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Short Report

Multiplex MassARRAY spectrometry (iPLEX) produces a fast and economical test for 56 familial hypercholesterolaemia-causing mutations

Wright WT, Heggarty SV, Young IS, Nicholls DP, Whittall R, Humphries SE, Graham CA. Multiplex MassARRAY spectrometry (iPLEX) produces a fast and economical test for 56 familial hypercholesterolaemia-causing mutations.
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Familial hypercholesterolaemia (FH) is a common single gene disorder, pre-disposing to cardiovascular disease, which is most commonly caused by mutations in the LDL-receptor (*LDLR*) gene. About 5% of patients carry the p.R3527Q (previously R3500Q) mutation in the apolipoprotein B (*APOB*) gene and 2% carry the p.D374Y mutation in the *PCSK9* gene, but the lack of high-throughput methods make routine genetic diagnosis difficult. In this study, we developed an iPLEX MassARRAY Spectrometry mutation test to identify 56 mutations (54 in the *LDLR* gene, 1 in the *APOB* gene and 1 in the *PCSK9* gene). The iPLEX test was verified by analysing 150 DNA samples from FH patients with a previously characterized mutation and 96 no-mutation control samples. Mutations were identified in all 150 FH mutation-positive samples using the iPLEX assay, with 96% directly called by the software. The false-positive rate in no-mutation control samples was 0.015%. The overall specific mutation assay failure rate was 2.1%. In the UK, this gives an average detection rate of 75%.The FH iPLEX test is not only designed for large-scale targeted population screening for FH mutations, such as lipid clinic patients, but can also be used for population screening. The assay can easily be developed further to include additional FH-causing mutations, thus increasing the sensitivity of the diagnostic assay.

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Key words: *APOB* – diagnostic – familial hypercholesterolaemia – FH – high-throughput mutation detection – iPLEX – *LDLR* – mass spectrometry – *PCSK9*

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Familial hypercholesterolaemia (FH) was first described in 1920 (1) and has a prevalence of around one in 500 individuals, making it one of the commonest single gene disorders (2). The condition is inherited as an autosomal co-dominant trait, and affected individuals have a defect in the clearance of low-density lipoprotein (LDL) receptors, which leads to accumulation of LDL cholesterol in plasma. The clinical consequence of FH is a marked pre-disposition to pre-mature vascular disease, especially coronary artery disease (3), but this can be treated with lipid-lowering drugs and this is known to reduce mortality considerably (4). FH is known to be caused by mutations in three different genes, most commonly in the gene coding for the LDL-receptor (*LDLR*), but mutations in the *APOB* gene encoding the ligand for the LDL-receptor cause a phenotypically identical condition (familial defective APOB) with the only common mutation being p.R3527Q (previously known as R3500Q) (5). Mutations in a third gene, *PCSK9*, has more recently been

reported to be involved in around 2% of FH cases in the UK (6).

The diagnostic physical sign of FH is tendon xanthomata (TX) (1), but these are not universally present even within members of one family, and seldom develop before the third decade. In our previous study (7), only 57% of FH patients attending the lipid clinic showed signs of TX, with even less (30%) being reported TX-positive in a recent UK survey (8). Identification and early treatment of affected individuals is clearly desirable, and in the absence of TX, a DNA-based diagnosis provides confirmation of the clinical diagnosis and enables early patient management. In addition, knowledge of the specific mutation within a family group facilitates the tracking of affected individuals and eliminates the problems associated with equivocal lipid profiles (9, 10).

The *LDLR* gene is encoded on chromosome 19p13.2, and so far over 1000 mutations have been reported (http://www.ucl.ac.uk/fh) of which over 200 have been found in patients in UK lipid clinics (11). The spectrum of disease-causing mutations is quite diverse in most multicultural populations (12). Genetic screening techniques such as single-strand conformational polymorphism, denaturing gradient gel electrophoresis, denaturing high-pressure liquid chromatography, and sequencing are expensive and time-consuming. In contrast, high-throughput genetic testing techniques, such as iPLEX genotyping, allow rapid and cost-effective testing for a large number of different mutations simultaneously (13). The purpose of this study was to develop an iPLEX diagnostic assay designed to identify the molecular basis of FH in patients in the UK and to evaluate its use as a diagnostic test. It could also be used for population screening if this becomes desirable for FH.

Materials and methods

Study subjects

DNA from 150 patients in whom the molecular cause of FH has been previously defined were re-analysed using the iPLEX MassARRAY assay. DNA was collected from 92 FH patients from the Regional Lipid Clinic, Belfast Health and Social Care Trust – Royal Victoria Hospital (14), and anonymized DNA samples from 58 FH patients with known mutations from the Simon Broome Register Study (6), were sent, with the laboratory blinded to mutation status. Ninety-six FH samples that did not contain any of the 56 included mutations were tested as mutation-negative controls to establish a false-positive rate.

iPLEX methodology

iPLEX MassARRAY system (Sequenom GmbH), based on the single base extension of an extend primer into the region of DNA variation, allows the detection of insertions, deletions, and single base substitutions in amplified DNA at multiplex levels of up to 40 DNA variants (Fig. 1). The extend primer is designed to hybridize adjacent to the variant being assayed and is extended by one of four mass-modified terminator molecules into the site of the nucleotide variation (mutation). The primer extension products are analysed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry and the genotypes differentiated on the basis on the mass of each allele (Fig. 2). This method provides an accurate, rapid and economical genotyping approach. Each test consists of four assay plexes run simultaneously on a 384-well chip array for 96 samples, alternatively four chips can be used to assay 384 samples or a combination of both.

The most common FH-causing mutations from the British population were selected and submitted to MASSARRAY Assay Design 3.1 software. The design process produced four assay plexes; the mutations included in these assays are shown in Table 1. All polymerase chain reaction (PCR) and iPLEX reactions were performed under standard conditions (15) on a 384-well plate, allowing the analysis of 92 samples (and four non-template controls) against the four assay plexes on the one plate (Fig. 1). The multiplex PCR was carried out using Qiagen HotStarTaq in a 5 μl volume on a MJ research PTC-200.

Results and discussion

The assays were tested by analysing 150 FH samples that had a known causative mutation discovered previously by sequencing (mutation-positive iPLEX controls) along with 96 samples which had been previously shown by various methods including single stranded conformational polymorphism and sequencing (6), not to contain any of the 56 tested mutations. Of the 150 mutation-positive samples, 144 (96%) were automatically called by the iPLEX MASSARRAY Typer 3.4 software (Sequenom, GmbH, Germany) using a three-parameter model that calculates the significance of each of the putative genotypes based on the relative confidence of the final genotype call. The remaining six mutation-positive samples did not reach the required confidence level threshold for

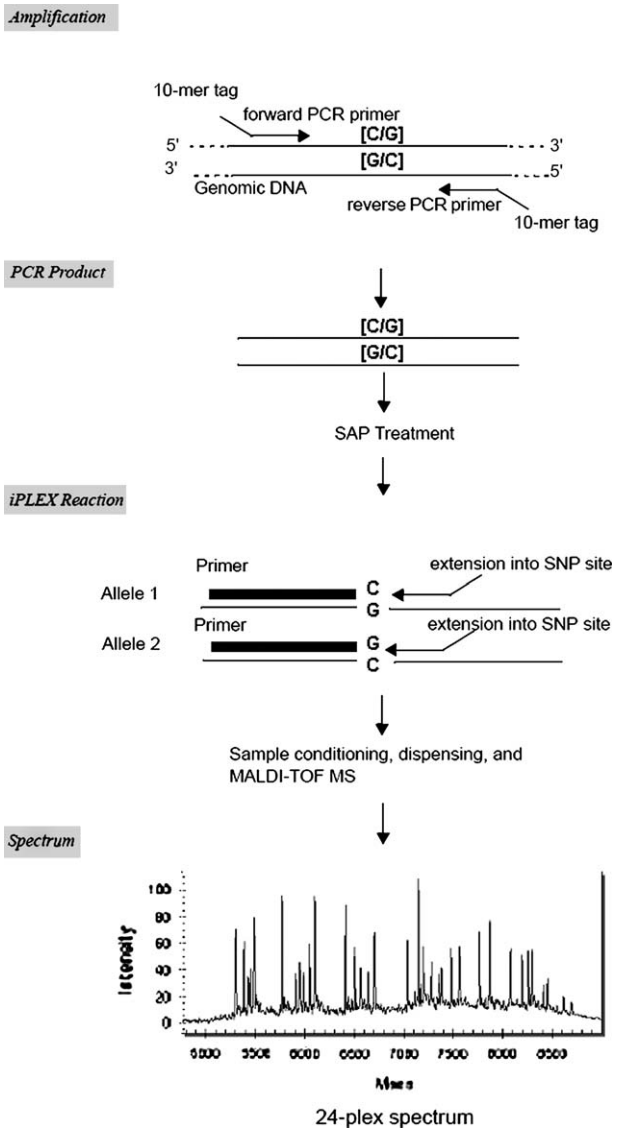

Fig. 1. Overview of the iPLEX FH diagnostic test process (the scheme depicts a single assay). The multiplex PCR is followed by SAP treatment to remove any excess dNTPs before undergoing single base primer extension (iPLEX reaction). The products are cleaned and transferred to the Sequenom Chip and analysed on the mass spectrometer.

automatic calling by the software, but the graphical overview indicated that manual inspection of the mass spectra was warranted. On inspection of their mass spectra, the mutations were identified and were included in the batch of samples to be sequenced for confirmation (four c.118delA and two c.652_654del3). All nomenclature is as suggested by the Human Genome Variation Society (http://www.hgvs.org/mutnomen/), with nucleotide number 1 being the A of the ATG methionine initiator codon and amino acid 1 being the methionine initiator codon. A further three samples (0.05% of genotypes) from the 96 mutation-negative controls had individual assays that were not called automatically by the software but looked like possible mutations on the mass spectra. Overall, 100% of the 150 mutation control samples were correctly identified of which 58 were assayed blind.

In the mutation-negative control group, one sample was called as a false positive for p.W483R and one mutation-positive sample was called as a false positive for an additional mutation, c.211delG. A total number of 13,776 assays were typed in the 246 samples, with two false positives identified, giving a false-positive rate of 0.015%. This demonstrates the need to sequence the positive calls to check for false positives, whose origins turned out to be noise spikes in the mass spectra.

For all 246 samples, 12 of the 984 plexes failed (1.2%) of which four occurred in a sample that had a mutation determined in one of the other three plexes. This leaves a plex failure rate of 8 (0.81%) for plexes that needed repeat. Individual assay 'no-calls' for a sample are repeated if there are more than three failures or if more than one common mutation has failed. Single common mutation failures were sequenced. For all 246 samples, there were 122 individual assay no-calls, which along with the 12 failed plexes (174 individual assays) equate to a specific mutation assay failure rate of 2.1%.

Sequenom GmbH state that up to 40 different single nucleotide polymorphisms can be multiplexed in one assay plex using the new iPLEX Gold reagents, which leaves the potential to further optimize this FH iPLEX test for clinical use. However, there are limitations to this technique because certain mutations cannot easily be tested with this multiplex assay due to the constraints of the surrounding sequence, e.g. p.Q384X previously known as Q363X is in a region that has high dimer potential for the unextended primer in either direction, and as such cannot be incorporated into the assay at this time. In addition, cross-homology between primers (both PCR and extend primers) can lead to interference in assaying mutations relatively close together within a gene of interest, e.g. p.D482N and p.D482H, which represent different nucleotide substitutions within the same codon. However, these were separated into two different assay plexes in the FH diagnostic test, thus avoiding the problem.

In its current form, the FH test covers 83% of the known Northern Irish point mutations and 78% of the point mutations from a population in the north-east of England, and around 65% of point mutations identified in the Simon Broome FH sample which included patients from

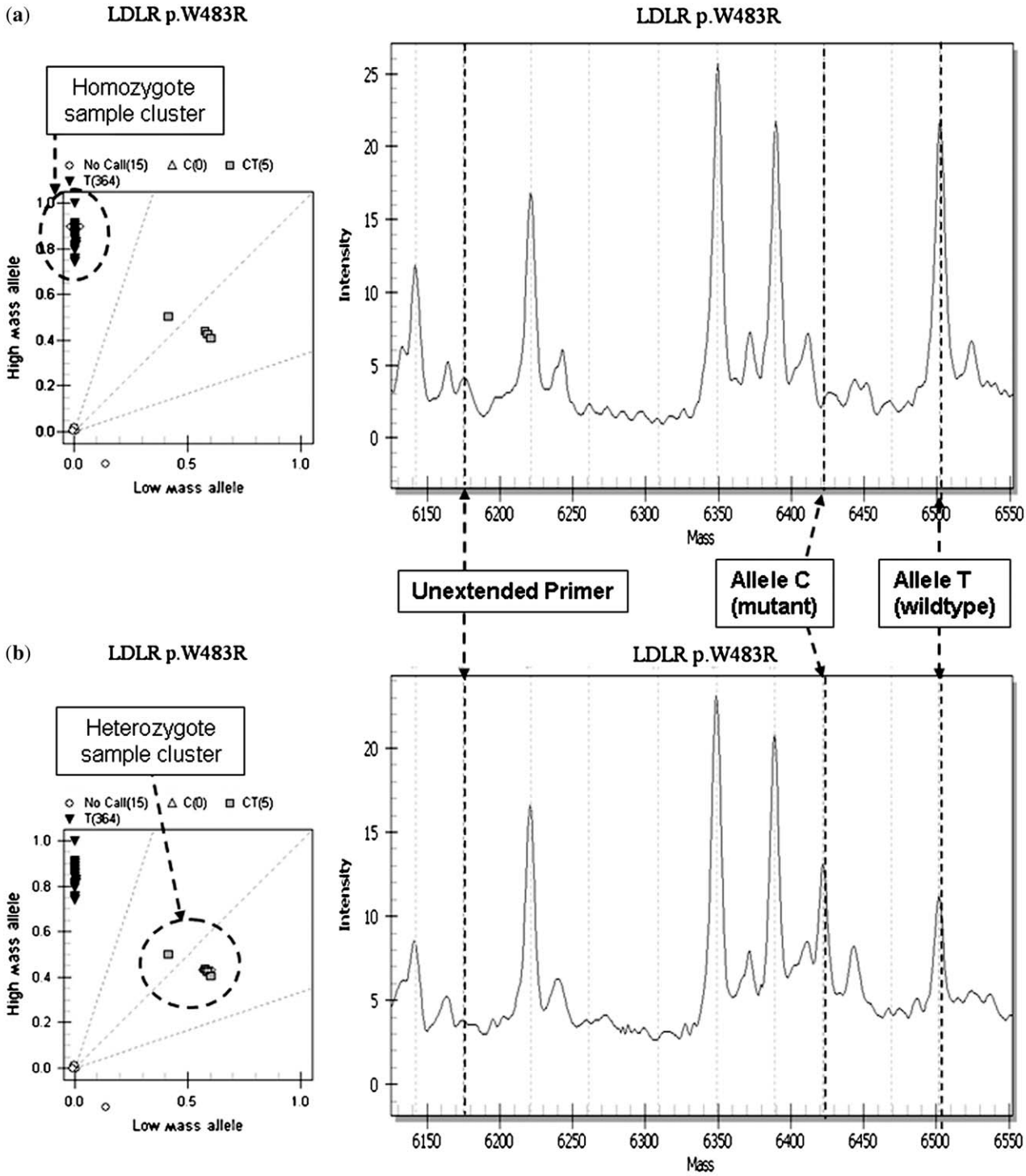

Fig. 2. Typical raw data output from the multiplex MassARRAY spectrometry iPLEX assay (LDLR p.W483R). The graph on the left shows the clusters of all samples on the chip, with the non-template controls at the bottom left. The highlighted sample spectrum is shown on the right, with the unextended primer (UEP) marked on the left with the dotted line, and the two potential products marked right with dotted lines. A homozygote wild type is shown in (**a**), while the heterozygote LDLR p.W483R is shown in (**b**).

London, Manchester and Oxford (6). Further work after the initial assay validation study has enabled the addition of the p.E228X and p.P685L mutations (previously known as E207X and P664L, respectively) into two of the existing plexes (A and B, respectively). These new

Table 1. The FH-causing mutations in *LDLR*, *APOB* and *PCSK9* that are included in the iPLEX test[a]

Mutation		Plex
LDLR c.-14C>A		C
LDLR p.E31X	E10X	D
LDLR c.118delA	FsI19	B
LDLR c.211delG	FsG50	C
LDLR p.R78C	R57C	D
LDLR p.W87G	W66G	B
LDLR p.E101K	E80K	A
LDLR p.P105S	P84S	D
LDLR c.313 + 1G>A	ivs3 + 1G>A	C
LDLR c.353delA	FsD97	B
LDLR p.D175N	D154N	D
LDLR p.C184Y	C163Y	C
LDLR c.585insT	FsS174	A
LDLR c.652_654del3	dG197	D
LDLR c.680del2	FsD206	B
LDLR p.D227E	D206E	B
LDLR p.E228X[b]	E207X	A[b]
LDLR p.C231X	C210X	D
LDLR p.E258X	E237X	C
LDLR p.E277K	E256K	A
LDLR c.925_931del7	FsE287	D
LDLR c.933delA	FsK290	B
LDLR p.C313Y	C292Y	C
LDLR p.R350X	R329X	A
LDLR p.Q366R	Q345R	D
LDLR p.C379R	C358R	B
LDLR p.K390X	K369X	C
LDLR c.1185delG	FsV374	D
LDLR p.A399D	A378D	C
LDLR p.E408K	E387K	A
LDLR p.R416W	R395W	D
LDLR p.R440G	R419G	C
LDLR p.D482H	D461H	C
LDLR p.D482N	D461N	B
LDLR p.W483R	W462R	D
LDLR p.V523M	V502M	A
LDLR p.P526S	P505S	C
LDLR p.N564H	N543H	A
LDLR p.G565A	G544A	D
LDLR c.1706-10G>A	ivs11-10G>A	A
LDLR c.1706-1G>A	ivs11-1G>A	C
LDLR p.L599S	L578S	D
LDLR p.P608L	P587L	B
LDLR c.1845 + 11C>G	ivs12 + 11C>G	C
LDLR c.1846-1G>A	ivs12-1G>A	A
LDLR p.D622A	D601A	C
LDLR p.R633C	R612C	B
LDLR c.1986_1987delAGinsC	FsG642	A
LDLR p.C667F	C646F	D
LDLR p.C677R	C656R	A
LDLR c.2061insC	FsI666	C
LDLR p.P685L[b]	P664L	B[b]
LDLR c.2292delA	FsI743	A
LDLR c.2389 + 1G>A	ivs16 + 1G>A	B
LDLR c.2458_2466del9	dS799-N801	A
LDLR p.G844D	G823D	C
APOB p.R3527Q	R3500Q	A
PCSK9 p.D374Y	D374Y	C

[a]Human Genome Variation Society nomenclature (http://www.hgvs.org/mutnomen/) is shown in column 1, while the previously used nomenclature is given in column 2. The third column of each mutation indicates which plex the mutation is in.
[b]A mutation that has recently been added to the iPLEX test.

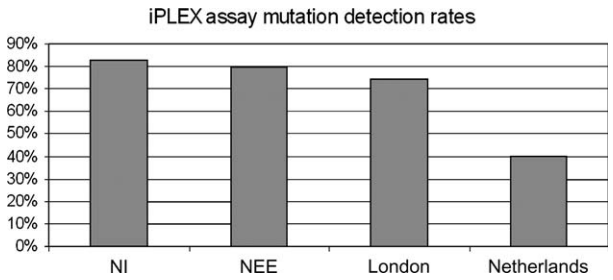

Fig. 3. FH iPLEX assay mutation detection rate in the Northern Irish (NI), North East of England (NEE), London and Dutch populations.

mutation assays were tested in 90 and 92 samples, respectively, and were successfully identified in four and five positive control samples, respectively. The p.E228X mutation was typed successfully in 89 of the 90 samples tested, while p.P685L gave a definitive signal in all of the 92 samples. The addition of these two mutations increase the coverage in the Simon Broome sample by 9% to around 74% (Fig. 3) and demonstrates the flexibility of the Sequenom iPLEX methodology for diagnostic analysis. There is still scope for the assay to be modified further, thereby increasing the coverage and diagnostic potential of this accurate and rapid test. Once DNA has been isolated, a single operator can amplify the samples in 3 h laboratory time, over 2 days, for loading on to the analyser. The mass spectrometry system can read the chip and produce a report within an hour. At the present time, the overall costs for an FH iPLEX screen including a diagnostic report is £50 per sample (based on a run of 84 samples), which equates to £0.86 per mutation tested. Thus, the test is very cost effective compared with other mutation screening methods.

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