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# ThalassoChip, an array mutation and single nucleotide polymorphism detection tool for the diagnosis of β-thalassaemia

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## Abstract

**Background:** The detection and diagnosis of β-thalassaemia for populations with molecular heterogeneity, or diverse ethnic groups, has increased the need for the development of an array high-throughput diagnostic tool that can deliver large scale genetic detection. We report on the update and validation of the ThalassoChip, a β-thalassaemia genetic diagnostic tool which is based on arrayed primer extension (APEX) technology.
**Methods:** ThalassoChip slides with new and redesigned probes were prepared for testing the microarray. Six hundred and sixty DNA samples collected from eight Mediterranean countries were used for standardisation, optimisation and validation of the ThalassoChip. The β-globin gene region was amplified by PCR, the products were hybridised to the probes after fragmentation and the APEX reaction followed.
**Results:** The ThalassoChip was updated with new probes and now has the ability to detect 57 β-globin gene mutations and three single nucleotide polymorphisms (SNPs) in a single test. The ThalassoChip as well as the PCR and APEX reactions were standardised and optimised using 500 DNA samples that were previously genotyped using conventional diagnostic techniques. Some probes were redesigned in order to improve the specificity and sensitivity of the test. Validation of the ThalassoChip performed using 160 samples analysed in blinded fashion showed no error.
**Conclusions:** The updated version of the ThalassoChip is versatile, robust, cost-effective and easily adaptable, but most notably can provide comprehensive genetic diagnosis for β-thalassaemia and other haemoglobinopathies.
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**Keywords:** arrayed primer extension technology; diagnostic test; microarrays; thalassaemia.

## Introduction

β-Thalassaemia is an autosomal recessive disorder caused by a reduction or lack of production of β-globin chains. β-Thalassaemia still remains one of the most significant single gene disorders as most thalassaemia patients require regular lifelong blood transfusions to survive, and early detection is essential to support the care of patients. In addition, molecular characterisation of carriers plays a central role in disease prevention, and is an absolute requirement when offering prenatal diagnosis to carrier couples (1). However, the current increase in migration patterns have complicated the identification of mutations due to the presence of a wider spectrum of mutations in most countries worldwide (2–4).

Current molecular diagnostic approaches for thalassaemia include PCR-based techniques capable of detecting the wide spectrum of α- and β-globin gene mutations. The methods include allele-specific oligonucleotide (ASO) probes, amplification refractory mutation system (ARMS) and sequencing for point mutations and gap-PCR for specific deletions. Other indirect methods include denaturing gradient gel electrophoresis (DGGE), as well as denaturing high performance liquid chromatography (dHPLC). More recently, several emerging techniques including multiplex ligation-dependent probe amplification (MLPA) and quantitative multiplex PCR short fluorescent fragments (QMPSF) for the detection of deletions, and real-time PCR and high-resolution melting

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curve analysis for the detection of point mutation have been used (5, 6).

Microarray technology is able to explore genetic abnormalities of polygenic disorders, such as heart disease, mental illness, and infectious diseases, and to distinguish pathological entities, such as acute myeloid leukaemia and acute lymphoblastic leukaemia (7, 8). Microarrays are used to study the therapeutic response to drugs and establish genetic profiles of treated and untreated patients. Microarrays can revolutionise the diagnosis of β-thalassaemia and Hb variants (9). Chan et al. in 2004 (10) developed a thalassaemia array covering the commonest mutations of α- and β-thalassaemias in Southeast Asia. Nanogen Microelectronic technology was used for the detection of β-thalassaemia mutations (11, 12). We developed and validated a microarray chip, called ThalassoChip, to serve as a high throughput, large scale β-globin mutation and polymorphism detection system (13, 14). The ThalassoChip is able to detect mutations and SNPs at low concentrations of genetic samples, and can show detectable signal with just 0.15 ng of human genomic DNA (15).

The ThalassoChip is based on arrayed primer extension (APEX) technology. It involves extension of oligonucleotides probes that are designed to stop a single nucleotide short relative to the position of the allelic variant (16). The probes are immobilised via the 5′ end on a glass surface, while their 3′ end is free for enzymatic extension. An amplicon containing the region which harbours the mutation(s) or single nucleotide polymorphisms (SNPs) under investigation is hybridised to the complementary probe sequence that is immobilised on the microarray chip. A single base extension of the probe is performed by incorporation of the appropriate dye-labelled dideoxynucleotide complementary to the variant base, followed by termination of the reaction. An automatic laser imaging system, Genorama QuattroImager, is used to read the APEX slide. Fluorescent labels (Fluorescein, Cy3, Cy5 and Texas Red) for the four dideoxynucleotides are read at four different emission wavelengths (17–20).

In this study, we report on the update and validation of the ThalassoChip, a β-thalassaemia genetic diagnostic tool which is based on APEX technology. This system has the ability to detect over 60 β-globin gene mutations and polymorphisms in a single step.

## Materials and methods

### Spotting

Probes (oligonucleotides) of 20–25 nucleotide bases in length were designed to be complementary to known SNPs and mutation regions, but terminating one base before the variant nucleotide position, were used for spotting. The microarray slides have a double-sided amino-silane coating onto which the oligos are electrostatically attached by their 5′ termini (21). The slide coating has the ability to preserve the probes anchored to the slide. Each spot has a diameter of 90–100 μm, with a minimum distance of 150 μm (optimal 250–375 μm) between spots.

New probes have been designed to update the ThalassoChip. The oligonucleotide probes were synthesised according to the wild type sequence, with an amino-linking group at the 5′ end attached to the glass slide. The 3′ end of the probe is free for enzymatic extension by DNA polymerase during the APEX reaction step. For each nucleotide variation, probes were designed to target both sense and antisense strands, and each probe was spotted in duplicate in order to increase the reliability of diagnosis. The β-globin gene sequence was derived from the NCBI database with accession number NG_000007.

The ThalassoChip routine screening process involved the following steps: a) DNA extraction and amplification of the appropriate region by PCR, b) purification of the amplified region followed by fragmentation, c) hybridisation of amplicons followed by APEX reaction, oligonucleotide probe extension by a single fluorescently labeled ddNTP, d) genotype determination using a four-channel imager.

### DNA samples

During the standardisation and validation process of the ThalassoChip, 660 human genomic DNA samples with various genotypes were tested. Five hundred DNA samples were extracted from whole blood using either the Puregene Blood Core Kit C (Qiagen, Hilden, Germany) or the QIAmp DNA Blood Mini kit (Qiagen, GmBh, Hilden, Germany). One hundred and sixty DNA samples were provided by the collaborating partners, from countries, such as Cyprus, Egypt, Morocco, Jordan, Saudi Arabia, Israel, Greece and Italy. The DNA sample concentration was adjusted to 50–100 ng/μL using pure sterile water. All samples used in this study were obtained with informed consent from the patients, and all work was performed in accordance to the declaration of Helsinki ethical guidelines as reflected in a priori approval by the Cyprus national Ethics Committee.

### Polymerase chain reaction

Four sets of primers were designed to amplify the β-globin regions that contain the majority of mutations and polymorphisms (Figure 1). Each set of primers covered an area approximately 200 bases long. The primers were designed taking into consideration their melting temperature ($T_m$), their propensity to form dimers with themselves or with other primers, and care was taken to avoid annealing of the primers to SNPs or mutations. Optimal PCR conditions were selected for each set of primers. Primer sequences were designed as follows: Primer set 1: Forward: 5′-CTAAGCCAGTGCCAGAAGAGCCAAG-3′; Reverse: 5′-TCAGTGCCTATCAGAAACCCAAGAG-3′. Primer set 2: Forward: 5′-GGGTTTCTGATAGGCACTGACTCTCTC-3′; Reverse: 5′-ACTGTACCCTGTTACTTCTCCCCTT-3′. Primer set 3: Forward: 5′-TTCAGGGCAATA-

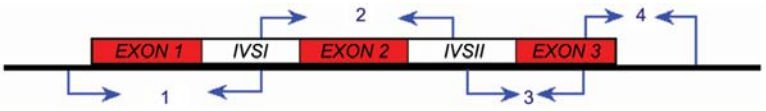


**Figure 1** β-Globin gene including positions of the four sets of primers used for PCR amplification.

ATGATACAATG-3′; Reverse: 5′-AAGTGATGGGCCAGCACACAG-3′. Primer set 4: Forward: 5′-GTGTGCTGGCCCATCATCTTG-3′; Reverse: 5′-CCTCCCACATTCCCTTTTTAG-3′.

The PCR reaction (25 μL) contained the following reagents: 10×PCR buffer (500 mM KCl, 15 mM $MgCl_2$ and 100 mM Tris-HCl, Amersham Pharmacia Biotech, Uppsala Sweden), 2 μL of deoxynucleoside triphosphate mix (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.6 mM dTTP, and 0.04 mM dUTP), 10 pmol/μL of primers, 2.5 U of Taq Polymerase (5 U/μL; Amersham Pharmacia Biotech, Uppsala, Sweden) and 100 ng of human genomic DNA. The PCR programme that produced the greatest yield followed the touchdown PCR protocol which also avoids amplification of non-specific sequences (22). The initial incubation was performed at 95°C for 5 min followed by 5 cycles of denaturation at 95°C for 30 s and primer annealing at 60°C and extension at 72°C for 20 s. The programme continued with another 30 cycles of annealing at 58°C for 30 s and then changed to 20 s for the final 8 cycles. The final extension was performed at 72°C for 5 min. PCR products were purified and concentrated (to 15 μL in volume) using the *Qiagen* QIAquick *PCR Purification Kit* (Hilden, Germany) as per the manufacturer's protocol. Each PCR product was tested by gel electrophoresis to ensure that each set of PCR reactions produced a single product of the expected size.

## Fragmentation

All PCR products amplified included 20% substitution of dTTPs with dUTPs. This enabled subsequent fragmentation with thermolabile uracil-N-glycosylase (by HK™, Madison, WI, USA) which hydrolyses the N-glycosidic bond between the deoxyribose sugar and uracil in DNA that contains deoxyuridine when it is heated to 38°C. During the fragmentation process shrimp alkaline phosphatase (SAP) was also added which catalyses the release of the 5′ phosphate group from the DNA. Dephosphorylation of the excess dNTPs that remain after PCR can interfere with the APEX reaction. The fragmentation mixture (30 μL) consisted of: 10×UNG buffer [500 mM Tris-HCl, pH 9.0; 200 mM $(NH_4)_2SO_4$], 0.03 U/μL of SAP (1 U/μL) (Amersham Bioscience Inc., Milwaukee, WI, USA), 0.03 U/μL of UNG (1 U/μL) (Epicentre Technologies, Madison, WI, USA) and approximately 75 ng/μL of purified PCR product. The fragmented PCR product was heated to 95°C for 10 min which enabled the deactivation of the SAP enzyme as well as denaturation of the double stranded DNA duplexes. Fragmentation efficiency was confirmed by electrophoresis.

## Amplicon hybridisation and the APEX reaction

During the APEX reaction, the microarray oligonucleotide probes were extended by a single fluorescently-labeled dideoxynucleoside triphosphate which was complementary to the DNA template. The DNA polymerase reaction was run at high temperature (58°C) to avoid or minimize undesirable secondary structure oligos, while permitting efficient hybridisation to the target site. Each slide was incubated for 15 min and any excess reagents were removed by washing the slides with boiling water, allowing annealed DNA to denature and wash away. This process can avoid background fluorescence and rehybridisation of unbound oligonucleotides.

The buffer reaction mixture (40 μL) that was used consisted of Tris-HCl/$MgCl_2$ buffer (260 nM, pH 9.5 and 65 mM, respectively; Amersham Pharmacia Biotech, Uppsala, Sweden), four different terminator nucleotides, each tagged with individual fluorophores: 12 μM of Fluorescein ddUTP, 19 μM of Cy3 ddCTP, 12 μM of Texas Red ddATP, 12 μM of Cy5 ddGTP (NEN, Boston, MA, USA, and Amersham Pharmacia Biotech, Uppsala, Sweden), 12 μM Thermosequenase DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden) and 70 ng/μL of denatured DNA.

## Workstation

The device used for the detection of mutations and polymorphisms during the validation of the chip was the Genorama QuattroImager detector 003 (Genorama Ltd., Tartu, Estonia). The detector is a four-channel imaging system composed of laser light sources and an integrated camera. The signal detection process is based on the recording of fluorescence emitted at the surface of the ThalassoChip that permits homologous exposure. The appropriate frequency of light source stimulates the labelled nucleotides to emit light that is captured by a charged coupled devised digital camera with 20 μm resolution (23).

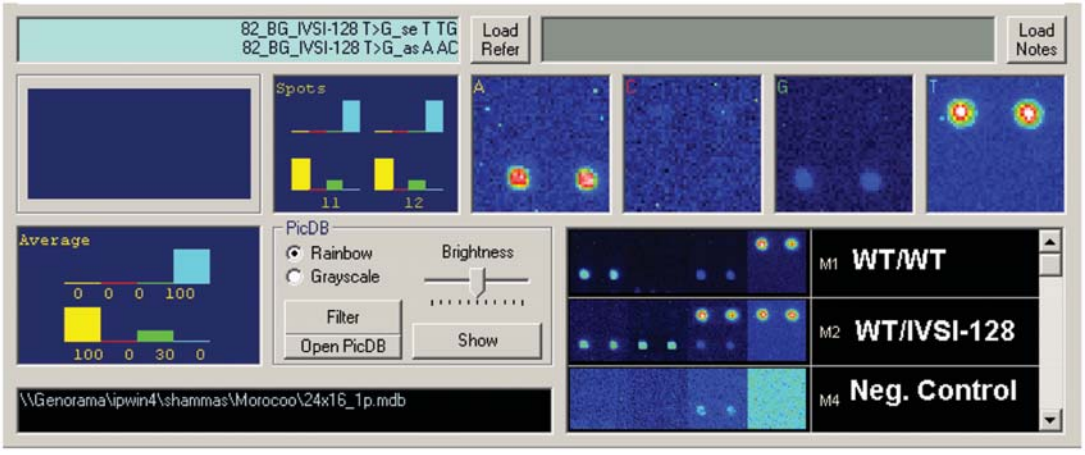


**Figure 2** Image from the BaseCaller 4.5 software used for the APEX analysis of sequence IVSI-128 (T→G).
The four-channel fluorescent images from each type of flurophore dNTP are shown in the top right boxes marked A, C, G and T, respectively. The sense strand (top) and anti-sense strand (bottom) are each determined twice to minimize errors. PicDB image from previous samples can be seen on the lower right hand corner. Sample observed in the PicDM images labeled M1 is WT, M2 is heterozygous for IVSI-128 and M4 is the negative control.

**Table 1** List of the β-globin mutations and polymorphisms that have been spotted on the ThalassoChip and validation results.

| | Common name | HGVS nomenclature | N/M | M/M |
|---|---|---|---|---|
| 1 | *–101 (C→T)* | *HBB:c.–151C>T* | 1 | |
| 2 | *–92 (C→T)* | *HBB:c.–142C>T* | | 1 |
| 3 | *–88 (C→T)* | *HBB:c.–138C>T* | 1 | |
| 4 | *–87 (C→G)* | *HBB:c.–137C>G* | 1 | |
| 5 | *–30 (T→A/C)* | *HBB:c.–80T>A/HBB:c.–80T>C* | 1 | |
| 6 | *–28 (A→C)* | *HBB:c.–78A>C* | 1 | |
| 7 | *+20 (C→G/T) (SNP)* | *HBB:c.–31C>T* | 17 | 2 |
| 8 | *+22 (G→A)* | *HBB:c.–29G>A* | 1 | |
| 9 | *+30 (T→C)* | *HBB:c.–21C>G* | 2 | |
| 10 | *+33 (C→G)* | *HBB:c.–18C>G* | 1 | |
| 11 | *Cd 1 delG* | *HBB:c.4delG* | 1 | |
| 12 | *Cd 2 (CAC→CAT) (SNP)* | *HBB:c.9C>T* | 6 | |
| 13 | *Cd 5 (CCT→del-CT)* | *HBB:c.17_18delCT* | 9 | |
| 14 | *Cd 6 (GAG → AAG) HbC* | *HBB:c.19G>A* | 1 | |
| 15 | *Cd 6 (GAG →del-A)* | *HBB:c.20delA* | 1 | |
| 16 | *Cd 6 (GAG →GTG) HbS* | *HBB:c.20A>T* | 1 | |
| 17 | *Cd 8 (AAG→del-AA)* | *HBB:c.25_26delAA* | 5 | |
| 18 | *Cd 8/9 insG* | *HBB:c.27_28insG* | 4 | |
| 19 | *Cd 9/10 insT* | *HBB:c.30_31insT* | 1 | |
| 20 | *Cd 15 (G→A)* | *HBB:c.48G>A* | 3 | |
| 21 | *Cd 22–24 delAAGTTGG* | *HBB:c.68_74delAAGTTGG* | 1 | |
| 22 | *Cd 24 (T→A)* | *HBB:c.75T>A* | 1 | |
| 23 | *Cd 24 delG/insCAC* | *HBB:c.74delinsCAC* | 1 | |
| 24 | *Cd 25/26 insT* | *HBB:c.78_79insT* | 1 | |
| 25 | *Cd 26 (GAG→AAG) HbE* | *HBB:c.79G>A* | 1 | |
| 26 | *Cd 27 (GCC→TCC) Hb Knossos* | *HBB:c.82G>T* | 1 | |
| 27 | *Cd 28 delC* | *HBB:c.85delC* | 1 | |
| 28 | *Cd 30 (A→G)* | *HBB:c.91A>G* | 5 | |
| 29 | *IVSI-1 (G→A/T)* | *HBB:c.92+1G>A / HBB:c.92+1G>T* | 9 | 1 |
| 30 | *IVS-2 (T→G)* | *HBB:c.92+2T>G* | 1 | |
| 31 | *IVSI (-3)/Cd 29 (C→T)* | *HBB:c.90C>T* | 1 | |
| 32 | *IVSI-5 (G→A/T/C)* | *HBB:c.92+5G>A/HBB:c.92+5G>T/HBB:c.92+5G>C* | 8 | |
| 33 | *IVSI-6 (T→C)* | *HBB:c.92+6T>C* | 12 | |
| 34 | *IVSI del17bp* | *HBB:c.93–17_93–1delTATTTTCCCACCCTTAG* | 1 | |
| 35 | *IVSI del25bp* | *HBB:c.93–21_96del* | 3 | |
| 36 | *IVSI del44bp* | *HBB:c.76_92+27del* | 1 | |
| 37 | *IVSI-110 (G→A)* | *HBB:c.93–21G>A* | 14 | 1 |
| 38 | *IVSI-116 (T→G)* | *HBB:c.93–15T>G* | 1 | |
| 39 | *IVSI-128 (T→G)* | *HBB:c.93–3T>G* | 1 | |
| 40 | *IVSI-130 (G→C/A)* | *HBB:c.93–1G>C/HBB:c.93–1G>A* | 3 | |
| 41 | *Cd 30 (G→C)* | *HBB:c.93G>C* | 3 | |
| 42 | *Cd 36/37 delT* | *HBB:c.112delT* | 2 | |
| 43 | *Cd 37 (G→A)* | *HBB:c.113G>A* | 9 | 2 |
| 44 | *Cd 39 (CAG→TAG)* | *HBB:c.118C>T* | 16 | 2 |
| 45 | *Cd 44 (TCC→del-C)* | *HBB:c.135delC* | 1 | |
| 46 | *Cd 76 (GCT→del-C)* | *HBB:c.230delC* | 0 | |
| 47 | *Cd 90 (G→T)* | *HBB:c.271G>T* | 1 | |
| 48 | *IVSII-1 (G→A)* | *HBB:c.315+1G>A* | 2 | 1 |
| 49 | *IVSII-74 (G→T) (SNP)* | *HBB: c.315+74G>T* | 2 | |
| 50 | *IVSII-654 (C→T)* | *HBB:c.316–197C>T* | 1 | |
| 51 | *IVSII-666 (T→C)* | *HBB:c.316–185T>C* | 1 | |
| 52 | *IVSII-705 (T→G)* | *HBB:c.316–146T>G* | 1 | |
| 53 | *IVSII-745 (C→G)* | *HBB:c.316–106C>G* | 17 | 2 |
| 54 | *IVSII-844 (C→G)* | *HBB:c.316–7C>G* | 0 | |
| 55 | *IVSII-848 (C→A)* | *HBB:c.316–3C>A* | 1 | |
| 56 | *IVSII-849 (A→C)* | *HBB:c.316–2A>C* | 1 | |
| 57 | *Cd106/107 insG* | *HBB:c.321_322insG* | 1 | |
| 58 | *Cd 121 (GAA→CAA) Hb D* | *HBB:c.364G>C* | 1 | |
| 59 | *Cd 121 (GAA→AAA) Hb O* | *HBB:c.364G>A* | 1 | |
| 60 | *+1570 (T→C)* | *HBB:c.*+96T>C* | 1 | |

### Data processing

Image analysis software was used to determine the genotype extension that has been produced during the APEX reaction, and each image was merged producing a single output file. Two types of software were used for the analysis, both of which are included in the Genorama™ Genotyping software. The BaseCaller software is used for data processing and single sample analysis (Figure 2) while the PicDB software was used for simultaneous analysis and comparison of multiple images.

## Results

### ThalassoChip update

New probes have been designed to update the ThalassoChip which can now detect 54 β-thalassaemia mutations that are common in Mediterranean countries, three haemoglobin variants (*HbS*, *HbE* and *HbC*) and three SNPs located in the β-globin gene (Table 1). The three SNPs that were included in the ThalassoChip are *Cd 2 (CAC→CAT)*, *IVSII-74 (G→T)* and *+20 (C→G/T)* polymorphism that is 100% associated with the *IVSII-745 (C→G)* $\beta^0$-thalassaemia mutation.

### Chip standardisation

Five hundred human genomic DNA samples were used for standardisation and optimisation of the ThalassoChip. All samples were previously genetically tested using conventional methods. The samples were used for the comprehensive determination and assessment of each probe, as well as for standardisation of the PCR reaction mixes, PCR programmes and optimisation of APEX conditions. Probes that showed low signal intensity were improved by changes made to the probe length or sequence that improved melting temperature. Changes in binding affinity had to be assured that did not induce changes in binding specificity.

### Chip validation

After the standardisation process, 160 human genomic DNA samples were run in blinded fashion. All samples were subsequently shown to be concordant with the conventional diagnosis that was made previously. All samples were screened against the 60 mutations and polymorphisms. As shown in Table 1, at least one sample from the pool was heterozygous (N/M) or homozygous (M/M) for each mutation and polymorphism with the exception of *IVSII-844 (C→G)* and *Cd 76 (GCT→del-C)*; due to their rarity in the Mediterranean region, only wild type alleles were tested. The normal allele for each probe was validated more than 140 times.

A general limiting factor in this study was the inadequate signal detection levels emitted from the Texas Red labelled ddATP. No changes were observed when the dye concentration was increased. Replacing the adenosine fluorophore with a higher intensity fluorescent dye could have improved the APEX analysis.

The relative fluorescent light intensity emitted when a sample is homozygous for an allele is usually twice as intense compared to a heterozygous sample, since the heterozygous sample has half of its probes extended with wild type nucleotide and the other half with the mutant nucleotide. This phenomenon can make peak assignment difficult when dealing with heterozygous samples.

## Discussion

The ThalassoChip potentially offers an alternative approach for the diagnosis of β-thalassaemia mutations and haemoglobin variants. The microarray is a diagnostic tool with low operating costs. It is estimated that each test costs 40 €, and can determine a wide spectrum of mutations and polymorphisms in a single experiment. This tool can provide diagnosis for populations with molecular heterogeneity. The current version of the ThalassoChip contains probes that can detect mutations common in the Mediterranean region, but it is being continuously updated to include more mutations that can cover more diverse ethnic groups. The APEX reactions are entirely reproducible under standardised conditions. These conditions include 1) the use of good quality human genomic DNA at the appropriate concentration, 2) optimised PCR amplification conditions, 3) successful DNA fragmentation 4) use of the optimised APEX reaction conditions.

The ThalassoChip can now serve as a diagnostic tool for β-thalassaemia and other haemoglobinopathies, but can also progress to an automated system that includes a greater number of mutations, even those underlying α- and δ-thalassaemia. The only disadvantage of this approach when compared to DNA sequencing is that the microarray chip is limited to detecting only known nucleotide changes for which the APEX probes have been designed, whereas previously undetected polymorphisms and new mutations will be missed.

## Conflict of interest statement

**Authors' conflict of interest disclosure:** The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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**Employment or leadership:** None declared.

**Honorarium:** None declared.

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