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Abstract: Non-deletional alpha plus thalassaemia is associated with a higher degree of morbidity and mortality than deletional forms of alpha plus thalassaemia. Screening for the common deletional forms of alpha-thalassaemia by Gap-PCR is widely practiced; however the detection of non-deletional alpha thalassaemia mutations is technically more labour intensive and expensive, as it requires DNA sequencing. In addition, the presence of four very closely homologous alpha globin genes and the frequent co-existence of deletional forms of alpha-thalassaemia present another layer of complexity in the detection of these mutations. With growing evidence that non-deletional alpha-thalassaemia is relatively common in the UK there is a demand for technologies which can quickly and accurately screen for these mutations. We describe a method utilising pyrosequencing for detecting the ten most common clinically significant non-deletional alpha-thalassaemia mutations in the UK. We tested 105 patients with non-deletional alpha-thalassaemia and found 100% concordance with known genotype as identified by Sanger sequencing. We found pyrosequencing to be simpler, more robust, quicker and cheaper than conventional sequencing, making it a good choice for rapid and cost effective diagnosis of patients with suspected non-deletional alpha-thalassaemia. The technique is also likely to help expedite prenatal diagnosis of pregnancies at risk of alpha-thalassaemia major.

***Conflict of interest**

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Title: -Screening for clinically significant non-deletional alpha thalassaemia mutations by pyrosequencing

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Key words: alpha thalassaemia, pyrosequencing, haemoglobinopathy screening, prenatal diagnosis

Abstract

Non-deletional α^+ -thalassaemia is associated with a higher degree of morbidity and mortality than deletional forms of α^+ -thalassaemia. Screening for the common deletional forms of α -thalassaemia by Gap-PCR is widely practiced; however the detection of non-deletional α -thalassaemia mutations is technically more labour intensive and expensive, as it requires DNA sequencing. In addition, the presence of four very closely homologous alpha globin genes and the frequent co-existence of deletional forms of α -thalassaemia present another layer of complexity in the detection of these mutations. With growing evidence that non-deletional α -thalassaemia is relatively common in the UK there is a demand for technologies which can quickly and accurately screen for these mutations. We describe a method utilising pyrosequencing for detecting the ten most common clinically significant non-deletional α -thalassaemia mutations in the UK. We tested 105 patients with non-deletional α -thalassaemia and found 100% concordance with known genotype as identified by Sanger sequencing. We found pyrosequencing to be simpler, more robust, quicker and cheaper than conventional sequencing, making it a good choice for rapid and cost effective diagnosis of patients with suspected non-deletional α -thalassaemia. The technique is also likely to help expedite prenatal diagnosis of pregnancies at risk of α -thalassaemia major.

Introduction

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4 α -Thalassaemia is the most common monogenic disorder in the world [1, 2]. It results from
5
6 reduced or absent synthesis of alpha-globin from one or both of the two alpha-globin genes
7
8 on chromosome 16. It is a heterogeneous condition ranging from asymptomatic carrier states
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10 to the lethal condition of Hb Bart's hydrops fetalis which results when an individual is
11
12 homozygous for a deletion which removes both alpha globin genes. Haemoglobin H disease
13
14 is the most clinically severe form of α -thalassaemia compatible with life, characterised by
15
16 reduced synthesis of α -globin from three of the four α -globin genes. This results in the
17
18 accumulation of β -globin tetramers (Hb H) which are relatively unstable, causing haemolytic
19
20 anaemia, Hb H also has a high oxygen affinity which results in tissue hypoxia. The
21
22 pathophysiology of Hb H disease is complex and can vary widely, even in patients with
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24 identical α -globin genotypes [3-5]. Most cases of Hb H disease are caused by α -globin gene
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26 deletions [6], however patients with non-deletional Hb H disease usually have more severe
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28 phenotypes and are more likely to require transfusions, some mutations can even result in the
29
30 lethal form of Hb H disease known as Hb H hydrops fetalis [3]. Mutations in the $\alpha 2$ globin
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32 gene are usually more severe than mutations in the $\alpha 1$ -globin gene as more globin is produced
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34 from $\alpha 2$ gene than the $\alpha 1$ gene [7].
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45 In the UK it is becoming evident that non-deletional α -thalassaemia mutations are found in a
46
47 wider spectrum of the population than previously thought, with the discovery of 30 different
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49 mutations out of the known published worldwide total of 67 non-deletion mutations (46%)
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51 [8]. This gives the UK the widest range of α -thalassaemia mutations of any country with a
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53 published spectrum of mutations. Couples at risk of having a fetus affected by Hb H
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55 Hydrops Fetalis and requesting prenatal diagnosis have also been identified in the UK [9].
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59 There are ten non-deletional α -thalassaemia mutations that are clinically significant and
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1 relatively common within the UK population [8]. These mutations consist of the $\alpha 2$
2 termination codon mutations (Hb Constant Spring, Hb Paksé, Hb Icaria, and Hb Seal Rock),
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4 the $\alpha 2$ polyadenylation signal mutations (Saudi, Turkish and Indian Mutations), and the
5
6 mutations which give rise to the hyper unstable alpha globin chain variants Sun Prairie,
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9 Quong Sze and Adana (table 1).
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14 There is therefore a need for a quick and inexpensive method to detect these mutations. Also,
15
16 the inception of the UK National Sickle Cell and Thalassaemia Screening Program in 2001
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18 [10, 11] has driven the development of faster and more accurate technologies. Unlike the
19
20 common deletional α -thalassaemia mutations which can be identified by simple and
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22 inexpensive techniques such as Gap-PCR, non-deletional mutations usually require
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24 identification by DNA sequencing which is more expensive and labour intensive.
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32 Pyrosequencing is a real-time sequencing technique based on the detection of light [12, 13]. It
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34 is rapid and inexpensive and has been used for the detection of a number of genetic disorders,
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36 including the assessment of disease burden in myeloproliferative disorders [14-16]. These
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38 reports have demonstrated the flexibility and accuracy of the technique; however these
39
40 reports have focused on single copy genes. The α -globin genes can be challenging to study
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42 as the two genes are highly homologous and reside in a GC rich cluster with other highly
43
44 homologous genes making specific gene amplification problematic. An added difficulty is the
45
46 extremely high population frequency of the deletional forms of α -thalassaemia [17]. This
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48 means that it is not unusual for individuals to have co-existing deletional and non-deletional
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51 α -thalassaemia mutations which can make data interpretation complex. Against these
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54 technical difficulties, we will investigate whether pyrosequencing can be used as a rapid
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1 screening method to accurately distinguish the 10 most common clinically significant
2 mutations in the $\alpha 1$ and $\alpha 2$ globin genes
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9 **Materials and Method**

10 Genomic DNA samples from 105 patients who had previously been identified as having non-
11 deletion α^+ -thalassaemia (by Sanger sequencing) were used in the study. Co-existing
12 deletional α -thalassaemia mutations were detected by Gap-PCR [18] or MLPA [19]. DNA
13 was extracted from 1ml of whole blood in EDTA using Chemagic Magnetic Separation
14 Module 1 or by standard phenol-chloroform procedures. Three pyrosequencing assays were
15 designed to screen for the ten most common clinically significant non-deletional mutations
16 within the $\alpha 2$ - and $\alpha 1$ -globin genes. Amplification of the polyadenylation and termination
17 codon mutation site was performed using a PCR primer pair specific for the $\alpha 2$ gene. Two
18 separate sequencing primers were then used in the pyrosequencing reaction (one to identify
19 the polyadenylation mutations and the other to identify the termination codon mutations).
20 The mutations for the haemoglobin variants Quong Sze and Sun Prairie in the $\alpha 2$ gene were
21 amplified using a 2nd set of primers and identified by the same sequencing primer as the
22 mutations are only 16bp apart. The sequencing primer was anti-sense and located one base
23 pair downstream from the mutation site for Hb Sun Prairie. Dispensation of 17 nucleotides
24 was programmed, covering 25 base pairs. The haemoglobin Adana mutation in exon 2 was
25 amplified in a third PCR reaction. Due to the sequence homology of the α -globin genes and
26 fragment size limitations of pyrosequencing, amplification primers could not be designed
27 which were specific to the $\alpha 1$ or the $\alpha 2$ gene. Pyrosequencing for Hb Adana would therefore
28 detect mutations on both the $\alpha 2$ and $\alpha 1$ genes. Sequencing was performed in a reverse (anti-
29 sense) direction. Primer sequences for all reactions are provided in table 2.
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2 Each 25µl PCR reaction contained 2x Qiagen Multiplex Mastermix, 10x Q-Solution and
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4 10µM each primer. All PCR conditions consisted of an initial hotstart denaturation at 94°C
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6 for 15 minutes, 40 cycles of 94°C for 30 seconds, 65°C for 1½ minute, 72 for 1½ minute; and
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8 final extension at 72°C for 10 minutes. Prior to pyrosequencing, 5µl of PCR product
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10 underwent electrophoresis on an agarose gel to confirm successful amplification. The PCR
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12 products (20µl) were then sequenced by Pyromark Q24 System (Qiagen) following
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14 manufacturers instructions. Briefly, a bead mix containing 2µl of Streptavidin Sepharose
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16 beads (Amersham Biosciences), 18µl of molecular grade water (Sigma) and 40µl of Binding
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18 Buffer (Qiagen) was mixed with the PCR product for 5 minutes on a plate shaker in order to
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20 retain the biotinylated PCR product onto the sepharose beads. The immobilized PCR
21
22 products were captured onto the filter probes on the Prep Tool by applying a vacuum, and
23
24 washed in 70% ethanol for 5 seconds, Denaturation Solution (Qiagen) for 5 seconds and 1x
25
26 Wash Buffer (Qiagen) for 10 seconds. The vacuum was then switched off and the single
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28 stranded PCR products released onto a Pyromark Q24 Plate (Qiagen) containing 25µl
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30 Annealing Buffer (Qiagen) and 0.8µl of sequencing primer (10µM). The sequencing plate
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32 was heated for 2 minutes at 80°C and allowed to cool at room temperature for 5 minutes to
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34 allow annealing of the sequencing primer. Sequencing was performed using programmed
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36 dispensation of nucleotides. The nucleotide dispensation order was designed to give the
37
38 clearest and most unambiguous sequencing pattern. The pyrogram sequence pattern was
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40 interpreted by PyroMark Q24 software (Qiagen).
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Results

Identification of polyadenylation and termination codon mutations

Pyrosequencing was 100% concordant with expected genotype as confirmed by Sanger sequencing, unequivocally identifying all heterozygotes, hemizygotes and homozygotes. (table 3, figure 1). Pyrosequencing is semi-quantitative as the detection of light is equal in molarity to the number of incorporated nucleotides; it therefore offers the advantage of providing allelic ratios at the target site of interest. Theoretically the ratio of wild type to mutant allele detected in heterozygotes should be 1:1, however we found that the ratios detected varied considerably depending on the mutation and this was particularly evident with polyadenylation signal mutations. This is probably because the polyadenylation signal has a homopolymeric run of three A nucleotides (ATAAA). The light emission from a run of the same nucleotides is non-linear which means the resulting allelic ratio will be skewed. However the software is mostly able to compensate to for this, as peak heights are still largely proportional to the number of incorporated nucleotides, allowing unequivocal determination of the sequence. This effect was also evident in the identification of heterozygotes for the termination codon mutation Hb Icaria, which was more difficult to resolve as the mutation also forms part of a homopolymeric repeat. However, the reduction in the normal allele and visual comparison of the pyrogram against normal patient samples made manual interpretation of the pyrogram unambiguous. Another factor was that the supplied Pyromark software was unable to score the Indian polyadenylation signal 2bp deletion mutation (ATA--), this is because the software cannot calculate the out-of-phase signal generated by a deletion. In spite of this technical limitation, visual examination of the pyrogram clearly showed that peak heights are still largely proportional to the number of incorporated nucleotides, allowing for unequivocal identification of the deletion when compared to wild type samples.

Identification of unstable alpha haemoglobin variant mutations

Pyrosequencing results were 100% concordant with conventional sequencing results (table 3). The allelic ratio for Hb Sun Prairie heterozygotes were marginally closer to the expected ratio of 1:1 than those obtained for Hb Quong Sze. This variation may be because of the longer read lengths employed in this assay. The read length was longer (25bp) to allow the Hb Sun Prairie and Hb Quong Sze mutations to be identified in the same sequencing reaction. However it is known that due to a reduction in enzyme efficiency and build up of enzyme inhibitors the quality of the sequence in a pyrosequencing reaction diminishes with increasing length [20]. This probably explains why the allelic ratio observed in Quong Sze heterozygotes (which is downstream of the Sun Prairie mutation) is more variable than that observed in Sun Prairie heterozygotes. However the quality of the sequencing at the Quong Sze mutation site was good enough to allow the mutation to be easily identified in all samples.

Due to technical constraints (see methods) the amplification primers designed to detect the Hb Adana mutation amplify both the $\alpha 1$ and $\alpha 2$ genes (figure 2). This means that unlike the other pyrosequencing reactions which only amplify the $\alpha 2$ gene the expected allelic ratio between the mutant and wild type allele is 1:3 for heterozygotes, 1:1 for homozygote's and 1:1 or 1:2 for hemizygotes (depending on whether it is a 1 gene or 2 gene α -globin gene deletion on the other allele). Four of the samples that were tested for Hb Adana were amniotic fluid samples that had been referred for prenatal diagnosis from 2 separate couples at risk of a pregnancy affected with Hb H Hydrops Fetalis. Both mothers had α^0 -thalassaemia trait ($-\text{FIL}/\alpha\alpha$) whilst both fathers were carriers of Hb Adana. Pyrosequencing

1 confirmed that all fetuses were compound heterozygous for both mutations and thus affected
2 with Hb H Hydrops Fetalis.
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6 7 *Mutation detection by allelic ratios*

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9 The quantitative information providing by pyrosequencing unexpectedly resulted in the
10 identification of a further mutation that would not have been detected by conventional
11 sequencing. Two samples which were correctly identified as carriers of the Indian
12 polyadenylation signal mutation gave an average ratio of wild type: mutant allele that was
13 closer to 2:1, rather than the ratio expected in a heterozygote of 1:1. These samples were from
14 a father and daughter with normal red cell indices. Gap PCR analysis revealed that both also
15 carried a triplicated α -globin gene on one allele and therefore had a total of five copies of the
16 alpha globin gene, with 4 genes functioning normally. This explains the observed normal red
17 cell indices and the greater than expected ratio of wild type to mutant allele observed on
18 pyrosequencing.
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36 **Discussion**

37 Growing evidence that clinically significant non-deletional α -thalassaemia mutations are
38 present in UK population at significant frequencies has driven the need to develop
39 technologies, which can quickly screen for the most common mutations. Here we have
40 described the diagnostic utility of pyrosequencing for the rapid detection of ten of the most
41 common non-deletional α -thalassaemia mutations. All mutations selected for screening are
42 clinically important and can result in haemolytic anaemia, Hb H disease or even Hb H
43 Hydrops Fetalis when homozygous or compound heterozygous with an α^0 -thalassaemia
44 thalassaemia deletion mutation. However detection of these mutations can be difficult as the
45 α -globin genes reside in GC rich cluster with high sequence homology, they are also often
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1 co-inherited with deletional forms of α -thalassaemia which can further complicate diagnosis.
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3 Pyrosequencing proved to be very robust, and was able to detect all 10 mutations in all
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5 possible allelic combinations (heterozygotes, homozygous and hemizygous), giving a specific
6
7 easily interpretable sequencing pattern. As the mutation of interest is detected in the context
8
9 of its surrounding sequencing, pyrosequencing offers a higher degree of accuracy and
10
11 therefore potentially has fewer pitfalls than gel-based detection methods. Pyrosequencing
12
13 also offers the flexibility of positioning the sequencing primer up to 10 base pairs away from
14
15 the mutation site. This is an advantage when sequencing difficult templates with a high GC
16
17 content or secondary loop structures (such as the α -globin gene cluster) and is probably the
18
19 reason that in our hands the method proved more robust than Sanger sequencing. In addition
20
21 we found the Pyromark Q24 software a useful tool in the rapid analysis of simple mutations,
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23 as it assigns an allelic score relative to the normal allele. Allelic quantification represents a
24
25 distinct advantage of pyrosequencing over conventional Sanger-dideoxy sequencing, which is
26
27 relatively insensitive and cannot reliably detect mutations when the proportion of mutant to
28
29 wild type allele is below 50%. Pyrosequencing however has been shown to detect alleles with
30
31 a frequency as low as 5% [14]. This feature allowed us to distinguish patients carrying an
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33 extra copy of the α -globin gene. The Pyromark software has not been designed to detect
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35 deletions; as a consequence, samples with the 2bp Indian polyadenylation signal deletion
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37 failed quality assessment, however visual comparison of the pyrograms with known wild type
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39 controls clearly shows a reduction in peak height of the wild type nucleotide which allows the
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41 mutation to be easily identified. It is therefore very important to include controls to overcome
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43 any difficulties in interpretation.
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56 Overall we felt that pyrosequencing offers a low cost, rapid method for screening for non-
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58 deletion α -thalassaemia mutations. The technique was robust and reliably identified non-
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1 deletion alpha thalassaemia even in the presence of co-existing α -globin gene deletions and
2 duplications, a situation which is very common. We also found the method more reliable than
3 Sanger sequencing and good results were obtained even in samples with low or poor quality
4 DNA which had given sub-optimal results on conventional sequencing. Pyrosequencing is
5 rapid in comparison to Sanger sequencing as there are fewer steps which means that results
6 can be obtained in half a day. This reliability and speed means that pyrosequencing would be
7 good method for robust prenatal diagnosis, especially as fetal samples can be of low DNA
8 concentration and fast turnaround times are required. The relatively low cost of the
9 consumables and instrumentation in comparison to conventional sequencing may also make
10 the method applicable in under-developed countries where haemoglobinopathies are
11 common.
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Table 1 – Ten most common clinically significant non-deletion α^+ -thalassaemia mutations in the UK population

Type	Mutation	Clinical Significance
$\alpha 2$ Poly A signal	Indian AATAAA→AATA--	HbH disease in combination with α^0 -thalassaemia or possible HbH Hydrops Fetalis in homozygous state [21]
	Turkish AATAAA→AATGAA	HbH disease in combination with α^0 -thalassaemia [22]
	Saudi AATAAA→AATAAG	Severe HbH disease when combined with α^0 -thalassaemia [23]. Risk of severe HbH disease and HbH Hydrops Fetalis in homozygous state [24]
$\alpha 2$ Termination Codons	Constant Spring cd 142 TAA→CAA	Haemolytic anaemia in homozygous state, unusually severe form of HbH disease in combination with α^0 -thalassaemia [25, 26]
	Icaria cd 142 TAA→AAA	Mild haemolytic anaemia in the homozygotes state, interacts with α^0 -thalassaemia to cause HbH disease [27].
	Seal Rock cd 142 TAA→GAA	Phenotype similar to Hb Constant Spring
	Pakse cd 142 TAA→TAT	Causes HbH disease of mild to moderate clinical severity in combination with α^0 -thalassaemia [5, 28]
Unstable α-chain variant	Quong Sze cd 125 CTG→CGG	Highly unstable, diverse clinical presentation in combination with α^0 -thalassaemia, possible fetal hydrops in the homozygous state [29]
	Sun Prairie cd 130 GCT → CCT	Severely unstable. Homozygotes have chronic haemolytic anaemia [30].
	Adana cd 59 GGC → GAC	In combination with α^0 -thalassaemia a can result in Hb H Hydrops Fetalis [6, 9]

Table 2. Primer sequences and nucleotide dispensation order used in pyrosequencing assays

Amplification Primers	Sequence (5'>3')
Poly A & Stop codon (F)	ATCTTCTGTGAGCACCGTGCTG
Poly A & Stop codon (R)	BIOTIN- GGCCTGGCACCTCTCAGGA
Quong Sze & Sun Prairie (F)	BIOTIN- GAGCCGCACTGACCCTCTT
Quong Sze & Sun Prairie (R)	AGGAGGAACGGCTACCGA
Adana (F)	BIOTIN- CCCACCACCAAGACCTACT
Adana (R)	GGTCCACCCGAAGCTTGT

Sequencing Primers	Sequence (5'>3')	Dispensation Sequence
Stop codon	GTGCTGACCTCCAAAT	GACAGTCGAGCT
Poly A	ACCGGCCCTTCCTGG	GCTGACTGAGCTAGTCT
Quong Sze & Sun Prairie	CACGGTGCTCACAGA	GAGCAGACTGTCAGAGC
Adana	GTTGGTCAGCGCGTC	CGCACTCTAGTCGT

Table 3. Genotypes of the 105 samples analysed in the study

Mutation	Heterozygotes	Homozygotes	Compound heterozygotes		
			with α^+ thalaessmia deletion	with α^0 thalaessmia deletion	with $\alpha\alpha$
Poly A Turkish	7		3		
Poly A Indian	4	3	2		2
Poly A Saudi	13	5	2		
Hb Quong Sze	8			2	
Hb Sun Prairie	8	6			
Hb Adana	5	1		4	
Hb Constant Spring	9	3	3	7	
Hb Icaria	1	1		1	
Hb Paksé	1			2	
Hb Seal Rock	1		1		

Figure 1. Pyrograms showing homozygosity for the $\alpha 2$ termination codon mutations

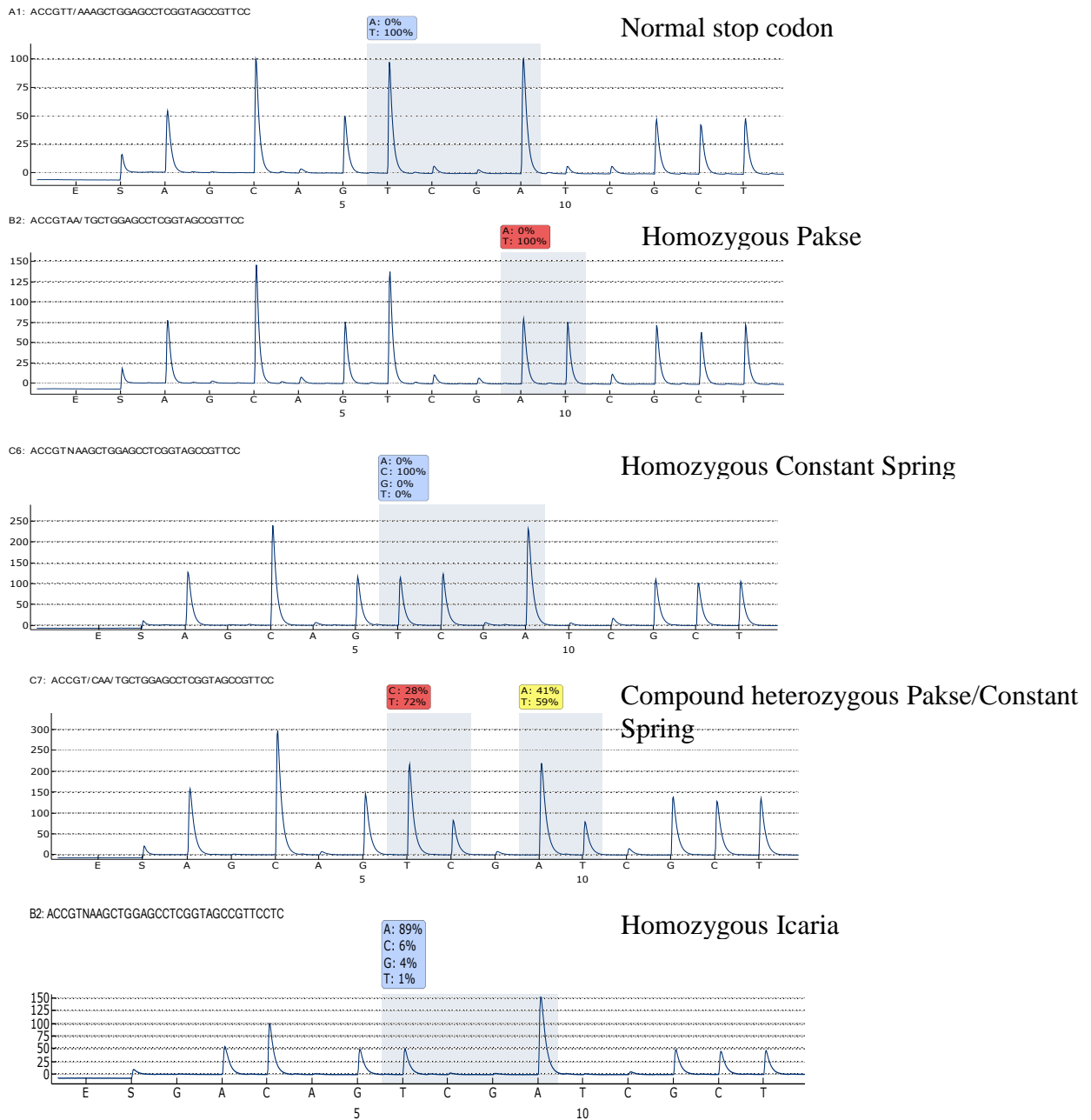
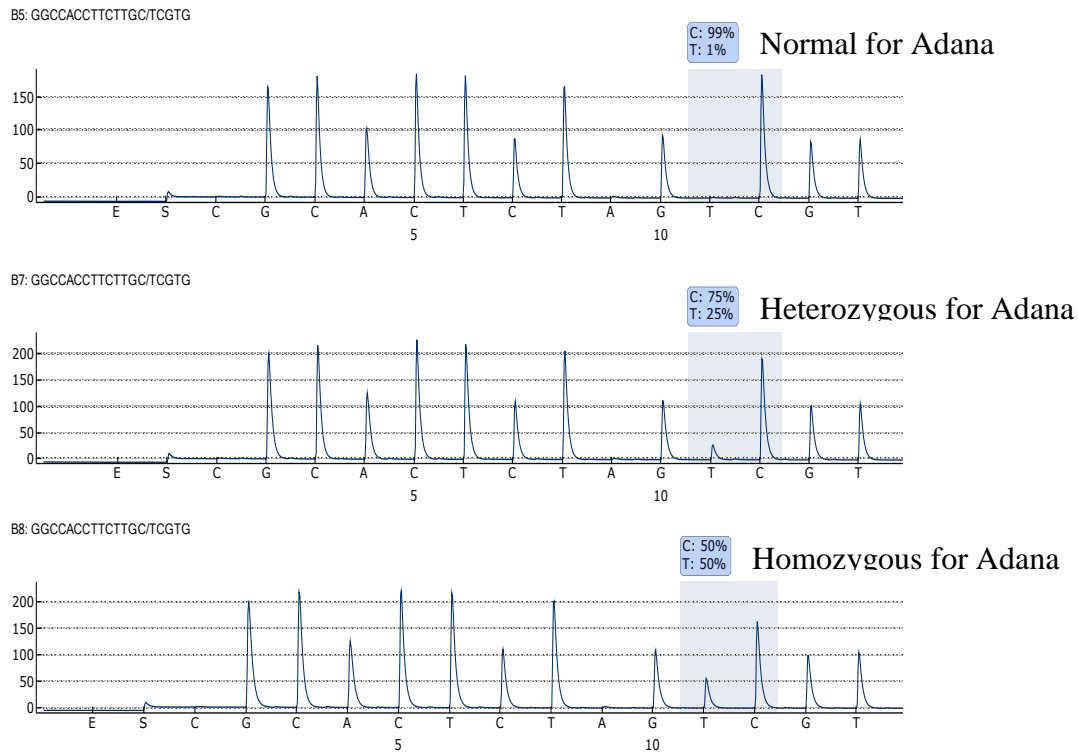


Figure 2. Pyrograms showing heterozygosity and homozygosity for the $\alpha 2$ globin gene mutation that results in the hyperunstable haemoglobin variant Adana.



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