

# Finger prick blood testing in Leber hereditary optic neuropathy

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## Abstract

**Individuals from 33 unrelated Australian families with optic atrophy were screened for 10 different single base alterations in mitochondrial DNA (mtDNA) associated with Leber hereditary optic neuropathy (LHON) using direct polymerase chain reaction amplification of blood spots collected on Guthrie cards. This method using blood spots allows easily accessible screening for LHON mtDNA mutations with minimal biohazard risk and reduced expense in the storage and transport of specimens.**

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Leber hereditary optic neuropathy (LHON) commonly presents as a bilateral sequential visual loss which may be associated with

characteristic optic disc swelling and peripapillary telangiectasia.<sup>1,2</sup> Diagnosis of LHON is difficult if the affected individual falls outside the classic presentation of a young adult male with a family history of similar visual loss. Fifty per cent of patients presenting with LHON are not men between 18 and 30 years.<sup>2</sup> DNA testing for the various single base alterations in mitochondrial DNA (mtDNA) that have been associated with LHON (Table 1) is helpful in the diagnosis even though the aetiological significance of these alterations is not yet clearly defined. Recently, direct polymerase chain reaction (PCR) amplification from Guthrie cards has been used in testing for phenylketonuria<sup>3</sup> and cystic fibrosis.<sup>4</sup> We used direct PCR amplification of blood from Guthrie cards to detect the described mtDNA base changes of LHON.

## Method

Informed consent was obtained from patients or, where relevant, parents. Approximately 0.25 ml of blood was placed on a Guthrie card and allowed to air dry. With young children Guthrie cards taken at birth were obtained from the neonatal screening laboratories. A 2.5 mm (approximately 3 µl) disc of dried blood was punched from the card for each PCR fragment amplification, using sterile laboratory methods. Each blood spot was soaked in a solution of 10 µl 1% Triton X-100 (Sigma, St Louis MO), PCR buffer [20 µl 5×PCR buffer (Perkin Elmer Cetus, Norwalk, CT)] and distilled water to a final total of 100 µl (including the volume for dNTPs, primers, and enzymes) at 37°C for 1-24 hours in a 1.5 ml microcentrifuge tube. The eluted Guthrie spot was then boiled for 5 minutes and the appropriate pair of primers<sup>5</sup> 1 µl of each, 10 µl 2 mmol dNTPs, and Taq polymerase 1 µl taq (PEC) were added. A PCR was run at 94°C for 90 seconds, 65°C for 150 seconds, and 72°C for 80 seconds for 25-35 cycles. After amplification, 16 µl were then digested with the appropriate enzyme (Table 1). The resultant digest was run alongside the undigested PCR product on a 3% gel and the fragments compared under ultraviolet light (Table 2).

## Results

More than 100 patients in 33 separate families have been studied by this method and the mutations confirmed by subsequent sequencing of extracted DNA. All but one of the DNA alterations of interest could be detected by restriction enzyme cleavage because they alter recognition sites of one or more enzymes (Table 2). The exception was the 4160 T to C mutation which does not alter any known restriction site.

Table 1 mtDNA base changes associated with LHON

mt base restriction enzyme sites altered gene mutation
*3460 G>A loss of HgaI, AclI, AhaII, BbiII, HinII <sup>7,8</sup>
ND1 A52T
4160 T>C loss of AluI (with mismatch primer) <sup>6</sup>
ND1 L285P
4216 T>C creation of NlaIII, NspHI <sup>9</sup>
ND1 Y304H
4917 A>G creation of MaeI, RmaI <sup>9</sup>
ND2 D150N
5244 G>A loss of HpaII, HapII <sup>10</sup>
ND2 G259S
7444 G>A loss of Xba <sup>10,11</sup>
Cox ter K
*11778 G>A loss of SfaNI, creation of MaeIII <sup>12-14</sup>
ND4 R340H
13708 G>A loss of BstNI, Fnu4HI, ApyI, EcoRII, MvaI <sup>9</sup>
ND5 A458T
15257 G>A loss of AccI <sup>10</sup>
CytB D171N
15812 G>A loss of Rsa, Csp6I <sup>10</sup>
CytB V356M

\*There is strong evidence that these mutations are causative.

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Table 2 PCR primers, products, enzymes, and fragments used in this study

Site gene	5' Primer	3' Primer	Frag	Enzyme	PCR fragments size
3460	3275-3295	3553-3573	299	AclI*	184+115
ND1	(21-mer)	(21-mer)			
4160	4137-4159	4281-4301	165	AluI*	22+143
ND2	(4158G, 23-mer)	(21-mer)			
4216	4137-4159	4281-4301	165	NspHI*	83+82
ND2	(4158G, 23-mer)	(21-mer)			
4917	4680-4700	4980-5000	321	MaeI*	106+90+39+86
ND2	(21-mer)	(21-mer)			
5244	5195-5212	5526-5546	352	HpaII†	48+304
ND2	(21-mer)	(21-mer)			
7444	7353-7373	7650-7670	318	XbaI†	230+88
Cox	(21-mer)	(21-mer)			
11778	11660-11683	11896-11919	260	SfaNI†	130+130
ND4	(24-mer)	(24-mer)		MaeIII*	115+131+14
13708	13567-13587	13869-13889	323	Fnu4HI†	139+184
ND5	(21-mer)	(21-mer)			
15257	15081-15101	15290-15310	230	Acc I†	175+55
CytB1	(21-mer)	(21-mer)			
15812	15749-15769	15919-15939	191	Rsa†	65+126
CytB1	(21-mer)	(21-mer)			

5' Primers are complementary to the H strand, 3' primers are complementary to the L strand of mtDNA. Numbering is as in the Cambridge sequence. LHON site is underlined.

\*Boehringer Mannheim, Indianapolis, IN, USA.

†New England Biolab, Beverly, MA, USA.

Table 3 Sequences around the 4160 mtDNA

	4	4	
	1	1	
	5	6	
	6	0	
Normal	CAACT		
LHON(Qld)	CAACC		
Mismatched primer	N-CAGG		
Sequence in normal PCR	N-CAGCT		AluI site
Sequence in LHON(Qld) PCR	N-CAGCC		loss of AluI site

We created an AluI site in DNA of normal individuals by using a mismatched 23 base primer altering an A to G at position 4158 (Table 3). Loss of this AluI site suggests the presence of the 4160 T to C mutation. (The primer was designed to avoid the additional mtDNA 4136 base change found in two patients.<sup>6</sup>)

The resultant fragment sizes are reported in Table 2. With SfaNI incomplete digestion was occasionally seen although this was easily checked as real heteroplasmy or as 'pseudo-heteroplasmy' by concurrent use of MaeIII.<sup>12 13</sup>

Finally, to test if DNA from old cards could be successfully amplified we processed seven cards from children with the 11778 mutation that had been stored from 2 to 9 years in neonatal screening laboratories. The cards were soaked for up to 2 days before amplification. Fresh blood samples from the same patients all showed 100% mutant DNA. However the cards taken at birth showed apparent heteroplasmy in six of seven patients.

### Discussion

PCR of Guthrie cards can be used to confirm the diagnosis of LHON in patients presenting with optic neuropathy. This is particularly important in atypical cases suspected of being LHON, multiple sclerosis, post-traumatic, or toxic nutritional amblyopia. The correct diagnosis allows adequate and correct genetic counselling.

This Guthrie card method could also be used to PCR amplify DNA for sequencing<sup>7</sup> or GC clamped denaturing gradient electrophoresis (DGGE) methods of detecting DNA mutations. The apparent heteroplasmy may be due to cross

contamination from adjacent cards during storage, or heteroplasmy may be present in the blood at birth and the normal mitochondrial genomes may disappear in time. This approach is useful in screening patients for further research. It also simplifies the technique for screening large numbers of people to establish the prevalence of these base changes in the general population.

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