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GenEye24: Novel rapid screening test for the top-3 Leber's Hereditary Optic Neuropathy pathogenic sequence variants

Sara Martins [a,b,c,1], Maria João Santos [a,b,d,1], Márcia Teixeira [a,b], Luísa Diogo [a,d,e,f], Maria do Carmo Macário [a,d,e,f], João Pedro Marques [d,f], Pedro Fonseca [a,d,f], Manuela Grazina [a,b,d,*]

[a] CIBB – Center for Innovative Biomedicine and Biotechnology (www.cibb.uc.pt/), Coimbra, Portugal
[b] Laboratory of Mitochondrial Biomedicine and Theranostics, CNC – Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal
[c] Biology Department, University of Aveiro, Aveiro, Portugal
[d] FMUC – Faculty of Medicine, University of Coimbra, Coimbra, Portugal
[e] Reference Centre of Inherited Metabolic Diseases - CHUC- Centro Hospitalar e Universitário de Coimbra, EPE, Coimbra, Portugal
[f] CHUC – Centro Hospitalar e Universitário de Coimbra, EPE, Coimbra, Portugal

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ABSTRACT

Leber's Hereditary Optic Neuropathy (LHON) has been mainly (90–95 %) associated to one of three variants: m.3460G>A, m.11778G>A, m.14484T>C. Herein, a screening method was developed for its detection, supporting clinical/therapeutics decision. It relies on real-time PCR with High-Resolution Melting (HRM) analysis. Variant classification is made using HRM Software and quality controls. A total of 101 samples were analyzed.

All samples were correctly assigned: 58 wild-type, 35 positive for m.11778G>A, 6 positive for m.14484T>C, 2 positive for m.3460G>A. Results presented sensitivity = 1, specificity = 1, Positive Predictive Value = 1 and Negative Predictive Value = 1.

A new Real-Time PCR/HRM screening method cost-efficient, simple, robust and quick, detecting LHON's top-3 is described.

1. Introduction

Leber's Hereditary Optic Neuropathy (LHON) is a rare maternally inherited disorder, clinically characterized by bilateral and painless vision loss, either simultaneously (25 %) or sequentially (75 %) (Bi et al., 2016). Commonly, the second eye becomes affected after six weeks to six months; within one year, 97 % of patients present bilateral blindness (Rasool et al., 2016; Yu-Wai_Man and Chinnery, 2000). The symptoms usually appear between 15 and 35 years of age, with higher prevalence in men – 5:1 (Table 1) (Bi et al., 2016; Rasool et al., 2016). Although typical LHON is restricted to the optic nerve atrophy, other symptoms, including neurological manifestations, may occur (Grazina et al., 2007).

LHON has been genetically associated with mitochondrial genome (mtDNA) variants, which are usually homoplasmic, with 10–15 % being heteroplasmic (Bi et al., 2016). Three mtDNA variants (top-3) represent 90–95 % of LHON cases with identified causing genetic variants (Table 1): m.3460G>A (*MTND1* gene, ND1 subunit); m.11778G>A (*MTND4* gene, ND4 subunit); and m.14484T>C (*MTND6* gene, ND6 subunit) – all encoding subunits of the complex I of the mitochondrial respiratory chain multienzymatic system (Yu-Wai_Man and Chinnery, 2000; Yu-Wai-Man et al., 2011). The m.3460G>A was associated to the most severe phenotypes and is less frequent; the m.11778G>A is the most prevalent and has been correlated with the lowest spontaneous recovery prognosis; finally, m.14484T>C has the mildest effect and the

Abbreviations: ARMS, Amplification-Refractory Mutation System; C_T, Cycle threshold; HRM, High-Resolution Melting; LHON, Leber's Hereditary Optic Neuropathy; mtDNA, Mitochondrial DNA/genome; *MTND1/4/6*, Mitochondrially encoded NADH dehydrogenase 1/4/6; N/A, Non-available; NC, Negative control; NGS, Next-Generation Sequencing; NTC, Non-template control; PC, Positive control; PCR, Polymerase Chain Reaction; RFLP, Restriction Fragment Length Polymorphism; T_m, Melting temperature.

* Corresponding author at: Faculty of Medicine, University of Coimbra, Pólo III – Subunit I, 2° andar, Gab 2.31., Azinhaga de Sta. Comba, Celas, PT-3000-354 Coimbra, Portugal.
E-mail address: mgrazina@ci.uc.pt (M. Grazina).
[1] These authors contributed equally to this work.

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Table 1
Prevalence of LHON's top-19 pathogenic sequence variants.

	LHON associated variants *	Relative %	Penetrance % Males *
TOP-3	m.3460G>A	13 * | 4–19 **	40–80
	m.11778G>A	69 * | 70–80 **	82
	m.14484T>C	14 * | 7–30 **	68
TOP-16	m.3376G>A	Rare*	N/A
	m.3635G>A		54 (range 25–100)
	m.3697G>A		N/A
	m.3700G>A		N/A
	m.3733G>A		36–44
	m.4171C>A		47
	m.10197G>A		N/A
	m.10663T>C		60
	m.13051G>A		63
	m.13094T>C		N/A
	m.14459G>A		N/A
	m.14482C>A		89
	m.14482C>G		N/A
	m.14495A>G		N/A
	m.14502T>C		11 [14502 + 11778: 47]
	m.14568C>T		N/A

*Data from *MITOMAP* 2022 (https://www.mitomap.org/foswiki/bin/view/MITOMAP/MutationsLHON); ** Data from Chun & Rizzo, 2017 (Chun and Rizzo, 2017); N/A: Non-available.

highest association with spontaneous recovery (Table 1) (Bi et al., 2016; Yu-Wai_Man and Chinnery, 2000; Yu-Wai-Man et al., 2021, 2011).

These genetic alterations create a bioenergetic dysfunction: depletion of energy production, increased reactive oxygen species content and oxidative stress (Bi et al., 2016; Carelli et al., 2004; Kogachi et al., 2019; Yu-Wai-Man et al., 2016). Idebenone, an ubiquinone analogue acting by a compensatory mechanism that restores bioenergetic capacity (Bi et al., 2016; Catarino et al., 2020; Chun and Rizzo, 2017; Finsterer et al., 2018; Gueven et al., 2017; Lopez-Sanchez et al., 2016; Rasool et al., 2016; Yu-Wai-Man, 2015), has been shown to have a positive impact on visual loss recovery. Experts, on the scope of International Consensus, have strongly agreed that "idebenone should be started as soon as possible", "in patients with disease less than 1 year" (Carelli et al., 2017, 2011; Klopstock et al., 2013; Pemp et al., 2021; Tonagel et al., 2021; Yu-Wai_Man and Chinnery, 2000). The timeframe seems to be critical for clinical action. Accordingly, the identification of the causative genetic variant is mandatory to delineate treatment. It is essential to quickly and reliably scan for the genetic alteration, in order to act timely and rescue function.

The LHON diagnosis is mainly established with the identification of one of the above mentioned pathogenic top-3 (Yu-Wai_Man and Chinnery, 2000), but current methods are labor-intensive and/or prolonged in either the protocol itself, batch-gathering necessities or bioinformatic analysis. The aim of the present work was to detect the pathogenic top-3 in LHON suspected patients, by a method which was developed as a diagnostic tool (GenEye24), to be quick, reliable and with a protocol that is easy to perform and interpret. The GenEye24 is planned to be implemented in clinical settings to provide strong results within 24 hours. Thus, the top-3 were investigated by Real-Time PCR and High-Resolution Melting (HRM) curve analysis for variant assignment. The results allow the achievement of an accurate and timely genetic diagnosis and, consequently, improve the chances for visual recovery.

2. Materials & methods

2.1. Study design and sample collection

The sample population in study included 101 subjects: 58 without a top-3 variant (10 samples of non-LHON patients [patients with suspicion of other mitochondrial cytopathies], 20 healthy controls, and 20 samples from individuals suspected of LHON but that previously tested negative for top-3, and 8 non-affected non-carrier relatives) and 43 with a top-3 (15 LHON patients and 28 non-affected carrier relatives). The low number of positive samples is both related to the rareness of LHON (European epidemiology 1:31,000–1:50,000 (Yu-Wai-Man et al., 2011)) and, consequently, to the number of cases registered at our Center so far. In addition, there is a high probability of underdiagnosis, since presently, LHON diagnostic is only considered if a known pathogenic variant is identified. However, novel causes may be still undiscovered.

Total DNA was previously extracted from several tissues (lymphocytes, fibroblasts) according to standard protocols (Ausubel et al., 1997). All samples were previously analyzed and confirmed by at least one additional method (Sanger Sequencing, Next Generation Sequencing (NG) or PCR-Restriction Fragment Length Polymorphism (RFLP)), in the scope of the clinical laboratorial diagnostic investigation. The study was performed with a single-blind approach and the samples were codified for comparison and confirmation of the veracity of the results.

This study was included within a broader project with approvals by the local Ethical Committee (#CE-071/2013 and #CE-032/2014) and National Committee of Data Protection (authorization #5484/2018). Informed consent was obtained from the participants or their legal representatives, following the Tenets of the Helsinki Declaration. The samples were completely anonymized, according to the European Union General Data Protection Regulation (Regulation (EU) 2016/679) and to Portuguese Law (DL 58/2019 and DL 59/2019).

2.2. Primer design and preparation

The functional role, or impact, of the genetic alterations under study was confirmed in literature and by consulting bioinformatic records, as follows. The genes harboring the variants were assessed in *MITOMAP*; the gene sequences were obtained for *Homo sapiens* in *NCBI Gene* (genes *MTND1*: ID 4535; *MTND4*: ID 4538; *MTND6*: ID 4541) and used for primer design.

The primers were designed using the *Primer3Plus* software (https://www.bioinformatics.nl/primer3plus) and the *WasP* software (https://bioinfo.biotec.or.th/WASP) was used to perform a Real-Time PCR HRM approach, warranting that each amplicon had only one of the top-3. Bioinformatic tools of UCSC (*In-Silico* and *BLAT*) and *EndMemo – DNA Tm Calculator* were also used (respectively: https://genome.ucsc.edu/cgi-bin/hgPcr; https://genome.ucsc.edu/cgi-bin/hgBlat; and https://endmemo.com/bio/tm.php) (see Supplementary Table S1 for *in-silico* data about the primers).

Chemical solutions were prepared to 50 μM (final concentration) and further dilutions were made with TE (1:10). This step was performed in a sterilized PCR workstation, in order to prevent contamination.

2.3. Real-Time PCR and HRM

The reactions were prepared in a sterile PCR workstation, pre-treated under UV-light for 15 min. The MeltDoctor™ HRM Master Mix (Applied Biosystems), featuring SYTO®9 dye, was used. The reactions were prepared according to manufacturer's recommendations: 10 μL of MeltDoctor™ HRM Master Mix, 0.3 μM of each primer, and 20 ng (1 μL) of genomic DNA, to a final volume of 20 μL per well. The DNA samples and primers were diluted as necessary with deionized water and TE (1:10), respectively, in sterile conditions.

The reactions were run in the Applied Biosystems™ 7500 Fast Real-Time PCR equipment, using the 7500 Software v2.0.6. The experimental conditions (Suppl. Table S2) were programmed following manufacturer's instructions, except for the annealing temperature, which was optimized to 55 °C. Manufacturer's instructions were followed for reagents/ equipment. The necessary quality controls for the assays were included, using the HRM approach to confirm the specificity of the obtained product.

Optimization was developed based on three positive controls (one

per variant), one negative control, one non-template control and a random patient sample. Screening was conducted after optimization and included 43 positive samples, 58 negative samples and one non-template control, to assess the three variants; all samples were analyzed in triplicates for quality assurance (result accepted with 2/3). The analysis of the samples followed a double-blind approach, concerning the presence/absence of pathogenic variant, performed by two independent users.

2.4. HRM analysis

The results were analyzed using two software packages: 7500 Software v2.0.6 and HRM v2.0.1. The amplification plot was observed, along with C_T values, as well as the normalized and derivative plots, to check for overall results and confirm the similarity of triplicates. Furthermore, for guarantee of quality in analysis assessment, the slopes in normalized curves are expected to appear as a sharp drop and T_m peaks in the derivative curves must be unique, in order to assure the specific identification of T_m by the software, allowing the correct and specific identification of each variant per sample.

The HRM technique enables the identification and differentiation of the specific genetic variants by amplicon's T_m. Thus, the software discriminates each well into mutant/wild-type, based on the T_m values. Additionally, the software calculates a corresponding confidence value. In the current study, the cut-off value considered was Confidence $\geq$ 80 % and at least 2 out of 3 triplicates in concordance). After identification of the variants by the HRM Software, the results were compared with existing sequencing and PCR-RFLP data.

3. Results

The samples were assessed by previously established orthogonal techniques, providing independent confirmation of results. The analysis identified all top-3 as follows: 58 negative samples, 35 positive samples for m.11778G>A, 6 positive samples for m.14484T>C and 2 positive samples for m.3460G>A. The samples were then analyzed by the Real-Time PCR approach presented, with an additional confirmation run, which allowed to gage intermediate precision. Repeatability precision was shown with the use of triplicates. Data obtained showed sensitivity = 1, specificity = 1, Positive Predictive Value = 1 and Negative Predictive Value = 1 (calculated as described elsewhere (Trevethan, 2017)).

The primers are specific, and the derived products have high dissimilarity. An annealing temperature of 55 °C (Suppl. Figs. S1-S2; Suppl. Table S2) was adopted after optimization, with optimal amplification of all targets and all samples (Suppl. Fig. 1), which was observed by analysis of the C_T values of all targets, as well as their plateau and fluorescence emission levels (Fig. 1A). A late fluorescence signal was observed in NTCs, due to primer-dimers, but it had no interference with the samples' assay. Both positive and negative controls achieved the quality parameters, both in the implementation process and in the amplification and HRM analysis, showing the specificity and the accuracy of the assay.

According to data shown in Table 2, the T_m value of a wild-type amplicon and a mutant amplicon are distinguishable enough for easy identification by the HRM Software (>0.3 °C apart). The T_m values of the Positive and the Negative Controls were calculated from averaging the observed T_m values in the software – the negative control samples were averaged into the Negative Control value, and the positives into the Positive Control value.

Table 2
Comparison of T_m values for each variant (wild-type vs mutant) of the LHON pathogenic top-3.

Target	PC/NC	Average observed T_m (°C)
m.3460G>A	PC	79.21
	NC	79.80
m.11778G>A	PC	74.31
	NC	76.55
m.14484T>C	PC	72.34
	NC	71.89

PC: positive control; **NC:** negative control; **T_m:** Melting temperature.

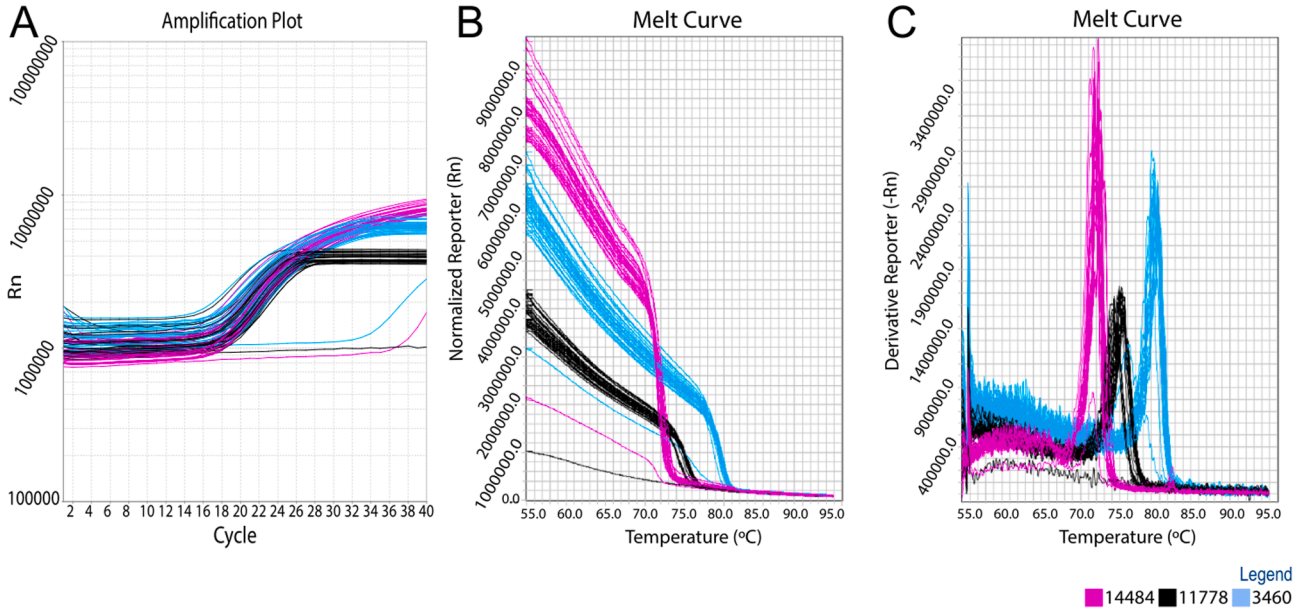

Fig. 1. Analysis of results from one 96-well plate in the 7500 Software. For a complete Real-Time PCR plate: (A) Amplification Plots; (B) Normative Melt Curve; (C) Derivative Melt Curve. Blue lines correspond to the curves of the m.3460G>A target; black lines to the m.11778G>A target; pink lines correspond to the m.14484T>C target. **1A:** NTCs show absence or very late amplification of primer dimers. The 3 targets appear aggregated and with similar amplification patterns for all samples. X axis: Temperature (°C); Y axis: Rn [Normalized Reporter]. **1B:** NTCs show curves with very low fluorescence. The 3 targets appear aggregated, and with similar values of melting temperature. X axis: Temperature (°C); Y axis: Normalized Reporter (Rn). **1C:** The aggregation of the 3 distinct peaks correspond to the 3 targets; each peak corresponds to the value of Tm, which will be analyzed in the HRM software. X axis: Temperature (°C); Y axis: Derivative Reporter (-Rn).

In Supplementary Fig. 1, the optimization's amplification curves (1A) and normalized (1B) and derivative (1C) melt curves from the 7500 Software are presented. In the derivative curves (Suppl. Fig. 1C), the peaks, which represent the T_m value of the amplicon, demonstrate that the three targets are easily distinguishable. Peaks from primer-dimers are also found, but at a much lower temperature (55 °C); these can be ignored, as they do not affect the HRM's analysis or results.

The results from the normalized (Fig. 1B) and derivative (Fig. 1C) curves obtained by using the HRM software were as expected: normalized curves exhibited a sharp slope and samples aggregated according to target (similar T_m); individual peaks were clearly observed in derivative curves. The normalized curves have definition (separation by target) and quality slopes; the derivative curves are distinctly separated by target, with only one peak per sample; together, these data allow the confident identification of results by the HRM software.

The samples that are positive for a genetic variant are clearly distinguishable from the samples which are negative for the same alteration (Suppl. Fig. 2). The aligned melt curves, which are produced by the HRM software, were automatically adjusted for the region of interest, as seen in Fig. 2. There is a clear distinction between negative and positive results, as evidenced by the obvious grouping.

There were no unexpected results, that is, none of the samples presented other than the expected variants, e.g., the appearance of a third variant. However, the single nucleotide variants possibly occurring in the amplified fragments were checked and their presence's impact on the HRM result was analyzed *in-silico*. This analysis is shown in Supplementary Table S3–S5, creating an overview of all the theoretically possible false positive results that could occur in a sample.

4. Discussion

This approach presents an easier and faster method to identify the top-3 LHON genetic variants, with the software resolving much of the identification process, providing the variant classification and its confidence value. The use of Real-Time PCR and HRM was chosen due to key features: it is simple, fast (quick turn-around), not labor-intensive, has low cost, high-sensitivity and -specificity, and is a closed-tube analysis (diminished contamination and processing errors). The observed T_m values (by single nucleotide variant) were averaged (Table 2) and the differences of these averages transmit the clear distinction between the different products/targets being assessed. These variances reassure that the differentiation of positive and negative samples is reliable, and that the correct variant is being identified. The referred distinction is observed throughout the analysis, reproduced in the amplification (Fig. 1A) and in the melt curves (Fig. 1B, 1C). The values and quality of curves provide high confidence in the results and warrant that the HRM software will predict variants correctly. Again, the clear distinction in variant calling is observed (Fig. 2), supported by the high confidence values (Mode 100 %; Median 98.18 %; Mean 96.66 %) reported by the HRM software (Suppl. Table S6).

The use of triplicates is recommended for any cases in which well omission is necessary, and, for our laboratory, it is essential to guarantee quality and confidence in the clinical molecular diagnosis that is provided daily. Omission may occur if issues that could disrupt the software variant classification are observed on the amplification curve (Fig. 1A; Suppl. Fig. 1A), such as: elevated starting fluorescence, absent or late amplification (absence of plateau stage), and/or low amplification (distinctly lower plateau). These events are rare (2.58 % of reactions) and, if it occurs, the reactions are rejected in order to avoid variant miscalling by the HRM software, which could assign the sample as having a different variant, confounding the deductive process. In HRM analysis (Fig. 2), if triplicates would show different variants, either i) the confidence value on one of the wells would be much lower, leading to its exclusion; or ii) as discussed, there were amplification issues (low/no amplification), seen in amplification curves and/or in very high C_T values. These variants would be discarded (6.13 % of reactions), considering the remaining two replicates' variant as the true result. The confirmation runs showed that the hardware and software have the capacity for accurate assignment of all samples, since all the results were correct, in reference to positive and negative controls (100 % of consistency).

The average time of preparation is 90 min, with further 135 min of Real-Time PCR run time, with each run allowing the assessment of eight samples in triplicate. This technique easily permits two different runs in one 8-hour workday. This enables, the screening of 16 samples, if necessary, or a confirmation screening of results to boost their robustness. However, and due the low-cost of this analysis, it is perfectly feasible to run only one sample, without great waste of resources (more empty wells) and without high economic burden (low-cost analysis). For example, this approach can be of common use in laboratories that

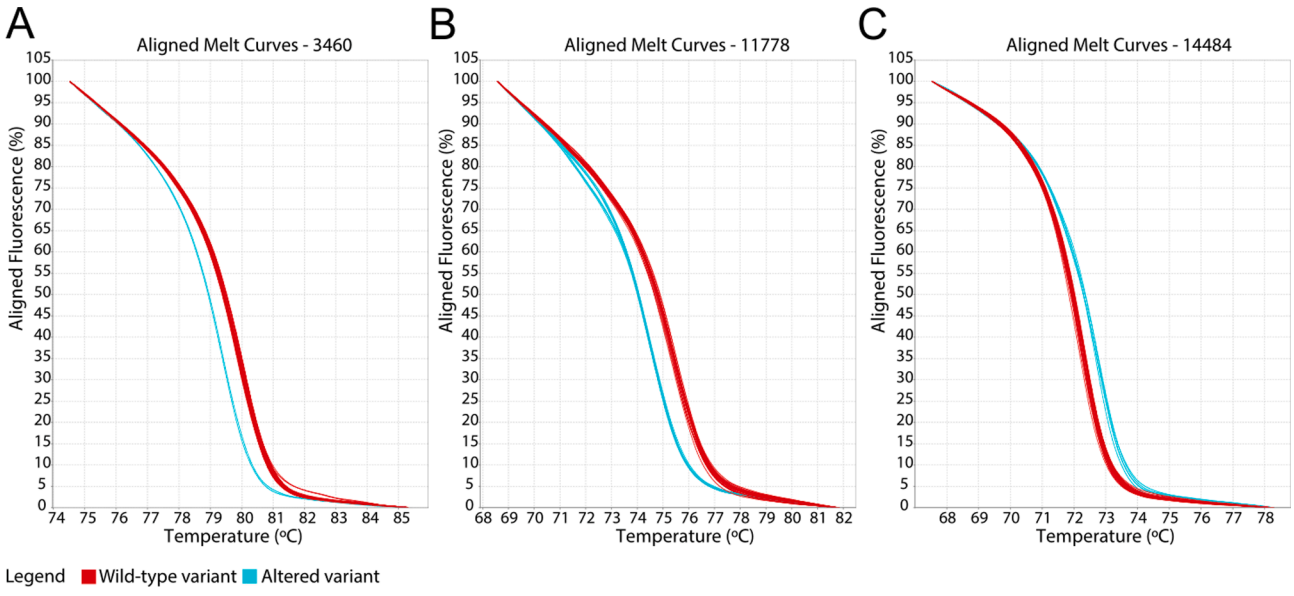

Fig. 2. Aligned Melt Curves, from the HRM Software, with variant calling (discriminated by color), from a complete 96-well plate. Wild-type variant (red) is negative, and variant (blue) is positive, for the target: **2A:** m.3460G>A; **2B:** m.11778G>A; **2C:** m.14484T>C. X axis: Temperature (°C); Y axis: Aligned Fluorescence (%).

struggle with batch gathering, for techniques like NGS (where analyzing only one / a few samples is a great waste of resources), or in situations with reduced funding and/or low resources (e.g., low-income situations).

The variants in study comprise 95 % of all pathogenic variants found in LHON cases, thus being useful in a considerable number of cases. The pathogenic top-3 screening is recommended by the well-established international experts of LHON, as a first step in determining diagnosis (Carelli et al., 2017; Yu-Wai_Man and Chinnery, 2000). A quick approach to this diagnostic investigation is herein presented, with the GenEye24 test. If the result is negative, other methods, such as NGS, can be used to search for other LHON variants, including rare alterations (Ryan et al., 2016), which are indicated in Table 1. The novel method proposed is very helpful, in order to streamline the LHON suspected cases that arrive in a laboratory, giving a faster answer to the common cases, which opens up time and labor for more complex theranostics' cases, and the corresponding economic impact of this line of action.

The amplified sequence may contain other genetic variants (benign or pathogenic single nucleotide variants), which possibly could translate in HRM output as a third variant (different from positive and negative controls) or as a false (positive or negative) result (Table 3; Suppl. Table S3–S5). Since the HRM analysis is based on the sample's T_m value, and this is obtained from the G + C/A + T ratio, the possibilities were theoretically analyzed in detail, in order to cover all potential occurrences and it can be classified (Table 3) as follows:

i. The third variant comes from any nucleotide change that is different from the one being screened, since it alters the T_m value to be either too high or too low in comparison to the values expected.
ii. False positives have the same T_m as the mutant sample; i.e. any other nucleotide variation that establishes the same GC/AT content of the mutant sample (e.g., the same nucleotide change but in a different position).
iii. False negatives might happen when mutant samples have the reverse change too (e.g., if the variant is G > A, then it would need a simultaneous A > G).

This can happen for the single nucleotide variants located in that amplicon span; in the Supplementary Table S3–S5, the nucleotide changes that could occur in these amplicons are presented, although *de novo* variants may happen. However, this screening technique, by definition, will be used on a cohort of LHON suspected cases, which necessarily will have a high probability of presenting one of the top-3, rather than if it were applied for population-wide research. Furthermore, according to *MITOMAP*, the majority of the alterations mentioned in Supplementary Table S3–S5 have very low frequencies and are very rare. Although this could be seen as a disadvantage/ limitation of this approach, the occurrence of those situations seems unlikely. Moreover, in a first phase of implementation, other confirmation methods, such as sequencing or RFLP could be used, since all techniques have limitations, in order to reassure higher confidence of the test in a timely screening, as presented. If it is the case, the authors recommend that the samples may be also analyzed alongside PCR-RFLP; this highlights that older methods may still be important in current genetic analysis, with a complementary role. The positive results can be confirmed by other methods; the negative results will proceed to the more usual methods of sequencing (e.g., NGS, which will also provide other interesting findings for diagnosis such as heteroplasmy), so the case would be resolved. The development of a second-line assay with the rarer top-16 LHON variants was contemplated; however, the cost-benefit would not be justifiable for diagnosis cases and, moreover, methods that, although having higher costs and taking longer for result-acquisition, give much more information are preferable as a follow-up to the negative cases from this top-3 analysis.

Table 3
Nucleotide changes that theoretically could be assigned by HRM analysis, as false negatives or positives in the LHON pathogenic top-3 genetic screening.

	m.3460G>A & m.11778G>A	m.14484T>C
false negative	A > G	C > T
	T > C	G > A
	G > T	G > T
	A > C	C > A
false positive	G > A	T > C
	C > T	A > G
	G > T	A > C
	C > A	T > G

The PCR-RFLP is surpassed in terms of robustness and time, since it is not specific for the genetic variant under analysis, although it is still important as a complementary method, including for confirmation of the positive results, if considered necessary. The novel approach presented gives a much higher confidence in results and greatly reduces issues like contamination due to its closed-tube analysis, in a shorter time span. Sequencing techniques give robust and accurate results, but they take much longer due to the arduous task of data analysis and present higher costs. Considering the months-long wait for clinicians and patients of NGS data analysis' results, due to its batch gathering necessity and the labor of bioinformatic data analysis, the 24-hour turnaround method is extremely helpful in creating faster clinical action, including treatment initiation. Furthermore, it is less labor intensive than all other mentioned techniques (Bi et al., 2010; Chambliss et al., 2017; Matsuda, 2017; Ryan et al., 2016; Stephenson, 2016). The GenEye24 test can be implemented by any laboratory with access to a real-time PCR machine capable of detecting the SYTO®9 dye (dsDNA binding dyes) and with HRM settings.

A few other similar techniques have been developed, relying on Amplification-Refractory Mutation System (ARMS) primers (Bi et al., 2010; Ryan et al., 2016) or TaqMan® probes (Martins et al., 2017). However, here, by using regular primers, an easier and less costly method is shaped. The primers used in the present study (in Suppl. Table S1) have a very high level of specificity, as verified by bioinformatics' tools, according to the information described in the Methods' section. Both other approaches will have higher costs, TaqMan® probes particularly. An ARMS method will use more plate wells (double), or develop the analysis in multiplex, both of which increase cost.

The development of the GenEye24 screening method was centered around the aim of ameliorating the conditions presented by other techniques (e.g., PCR-RFLP, pyrosequencing, other PCRs, Sanger sequencing and NGS (Chambliss et al., 2017; Ryan et al., 2016)), comparing time/cost/labor; time was determined as particularly relevant, since this is an essential issue for starting therapy (van Everdingen et al., 2021). Additionally, time is vital for patients and their families; it is also in the interest of the professionals, both clinicians and laboratory technicians, to have access to this time-saving screening technique. Clinicians wish to start the correct course of therapy as soon as possible; for the laboratory team, this fast technique means less labor, which will allow more time dedicated to cases with a more challenging molecular diagnosis, as well as advancing research and development. Indeed, it has been stated by clinicians and experts that timely/rapid diagnosis is as an essential issue in LHON and that idebenone should be started rapidly (Carelli et al., 2017; Pemp et al., 2021; van Everdingen et al., 2021).

4.1. Conclusions

The method is robust, simple, of easy-analysis, fast and cost-efficient. The LHON top-3 comprise about 95 % of all genetic positive LHON cases, making this novel approach very useful in a clinical setting. This detection is reliable enough to inform the medical decision on diagnosis and treatment, and its implementation allows timely action, which is essential for recovery of eye function and vision, whenever possible. This is a pioneering study with a notable contribution to make

personalized and precision medicine a reality, which is closer to the patients' needs, who may benefit from this study worldwide.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Contributions of individual authors

Manuela Grazina is the guarantor of this work and planning and, as such, had full access to all data and takes responsibility for data integrity and data analysis' accuracy. Sara Martins and Maria João Santos were main co-authors for this work, participating in phases of execution. Márcia Teixeira was involved with confirmation analysis and writing. Luísa Diogo, Maria do Carmo Macário, João Pedro Marques and Pedro Fonseca made contributions concerning patient management and text revision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mito.2023.01.006.

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