTTGGTGGTGAGGCCC	6870.5	7158.5(+ddT)	7143.5(+ddC)
CD43 G > T	CCTTGGACCCAGAGGTTCTTT	6387.2	6700.2(+ddG)	6675.2(+ddT)
CD17 A > T	AACTTCATCCACGTTCACCT	5970.9	6258.9(+ddT)	6267.9(+ddA)
–28 A > G	ATGGCTCTGCCCTGACTT	5440.6	5728.6(+ddT)	5713.6(+ddC)

[a]Average molecular weight

Table 1 Expected masses of the primer and extension products for the eight human β-globin (HBB) gene target mutation sites by PinPoint assay