Molecular Diagnosis in β-thalassemia

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31 July, 2009

Thalassemia

- Thalassa (Greek letter)= the sea
- Defective globin synthesis,
- Normal $\alpha = \beta$ ($\alpha/\beta = 1$)
- The alpha (α) thalassemias are concentrated in Southeast Asia, Malaysia, and southern China.
- The beta (β) thalassemias are seen primarily in the areas surrounding Mediterranean Sea, Africa and Southeast Asia.
- α-thalassemia: usually caused by gene deletion, 3-5% in Taiwanese
- β-thalassemia: usually caused by gene mutations, 1-3% in Taiwanese

- 200 mutations in β-hemoglobin genes lead to β-thalassemias.
- 80 deletions and point mutations in α-globin genes result in α-thalassemias.

Weatherall, D. J. & Clegg, J. B. *The Thalassaemia Syndromes* 4th edn (Blackwell Science, Oxford, 2001).

HbVar: A Database of Human Hemoglobin Variants and Thalassemias
Summaries of mutation categories

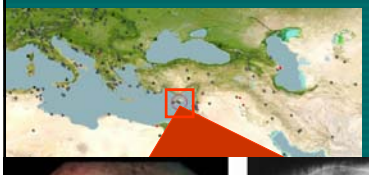
Query	Count of results	Button to view results
Total entries in database	1361	View summary table
Total hemoglobin variant entries	1018	View summary table
Total thalassemia entries	393	View summary table

http://globin.bx.psu.edu/cgi-bin/hbvar/counter

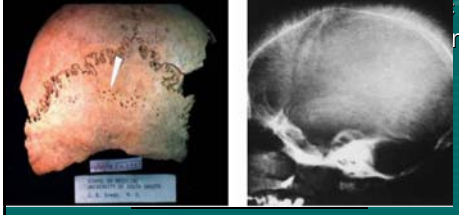
Name	Mutation	Mutation, HGVS nomenclature
Hb F-Ibeni Khirane	Agamma 22(B4) Asp>Asn	HBG1:c.67G>A
Hb F-Oman	Agamma 131(H9) Gln>His	HBG1:c.396G>T
Hb La Pommeraie	beta 133(H11) Val>Met	HBB:c.400G>A
Hb Alperton	beta 135(H13) Ala>Val	HBB:c.407C>T
Hb Gorée	alpha2 4(A2) Pro>Arg	HBA2:c.14C>G
Hb Meulan	alpha2 84(F5) Ser>Asn	HBA2:c.254G>A
Hb Jura	alpha2 114(GH2) Pro>Thr	HBA2:c.343C>A
Hb Kenton	alpha1 or alpha2 134(H17) Thr>Ser	HBA1:c.[404C>G (or HBA2) or 403A>T (or HBA2)]
Hb Les Lilas	alpha1 74(EF3) Asp>Val	HBA1:c.224A>T

http://globin.bx.psu.edu/hbvar/menu.html June 13, 2009

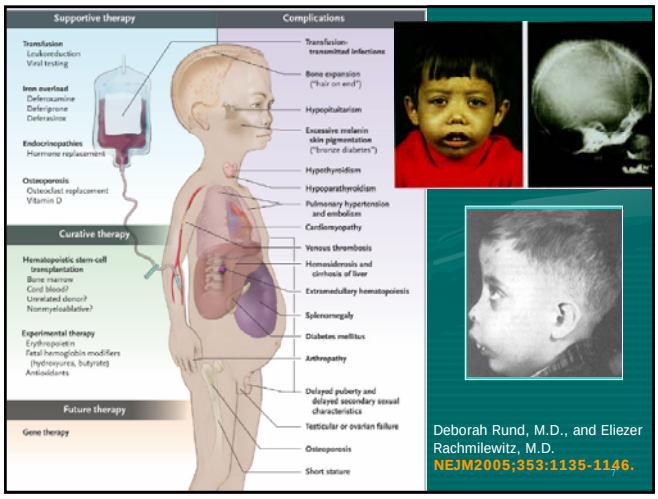
Thalassemia in Antiquity

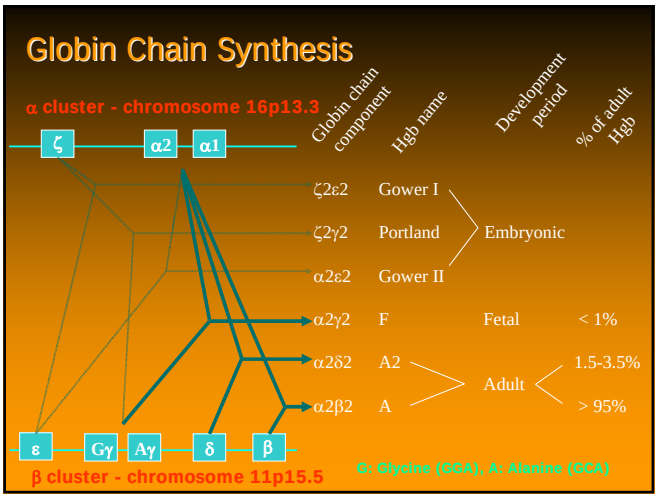


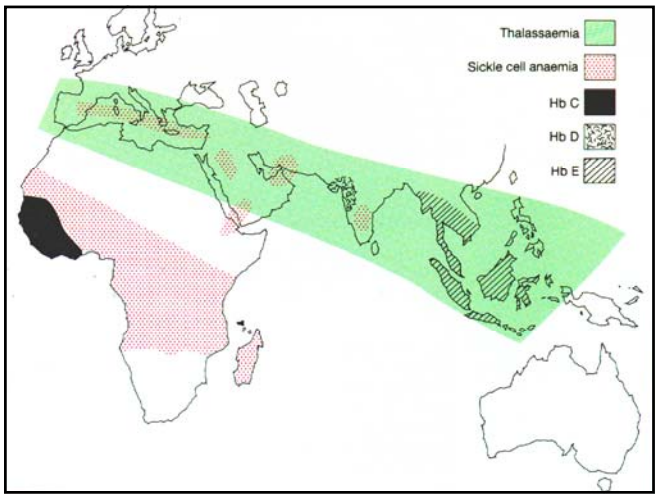
Choirokoitia(喬伊魯科蒂亞)
7000 BC: Over 150 graves, 47% of children. Died of disease of nd thalassemia.

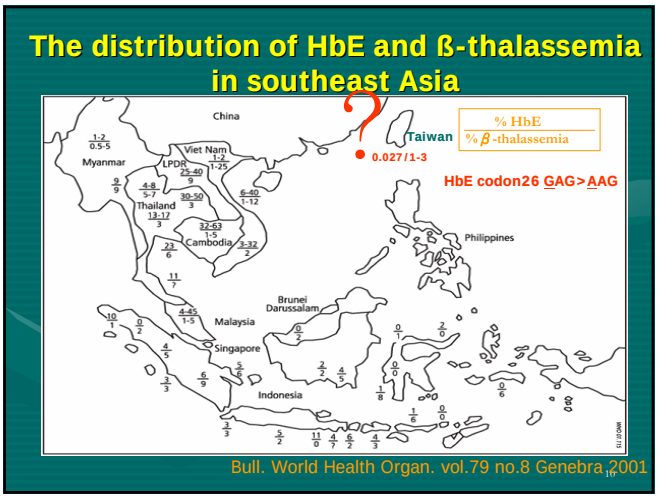


Porotic hyperostosis on parietal bone (UIOWA). Hair-on-end appearance (eMedicine).

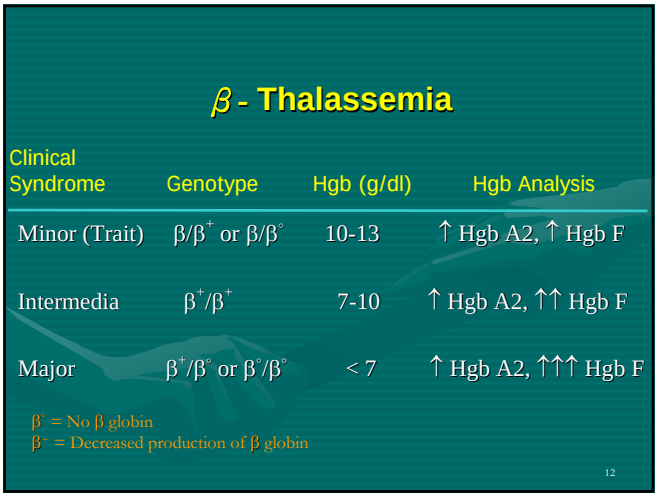

Supportive therapy
Complications
Curative therapy
Future therapy
Gene therapy
Deborah Rund, M.D., and Eliezer Rachmilewitz, M.D.
NEJM2005;353:1135-1146.


Globin Chain Synthesis
α cluster - chromosome 16p13.3
ζ
α2
α1
Globin chain component
Hgb name
Development period
% of adult Hgb
ζ2ε2 Gower I
ζ2γ2 Portland
Embryonic
α2ε2 Gower II
α2γ2 F
Fetal
< 1%
α2δ2 A2
1.5-3.5%
α2β2 A
Adult
> 95%
ε
Gγ
Aγ
δ
β
β cluster - chromosome 11p15.5
G: Glycine (GGA); A: Alanine (GCA)


Thalassaemia
Sickle cell anaemia
Hb C
Hb D
Hb E


The distribution of HbE and ß-thalassemia in southeast Asia
China
Taiwan
0.027 / 1-3
% HbE
% β -thalassemia
HbE codon26 GAG>AAG
Myanmar
LPDR
Viet Nam
Thailand
Cambodia
Philippines
Brunei Darussalam
Malaysia
Singapore
Indonesia
Bull. World Health Organ. vol.79 no.8 Genebra 2001


血紅素基因病變之相關疾病
血紅素基因病變
量的變化
質的變化
質+量的變化
海洋性貧血
變異血紅素
Thalassemic hemoglobinopathy
α -thalassemia
β -thalassemia
γ -thalassemia
δ -thalassemia
Stable
Unstable


β - Thalassemia
Clinical Syndrome
Genotype
Hgb (g/dl)
Hgb Analysis
Minor (Trait) β/β+ or β/β° 10-13 ↑ Hgb A2, ↑ Hgb F
Intermedia β+/β+ 7-10 ↑ Hgb A2, ↑↑ Hgb F
Major β+/β° or β°/β° < 7 ↑ Hgb A2, ↑↑↑ Hgb F
β° = No β globin
β+ = Decreased production of β globin
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Incidence of Hemoglobin Variants in Taiwan

- Hb CS≒ $\alpha^{3.7}$ deletion 0.5%
- Hb J-Meinung (J-Bangkok, Korat) 0.065%
- Hb G-Taichung 0.049%
- Hb E 0.027%
- Hb Kaohsiung (Hb New York) 0.022%
- Hb G-Taiwan-Ami 0.57% in Ami tribe

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Clinical Diagnosis of Hemoglobin Gene Mutation

	α - thalassemia	β - thalassemia	Hb variant
MCV	≦80 fl	≦80 fl	Normal
MCH	≦27 pg	≦27 pg	Normal
Hb EP	A2<3.5	A2≧3.5	α ≒25% β ≒50%
Ferritin	Normal	Normal	Normal

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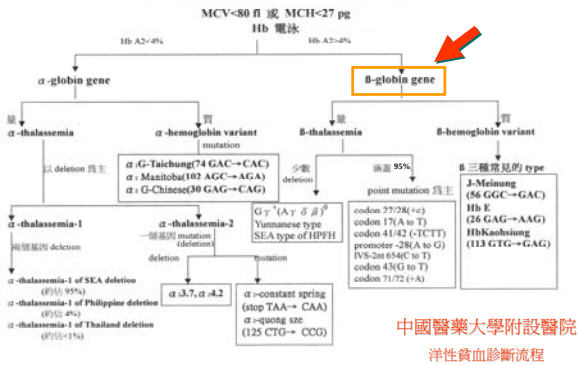
海洋性貧血基因檢驗流程圖



中國醫藥大學附設醫院
洋性貧血診斷流程

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Hemoglobin

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597254

Hb Hekinan in a Taiwanese Subject: A T Substitution at Codon 27 of the α1-Globin Gene Abolishes an HaeIII Site

Hung-Chang Shih [ab]; Mu-Chin Shih [a]; Yu-Chang Chang [a]; Ching-Tien Peng [ac]; Tien-Jye Chang [b]; Jan-Gowth Chang [d]
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[b] Department of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan
[c] Department of Biotechnology and Bioinformatics, Asia University, Taichung, Taiwan
[d] Department of Laboratory Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan

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To cite this Article: Shih, Hung-Chang, Shih, Mu-Chin, Chang, Yu-Chang, Peng, Ching-Tien, Chang, Tien-Jye and Chang, Jan-Gowth (2007) 'Hb Hekinan in a Taiwanese Subject: A T Substitution at Codon 27 of the α1-Globin Gene Abolishes an HaeIII Site', Hemoglobin, 31:4, 495 - 498
To link to this article: DOI: 10.1080/03630260701590368
URL: http://dx.doi.org/10.1080/03630260701590368

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- Hemoglobin (Hgb) Hekinan [alpha 27(B8); Glu--Asp] is a rare α-chain variant found mainly in Black, Japanese, and Thailand.

Occurrence Comment:
Found in a Japanese male; in a woman from French Guyana (of Chinese-Black descent), and in three Chinese families from Macao

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- CBC data was collected using a full automated blood cell counter (Sysmex XE-2100 combine with SP-1000i series; Sysmex Co., Chuo-Ku, Kobe, JAPAN).
- Hemoglobin (Hgb) analysis was used electrophoresis by automated HPLC (PRIMUS CLC385; Kansas city, Missouri, USA) as shown in Fig. 2A.

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CBC and Ferritin data

Date: 09/May/2006

Items / Name	RBCx10*3/ul M:4.5-5.5 F:4.0-4.5	Hgb gm/dl M:14.0-18.0 F:12.0-16.0	Hct % M:39-52 F:35-48	RDW 11.5-14.5	MCV fl 80-99	MCH pg 27-31	MCHC g/dl 33-37	Ferritin ng/ml M:17.9-464 F:*
楊X龍	5.73 ↑	12.8 ↓	39.0	15.2 ↑	68.1 ↓	22.3 ↓	32.8 ↓	218
楊XX秋	4.94 ↑	10.2 ↓	34.1 ↓	17.4 ↑	69.0 ↓	20.6 ↓	29.9 ↓	521 ↑
楊X穎	4.57	9.9 ↓	33.3 ↓	17.0 ↑	72.9 ↓	21.7 ↓	29.7 ↓	2.58 ↓

*:<50 yrs 6.24-137, >50 yrs 11.1-264

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楊X穎10 yr-old

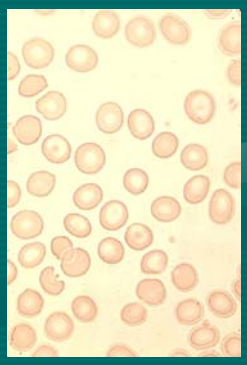
Peripheral smear

Note:

Hypochromia +

Microcytosis +

Anisocytosis +



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Automated HPLC (PRIMUS CLC385; Kansas city, Missouri, USA)

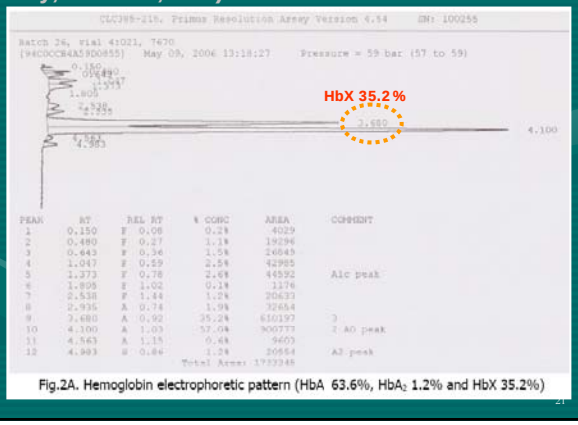

Fig.2A. Hemoglobin electrophoretic pattern (HbA 63.6%, HbA_2 1.2% and HbX 35.2%)

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High-Performance Liquid Chromatography, HPLC

Items / Name	HbA 96-98%	HbA2 1.5-3.5%	HbF <1.0%	Hb X –
楊X龍	63.5	1.2	0.1	35.2
楊XX秋	91.1	6.0	2.9	–
楊X穎	82.5	2.5	0.3	14.7

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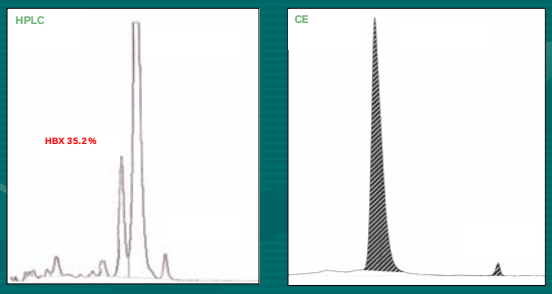
HPLC and Capillary Eletrophoresis



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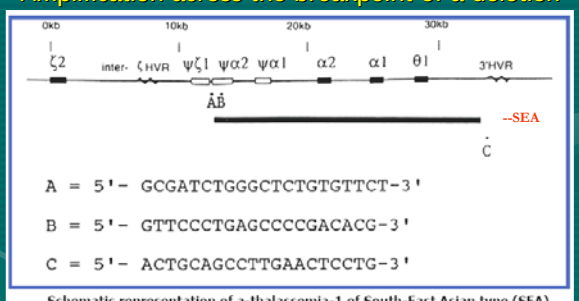
Amplification across the breakpoint of a deletion



Schematic representation of a-thalassemia-1 of South-East Asian type (SEA). The positions of primer A and B are used to amplify the normal area, A and C amplify the breakpoint.

Design by Chang Jan-Gowth, MD

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- Selective amplification of the α1-globin and α2-globin exon1 genes was performed using specific primers:
  P1F (5'-CTC TTC TGG TCC CCA CAG AC-3')
  P4R (5'-CAG GAC GGT TGA GGG TGG CCT-3')
- PCR products were analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems Co., Foster City, California, USA)

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Restriction enzyme

*Hae*III crystals

Enzyme	Sequence	Cut Site
HaeIII	GGCC	G G/C C C C/G G

Source:
A *E. coli* strain that carries the *Hae*III gene from *Haemophilus aegypticus* (ATCC 11116).

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PCR-RFLP

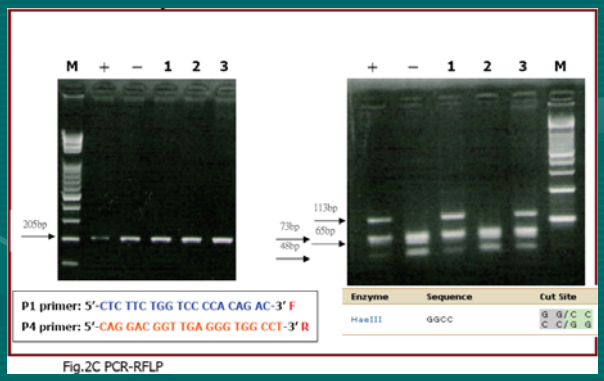

Fig.2C PCR-RFLP

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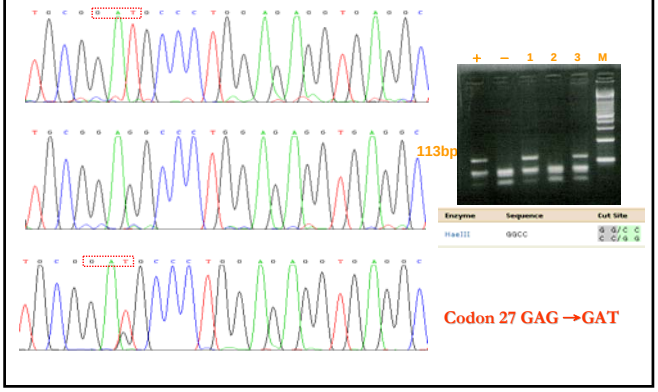
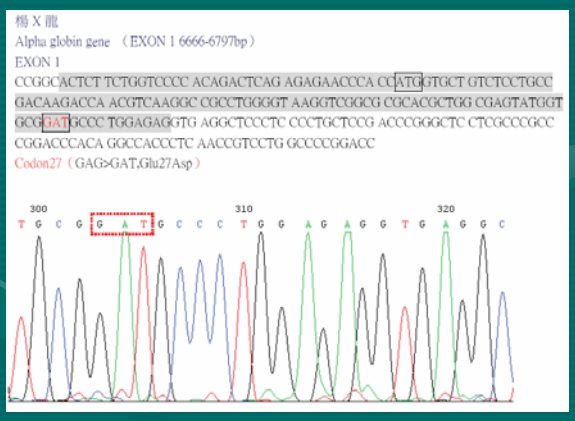
Molecular analysis of Hb Hekinan





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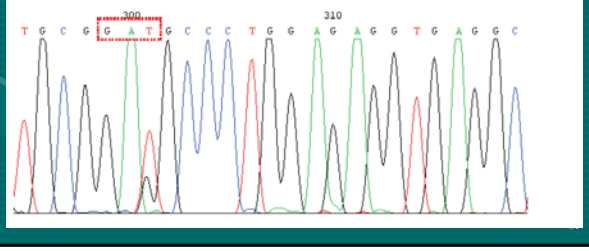
楊 X 新
Alpha globin gene （EXON 1 6666-6797bp）
EXON 1
CCGGCACTCT TCTGGTCCCC ACAGACTCAG AGAGAACCCA CCATGGTGCT GTCTCCTGCC
GACAAGACCA ACGTCAAGGC CGCCTGGGGT AAGGTCGGCG CGCACGCTGG CGAGTATGGT
GCGGATGCCC TGGAGAGGTG AGGCTCCCTC CCCTGCTCCG ACCCGGGCTC CTCGCCCGCC
CGGACCCACA GGCCACCCTC AACCGTCCTG GCCCCGGACC
Codon27（GAG>GAT,Glu27Asp）



Rapid molecular identification of β-thalassemia using high resolution melting analysis

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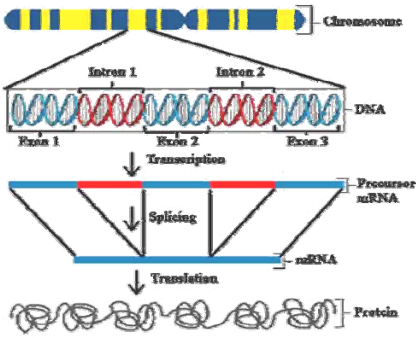
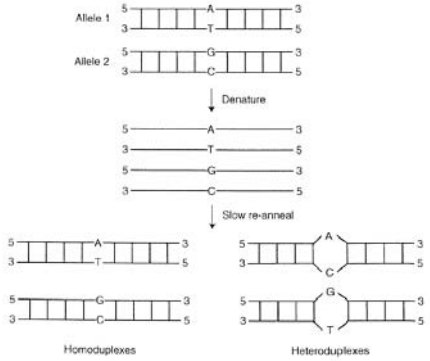
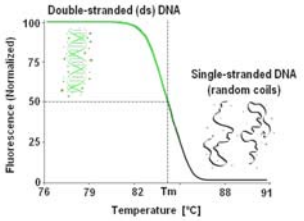
Central Dogma of Molecular Biology



Schematic representation of homo- and heteroduplexes of PCR amplicons



Expert Rev Mol Diagn. 3:811-8,2003



What's HRM?

- High-resolution melting analysis is a new method introduced in 2003
- HRM characterizes nucleic acid samples based on their disassociation (melting) behavior
- Samples can be discriminated according to their sequence, length, GC content or strand complementarity.
- Even single base changes such as SNPs (single nucleotide polymorphisms) can be readily identified.
- A closed-tube, post-amplification method done that uses simple and inexpensive generic dye chemistry.

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HRM Applications

- Mutation discovery (gene scanning)
- Screening for loss of heterozygosity
- DNA fingerprinting
- SNP genotyping
- Characterization of haplotype blocks
- DNA methylation analysis
- DNA mapping
- Species identification
- Somatic acquired mutation ratios
- HLA compatibility typing
- Association (case/control) studies
- Allelic prevalence in a population
- Identification of candidate predisposition genes

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Rotor-Gene™ 6000, corbettlifescience

LC480®, Roche

LightScanner, Idaho Technology

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HRM Workflow in the LC480®

- In a Gene Scanning experiment, sample DNA is first amplified via real-time PCR in the presence of a proprietary saturating DNA dye.
- A melting curve is then performed using high data acquisition rates, and data are finally analyzed using a Gene Scanning Software, by three basic steps

Normalization / Temperature shifting / Difference Plot

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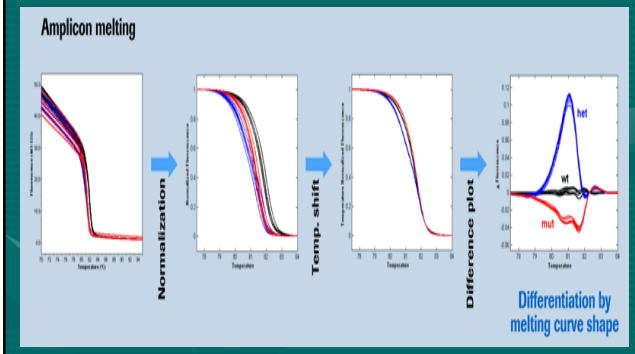

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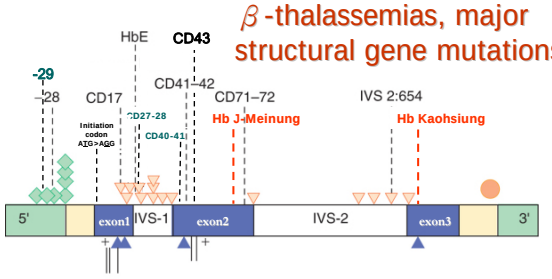
β-thalassemias, major structural gene mutations



IVS = intervening sequence

(From Stamatoyannopoulos G, Neinhuis AW, Leder P, Majerus PW: The Molecular Basis of Blood Disease. Philadelphia, WB Saunders, 1987.)

Primers use for HRM analysis of *HBB* gene mutations

Detection for	Sequence (5' to 3')	Length of PCR amplicon (bp)	Annealing Temp. (°C)
Promoter and Exon 1	P1 5'-CCAATCTACTCCCAGGAGCA-3' (forward) P2 5'-GGCAGAGAGAGTCAGTGCCTA-3' (reverse)	323	52
*Promoter and Initiation codon	P3 5'-ACTTCTCCTCAGGAGTCAGGT-3' (reverse)	154	56
*Exon 1	P4 5'-AGACACCATGGTGCACCTGAC-3' (forward)	204	56
Exon 2	P5 5'-GAAGACTCTTGGGTTTCTGA-3' (forward) P65'-TCATTCGTCTGTTTCCCATTCTAAAC-3' (reverse)	404	52
*Exon 2	P7 5'-GAGCCTTCACCTTAGGGTTT-3' (reverse)	164	56
*Exon 2	P8 5'-CTCCTGATGCTGTTATGGGC-3' (forward) P9 5'-AGAAAACATCAAGGGTCCCA-3' (reverse)	193	56
Intron 2	P105'-GTGTACACATATTGACCAAATCAGGGTA-3' (forward) P11 5'-GGTAGCTGGATTGTAGCTGC-3' (reverse)	293	56
*Intron 2	P12 5'-ATTTATATGCAGAAATATTG-3' (reverse)	223	52
Exon 3	P13 5'-CTGGATTATTCTGAGTCCAAGC-3' (forward) P14 5'-ATTAGGCAGAATCCAGATGCTC-3' (reverse)	309	52

*Block single nucleotide polymorphisms (SNPs)

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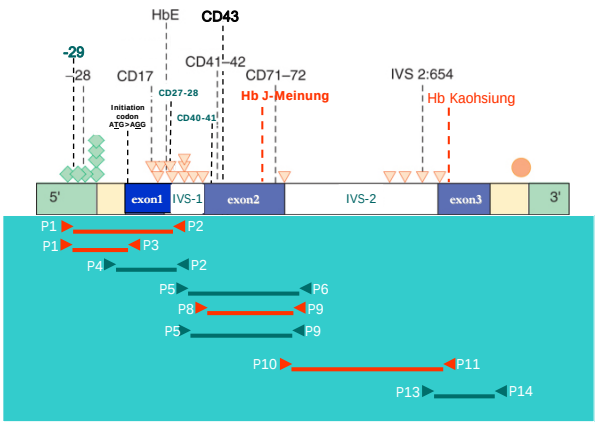

Screening of the *HBB* genes mutations: promoter and exon 1 regions

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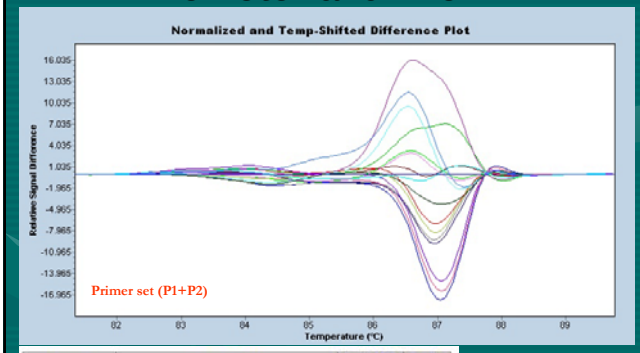
Promoter and Exon 1



Detection for	Sequence (5' to 3')	Length of PCR amplicon (bp)	Annealing Temp. (°C)
Promoter and Exon 1	P1 5'-CCAATCTACTCCCAGGAGCA-3' (forward) P2 5'-GGCAGAGAGAGTCAGTGCCTA-3' (reverse)	323	52

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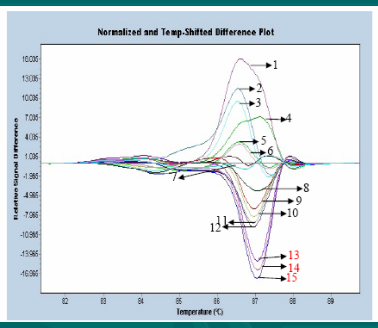


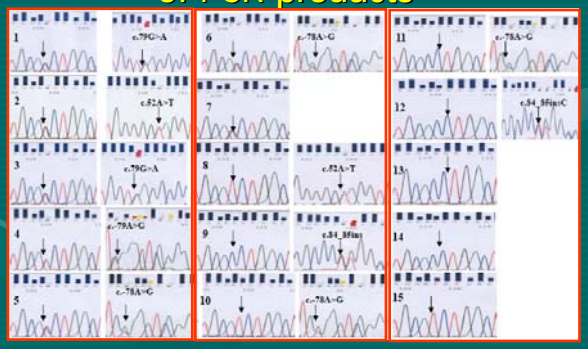
1. c.79G>A+ c.9C>T (heterozygous)
2. c.52A>T+ c.9C>T (heterozygous)
3. c.79G>A+ c.9C>T (heterozygous)
4. c.-79A>G+ c.9C>T (heterozygous)
5. c.-78A>G+ c.9C>T (heterozygous)
6. c.-78A>G+ c.9C>T (heterozygous)
7. c.9C>T (heterozygous)
8. c.52A>T+ c.9C>T (homozygous) (T/T)
9. c.84_85insC+ c.9C>T (homozygous) (C/C)
10. c.-78A>G+ c.9C>T (homozygous) (T/T)
11. c.-78A>G+ c.9C>T (homozygous) (C/C)
12. c.84_85insC+ c.9C>T (homozygous) (C/C)
13. c.9C>T (homozygous) (C/C)
14. c.9C>T (homozygous) (C/C)
15. c.9C>T(homozygous) (T/T)

Screening of the *HBB* genes mutations in promoter and exon 1. We are unable to distinguish the *HBB* mutations in promoter and exon 1 from the melting curve due to the interference of 1 SNP.

c.9C>T

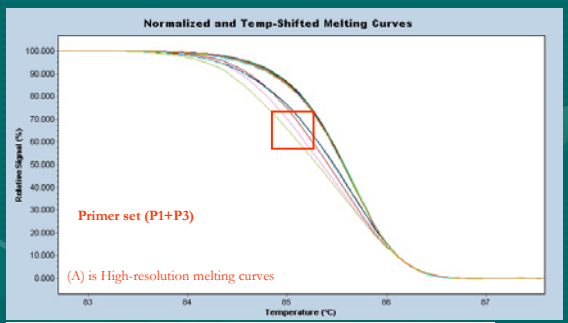
45

Confirmed by direct sequencing of PCR products



46

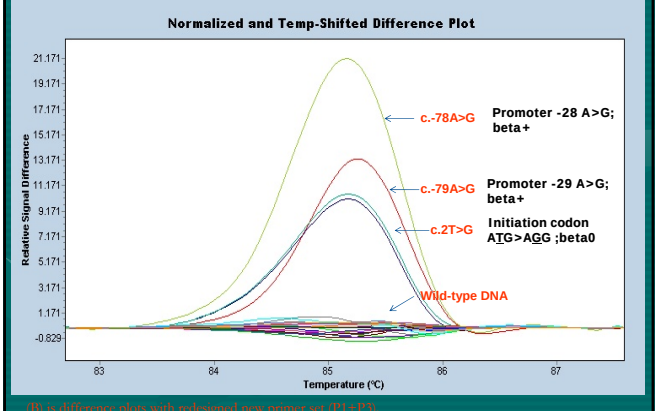
Promoter and Initiation codon



(A) is High-resolution melting curves

Detection for	Sequence (5' to 3')	Length of PCR amplicon (bp)	Annealing Temp. (°C)
Promoter and Exon 1	P1 5'-CCAATCTACTCCCAGGAGCA-3' (forward) P2 5'-GGCAGAGAGAGTCAGTGCCTA-3' (reverse)	323	52
Promoter and Initiation codon	P3 5'-ACTTCTCCTCAGGAGTCAGGT-3' (reverse)	154	56

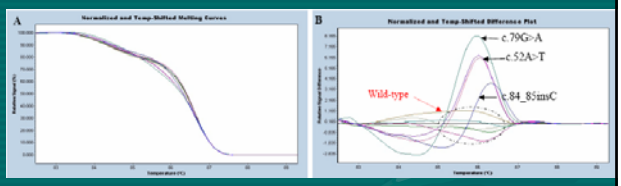
47



(B) is difference plots with redesigned new primer set (P1+P3).

48

Screening of the *HBB* genes mutations in exon 1

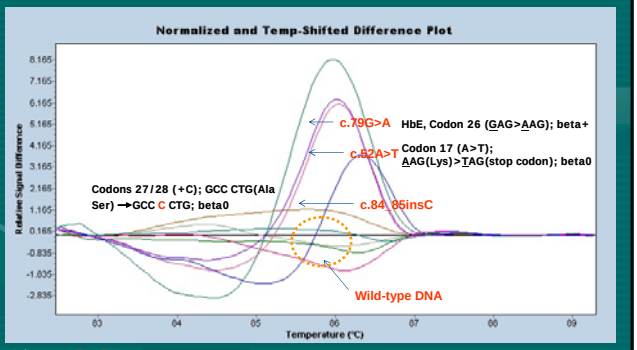


(A) is High-resolution melting curves and (B) is difference plots with redesigned new primer set (P4+P2). The c.79G>A, c.52A>T and c.84_85insC are easily to be distinguished in the normalized and temp-shifted difference plot.

Detection for	Sequence (5' to 3')	Length of PCR amplicon (bp)	Annealing Temp. (℃)
Promoter and Exon 1	P1 5'-CCAATCTACTCCCAGGAGCA-3' (forward) P2 5'-GGCAGAGAGAGTCAGTGCCTA-3' (reverse)	323	52
Promoter and Initiation codon	P3 5'-ACTTCTCCTCAGGAGTCAGGT-3' (reverse)	154	56
Exon 1	P4 5'-AGACACCATGGTGCACCTGAC-3' (forward)	204	56

49

Exon 1

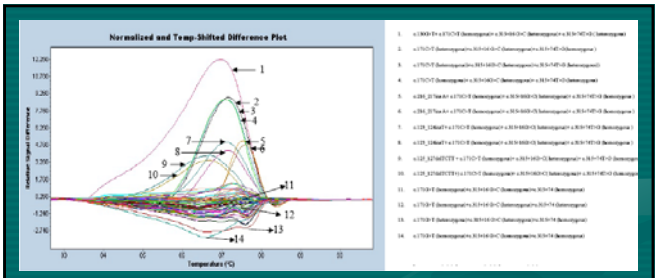


Primer set (P4+P2)

50

Screening of the *HBB* genes mutations: exon 2 region

51

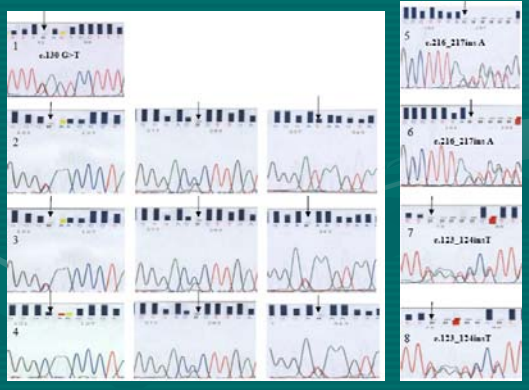


We are unable to distinguish the *HBB* gene mutations from the complicated melting curve due to the interference of 3 SNPs.

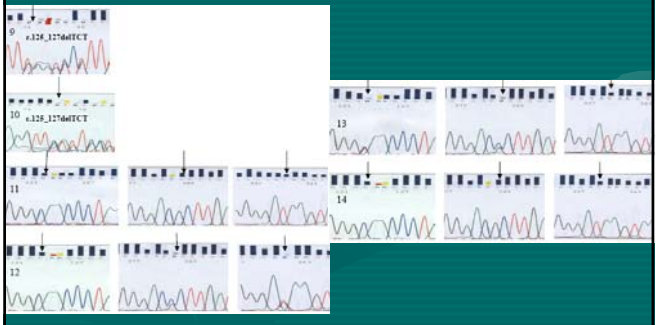
(c.171C>G, c.315+16G>C and c.315+74T>G)

Exon 2	P5 5'-GAAGACTCTTGGGTTTCTGA-3'(forward) P65'-TCATTCGTCTGTTTCCCATTCTAAAC-3' (reverse)	404	52

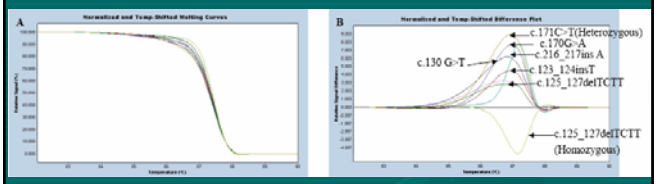
52



53



54



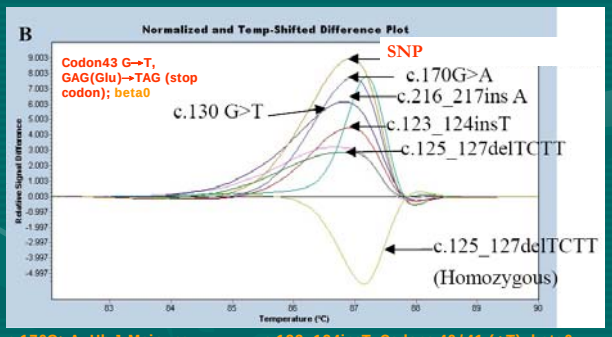
we redesigned a new primer set (P5+P9) which overlaid the 2 SNPs in order to block the SNPs interference.

(c.315+16G>C and c.315+74T>G)

Exon 2	P5 5'-GAAGACTCTTGGGTTTCTGA-3'(forward) P65'-TCATTCGTCTGTTTCCCATTCTAAAC-3' (reverse)	404	52
*Exon 2	P7 5'-GAGCCTTCACCTTAGGGTTT-3' (reverse)	164	56
*Exon 2	P8 5'-CTCCTGATGCTGTTATGGGC-3' (forward) P9 5'-AGAAAACATCAAGGGTCCCA-3' (reverse)	193	56

55

Exon 2

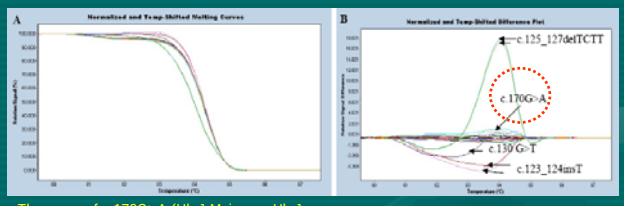


c.170G>A: Hb J-Meinung **c.123_124insT: Codons 40/41 (+T); beta0**

c.216_217ins A: Codon 71/72 +A; beta0

c.125_127delTCTT: Codon 41/42 –TCTT; beta0

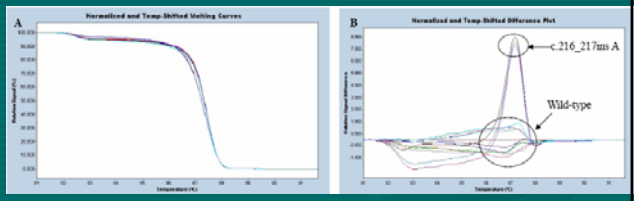
56



The curve of c.170G>A (Hb J-Meinung, Hb J-Bangkok, Korat) shift to the group of wild type DNA by using the primer sets (P5 +P7)

Exon 2	P5 5'-GAAGACTCTTGGGTTTCTGA-3'(forward) P65'-TCATTCGTCTGTTTCCCATTCTAAAC-3' (reverse)	404	52
*Exon 2	P7 5'-GAGCCTTCACCTTAGGGTTT-3' (reverse)	164	56

57



(A) is High-resolution melting curves and (B) is difference plots with redesigned new primer set (P8+P9). The c.216_217insA is easily to be distinguished in the normalized and temp-shifted difference plot.

*Exon 2	P8 5'-CTCCTGATGCTGTTATGGGC-3' (forward) P9 5'-AGAAAACATCAAGGGTCCCA-3' (reverse)	193	56

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Screening of the *HBB* genes mutations: intron 2

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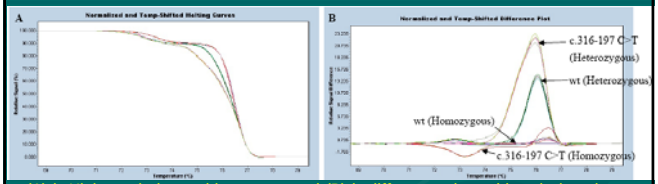


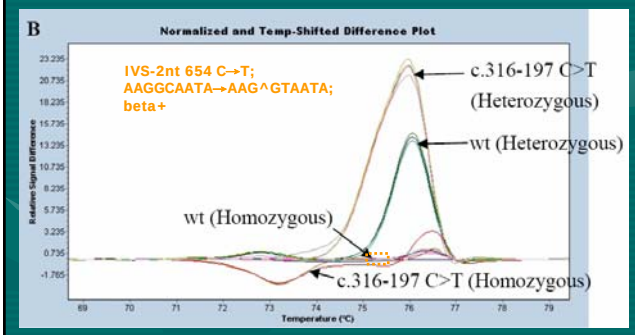
(A) is High-resolution melting curves and (B) is difference plots with redesigned new primer set (P10+P11). The c.316-197 C>T (heterozygous), wild-type (heterozygous), wild-type (homozygous) and c.316-197 C>T (homozygous) are easily to be distinguished in the normalized and temp-shifted difference plot.

wt: c.316-185C>T

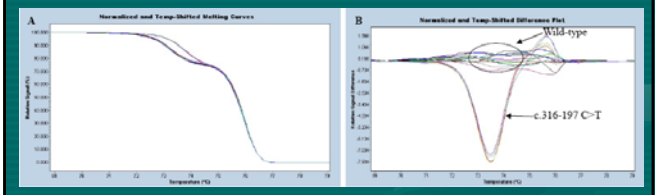
Intron 2	P105'-GTGTACACATATTGACCAAATCAGGGTA-3' (forward) P11 5'-GGTAGCTGGATTGTAGCTGC-3' (reverse)	293	56

60

Intron 2



61



(A) is High-resolution melting curves and (B) is difference plots with redesigned new primer set **(P10+P12)** for blocking the SNP. The c.316-197 C>T is easily to be distinguished in the normalized and temp-shifted difference plot.

Intron 2	P10 5'-GTGTACACATATTGACCAAATCAGGGTA-3' (forward) P11 5'-GGTAGCTGGATTGTAGCTGC-3' (reverse)	293	56
Intron 2	P12 5'-ATTTATATGCAGAAATATTG-3' (reverse)	223	52

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Screening of the c.341T>A (Hb Kaohsiung or New York): Exon 3

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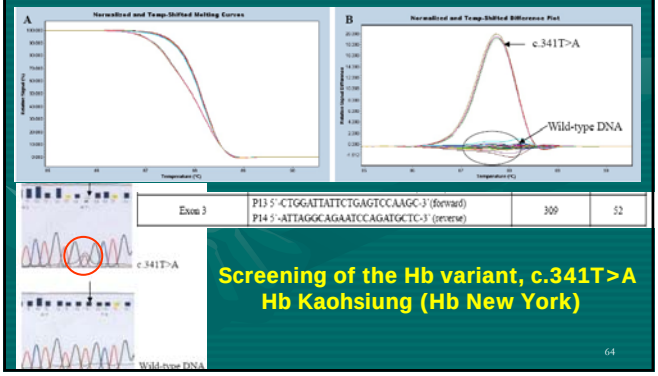
Exon 3



Exon 3	P13 5'-CTGGATTATTCTGAGTCCAAGC-3'(forward) P14 5'-ATTAGGCAGAATCCAGATGCTC-3' (reverse)	309	52

Screening of the Hb variant, c.341T>A Hb Kaohsiung (Hb New York)

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Panel	Sequence
Panel A	5'-CCAATCTACTCCCAGGAGCA-3' (Forward)--P1 5'-ACTTCTCCTCAGGAGTCAGGT-3' (Reverse)--P3
Panel B	5'-AGACACCATGGTGCACCTGAC-3' (Forward)--P4 5'-GGCAGAGAGAGTCAGTGCCTA-3' (Reverse)--P2
Panel C	5'-GAAGACTCTTGGGTTTCTGA-3'(Forward)--P5 5'-AGAAAACATCAAGGGTCCCA-3' (Reverse)--P9
Panel D	5'-GTGTACACATATTGACCAAATCAGGGTA-3'(Forward)--P10 5'-GGTAGCTGGATTGTAGCTGC-3' (Reverse)--P11

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Discussion

- HRM analysis offers several benefits including lowering manpower, time-saving, and decreasing the risk of PCR carryover contamination.
- The HRM analysis is the most cost-effective in diagnostic laboratories with moderate to high patient sample volumes. This is because up to 96 or 384 DNA samples can be analyzed within 2 h by a single medical technologist (including data interpretation).
- Our results suggest that HRM is a feasible and highly accurate method for the screening and identification of β-thalassemia, therefore, it could replace the currently methods applied in the screening of β-thalassemia and prenatal diagnosis

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Thanks for your attention!