

ORIGINAL ARTICLE

Multiplex ligation-dependent probe amplification: a novel approach for genetic diagnosis of Porphyria

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Porphyrias are disorders caused by the genetic defects of enzymes of the heme pathway and are characterized by such a wide genetic heterogeneity that even the molecular analysis is not always decisive for a correct diagnosis. In the past few years, deletion with a size range of few kilobase pairs have been reported. These peculiar mutations, missed by both sequencing and cytogenetic techniques, have been identified by time consuming and technically demanding methods. To provide a rapid and sensitive method for the detection of deletions responsible for porphyria, we successfully designed and tested seven chemically synthesized probe sets specific for each heme gene and their flanking regions, to be used in multiplex ligation-dependent probe amplification technique.

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INTRODUCTION

Porphyrias (mim 125270, 176000, 263700, 176100, 121300, 176200 and 177000) are rare diseases caused by genetic defects of the enzymes involved in the heme pathway (EC: 4. 2.1.24, 2.5.1.61, 4.2.1.75, 4.1.1.37, 1.3.3.3, 1.3.3.4, and 4.99.1.1, respectively). Porphyrias are classified as 'acute' and 'chronic' based on their clinical manifestations, or as 'hepatic' and 'erythropoietic' according to the two main sites in which the excess metabolites are produced.¹ Acute porphyrias are characterized by mild to severe acute attack with neuromuscular dysfunctions affecting the autonomic, peripheral or central nervous system, that can lead to death if not detected. In approximately 10% of cases, acute liver failure has been reported.^{2,3} Chronic Porphyrias are characterized by mild to severe cutaneous photosensitivity and chronic involvement of the liver with increased risk of hepatocarcinoma.⁴ Nowadays, the diagnosis of Porphyria is based on clinical symptoms supported by specific pattern of porphyrins in blood, urine and stools; however, because of physiological fluctuations, the measurements of porphyrinic metabolites are not always reliable.⁵ The erythrocyte enzymes' activity confirms the diagnosis. However, the normal and pathological values might overlap, becoming misleading.⁶ Genetic analysis would be the only reliable tool to establish a correct diagnosis of Porphyria even though the high genetic heterogeneity makes this approach difficult. Although restricted geographic areas seem to have a high prevalence of specific molecular abnormality,⁷ most of the reported mutations have been detected in a single case or a family.^{8–10} Moreover, approximately 10% of porphyria cases remain devoid of molecular diagnosis. The reported molecular abnormalities of the different forms of Porphyrias are so far limited to point

mutations or small deletions/insertions in the coding regions and in the splicing junctions of the seven genes involved in the heme pathway: ALAD (GeneID: 210); HMBS (GeneID: 3145); UROS (GeneID: 7390); UROD (GeneID: 7389); CPOX (GeneID: 1371); PPOX (GeneID: 5498); and FECH (GeneID: 2235), which are readily detected by PCR and sequencing techniques (<http://www.hgmd.cf.ac.uk/ac/index.php>). Recently, single gene or long portion gene deletion have also been reported.^{11–13} These peculiar mutations of the size of a few kilobase pairs are missed by both PCR and by cytogenetic techniques, including fluorescent *in situ* hybridization. Long PCR, absence of Mendelian segregation of polymorphisms and gene dosage, commonly used to identify large deletion, are time consuming and technically demanding. Moreover, the feasibility of these techniques depends on the availability of DNA from biological parents, good quality DNA, and polymorphic sites with high frequency and efficiency of multiplex PCR.^{14,15} For all these reasons, it is possible that long gene deletions have gone undetected in patients with clinical symptoms and biochemical signs of porphyria. Recently, a rapid and relatively easy technique for DNA quantitative analysis, named multiplex ligation-dependent probe amplification (MLPA), has been described.¹⁶ This method, based on the hybridization and ligation of two adjacently annealing oligonucleotides, is suitable for detecting single gene or long sequence deletion. Each oligonucleotide pair (probe) is designed to have common ends, which means that all probes can be simultaneously amplified with one universal primer pair. By using a single fluorescently labelled universal primer, the resulting products can be easily separated based on size and then quantified. To improve the molecular diagnosis of porphyria and to

simplify the detection of deletions in the heme genes, we designed and tested seven chemically synthesized probe sets specific for each heme gene and their flanking regions covering genomic regions from 39 to 152 kb. In this paper, we report the validation of the MLPA using our set of probes and we confirm that MLPA is a very useful and reproducible method for improving molecular diagnosis of Porphyria.

MATERIALS AND METHODS

Probe design

A total of 144 probes were designed to detect the rearrangements on the heme genes: 16 for ALAD; 23 for HMBS; 20 for UROS; 18 for UROD; 24 for PPOX;

16 for CPOX; 24 for FECH; and 3 for internal control genes (ILKAP, DACH and DEFB129). The sequences of the probes are shown in Table 1. The probes were split into 7 sets, each containing the specific probes for a single heme gene and 3 control probes for a maximum of 27 probes. Each probe sets covered the following genomic regions, respectively: gene ALAD: NC_000009:115174000–115213000 (reverse complement); gene HMBS: NC_000011:118442000–118494000; gene UROS: NC_000010:127443000–127533000 (reverse complement); gene UROD: NC_000001:45225000–45320000; gene CPOX: NC_000003:99733000–99863000 (reverse complement); gene PPOX: NC_000001:159334500–159451000; gene FECH: NC_000018:53290000–53442000 (reverse complement). These regions contained at least the target gene with upstream and downstream genes. In the presence of small genes, close to one another,

Table 1 Names and sequences of probes

| Name | Upstream hybridizing sequence | Downstream hybridizing sequence | s.d. |
|-------------|---|--|------------------|
| ALAD | | | |
| 1 POLE3_4 | CTCAGTGCTAACAACTTTGCAATGAAAGGAAAGCGGAAGACG | CTGAATGCCAGTGATGTGCTCTCAGCCATGGAAG | 0.08 (0.90–1.14) |
| 2 ALAD_Prom | CTAGGCTGGGCAAAGGAATGCTGT | GAAGTGAAACCTGGGCTGTAACCGCCTCCGAG | 0.08 (0.80–1.04) |
| 3 ALAD_2 | GGAGTTAACACACCAGGGTCTAGT | CCTTTTGTCTGCTGTGTGGCT | 0.05 (0.94–1.08) |
| 4 ALAD_3 | TCCAACCTCATCTACCCATCTTTG | TCACGTGAGTCTCCAAGAATGGG | 0.06 (0.88–1.03) |
| 5 ALAD_4 | CTTCCCACAGCTACCAATCCATATCCCACCCCGCTCTTGCCAG | GGATGTTCTGATGACATACAGCCTATCACCAGCC | 0.05 (0.90–1.03) |
| 6 ALAD_5 | GCCTACGCTGTGCTTGTATCTTTGGCGTCCCAAGCAGAGTT | CCCAAGGTGAAGAATCAAAGGAAGGGCTAAG | 0.06 (0.88–1.08) |
| 7 ALAD_6 | CGAGGAGTCCCAGCTATTGAGGCAATCCATCTGTTGAG | GAAGACCTTCCCACCTCCTGGTG | 0.02 (0.98–1.04) |
| 8 ALAD_7A | GCTCCCCAAAACCCAGTCACTG | TCCTGAAGGGCTCCTGAGTGAAAACGG | 0.06 (0.86–1.02) |
| 9 ALAD_7B | CAAGGCAGGTGAGTGAACCACCAGCAGGGATGGGCACC | TCTGGGTCAAGGGTGGCAGAGTG | 0.11 (0.92–1.21) |
| 10 ALAD_8 | GATGTCAGGTGGTAGCCCGTCGGACATGATGGATGGACG | CGTGAAGCCATCAAAGAGGCCCTGATG | 0.07 (0.88–1.10) |
| 11 ALAD_9 | CATAGGTATCGGTGATGAGCTACAGTGCCAAATTTG | CTTCTGTTTCTATGGCCCTTTCCGGTGAG | 0.05 (0.90–1.06) |
| 12 ALAD_10 | CACCCTGATGACTCTGCTTTGCGAG | GATGCAGTAAGTCAAGCCAGCTTTTGG | 0.09 (0.86–1.14) |
| 13 ALAD_11 | GACATGCTCATGTTGAAGCCGGGAATGC | CCTACCTGGACATCGTGGGGAGGTAAGG | 0.01 (0.97–1.01) |
| 14 ALAD_12 | CATTTCCAGCTGTCTCCCTGTCTGCTTCCCTGCAGCACCTGAC | CTCCCTCTCGCGGTGACACGCTCTCTGGAGAGTTTGCCATG | 0.07 (0.96–1.15) |
| 15 ALAD_13 | GCAACTTTCTGCATCTCTCCCAACACAGGTGCTGACATCATCATC | CTACTACACCCGAGCTGCTGC | 0.02 (0.95–1.02) |
| 16 HDHD3_2 | CTGACCCACAAGGCCTGGAAAGAG | GTGATTGTTAGGTTGCGCAGAGGTGGTCTTATCCAG | 0.08 (0.95–1.19) |
| HMBS | | | |
| 1 VPS11_1 | GCCACACCTGCTTCTGGATCCGCTGCTTCCAAGTTCCTTTGC | CTCCCTCTGGCATCACTGTCTGCGACTCAGG | 0.02 (0.99–1.05) |
| 2 VPS11_6 | CTGTGCAACAAGTTCATAGCCTATAGCACCGTCTTTGAGG | ATGTAGTGGATGTGCTTGTGAGTGGGGCTCCCT GTACGTGTGACGC | 0.03 (0.97–1.04) |
| 3 VPS11_16 | CTGGCTGACATGCCAGGGCTCCACTCTCATCT | AATGTCACAGCCCTCAGAACTAAAGCG | 0.04 (0.96–1.06) |
| 4 HMBS_1 | CACCCACACACAGCCTACTTTCCAAG | CGGAGCCATGTCTGGTAACGGCAATG | 0.03 (0.98–1.06) |
| 5 HMBS_2 | CTGAGGAGGGCAGAAGTACTGAGGAAGTTAAAGG | GACCAAGCCTGGAGTATTTCCCACTGTGAGACTCAGCTGGC | 0.04 (0.96–1.07) |
| 6 HMBS_3 | CAGGAAGAAAACAGCCCAAAGATGAG | AGTGATTCGCGTGGGTACCCGCAAGAGC | 0.04 (0.93–1.05) |
| 7 HMBS_4 | CAGCTTGTCTGCATACAGACGGACAGTGTGGTGGCAACA | TTGAAAGCCTCGTACCCTGGCCTGC | 0.04 (0.96–1.06) |
| 8 HMBS_5 | CTCTCTCCTCAGTTGCTATGTCCACCACAGG | GGACAAGATTCTTGATACTGCACCTCTCTAAG | 0.02 (0.98–1.05) |
| 9 HMBS_6 | CTGTCCGGCAGATTGGAGAGAAAAGCCTGTTTAC | CAAGGAGCTTGAACATGCCCTGGAGAAGAATG | 0.04 (0.99–1.10) |
| 10 HMBS_7 | CCATCTCTATAGAGTGACCTGGTTGTTCACTCCTTGAAGG | ACCTGCCCACTGTGCTTCTCTGGCTTCCAC | 0.03 (0.98–1.07) |
| 11 HMBS_8 | GGGAAAACCCCTCATGATGCTGTTGCTTTACCCAAAAA | TTTGTGGGAAGACCCCTAGAAAACCTGCCAGAGAAGAG | 0.05 (0.93–1.07) |
| 12 HMBS_9 | GTAACCTCTCTGGGCGAGTGGTGG | GAACCAGCTCCCTGCGAAGAG | 0.04 (0.94–1.07) |
| 13 HMBS_10 | CCGACACTGTGGTCTTAGCAAC | TCTCCACAGCGGGAAACCTCAACACC | 0.03 (0.98–1.05) |
| 14 HMBS_11 | CAGATCCTGCACCCCTAGGAATG | CATGTATGCTGTGGGCCAGGTAC | 0.04 (0.91–1.04) |
| 15 HMBS_12 | CATCTTGGATCTGGTGGGTGTGCTGCACGATCCCGAGA | CTCTGTTCTGCTGCATCGCTGAAAGGGCCTTC | 0.03 (0.99–1.09) |
| 16 HMBS_13 | GGCTGCAGTGTGCCAGTAGCCGTGCATA | CAGCTATGAAGGATGGGCAAGTAAGTGG | 0.01 (0.98–1.02) |
| 17 HMBS_14 | CTGGAGGAGTCTGGAGTCTAGACGGCTCAGATAGCATA | AAGAGACCATGACGGCTACCATCCATGTC | 0.03 (0.95–1.06) |
| 18 HMBS_15 | CATGAAGATGGCCCTGAGGATGACCCACAGTTGGTAGG | CATCACTGCTCGTAACATTCCACGAGGGCCCAAGTTGGCTGC | 0.06 (0.95–1.12) |
| 19 DPAGT1_9 | CAATGATCAAGGGACTCAGACATCATAGAA | GAGTCAAGCAGCTGATATCGAATGGAG | 0.03 (0.97–1.06) |
| 20 DPAGT1_4 | CTCTACCAGTGAAGACAATGATGGAAGCAGA AATGACTAGTACTGGCCA | GCCTTAGGCCGTTAATCTCTGCTAGGATATTGATGGC | 0.07 (0.92–1.13) |
| 21 DPAGT1_1 | CCTCTCTAAGGCAACCTATGTTCTGCCCGCTGCACCCGCCTA | TCTACTGTTTCCGCCCGGATCTTGTCTGCTGAACAACCATTAC | 0.05 (0.94–1.07) |
| 22 TMEM_1 | CTCTTCCGCTTCAAGTCTTTCCGGGAGAACTGGCAGCG GGCTTGGGTGCGAGC | GCTGAACGAGCAGGCCTGCAGAAACGGGGTGGAGTTGGAC | 0.03 (0.98–1.05) |
| 23 TMEM_14 | CTTTTCTATTATATGTGTGGCTTAGGACCCCTCCGTGAA CAGATGATAG | AGGGCATCTCTCCAGGTGACCCCTCTTTTCTGTCT | 0.03 (0.98–1.08) |

Table 1 Continued

| Name | Upstream hybridizing sequence | Downstream hybridizing sequence | s.d. |
|-------------|---|---|------------------|
| UROS | | | |
| 1 | BCC1P_8 CTTATTATGACAGAGCAATTCACACAGTCATGAAGTTCC | CTGGAATCACTTAGAGTTGATCTTGATTGAATTAGTC | 0.06 (0.91–1.10) |
| 2 | DHX32_8 CAAATTGCTTTTCACATGTGCCACATGGAGCTGAAGAG | GCTGCCTTGACTTGTGGGAAGACATTTTACATCCC | 0.09 (1.00–1.18) |
| 3 | BCC1P_6 CTTCACAAATGTCTTACTAATCAGAAGGTAAAAGTAG | CACCTCCACATGGCTTATTGGTTCTGTGTGC | 0.05 (0.90–1.06) |
| 4 | BCC1P_3 CACACTCCCAATATGGTTCTGTGAATTAAGAGATCTG | TTAGTTCTGCAGTGTTCACAGGAGCC | 0.09 (1.00–1.19) |
| 5 | UROS_Prom CTTACAGTCACGTCCAGCTTGC | GGTCCCATCTTGCTTTTTCTTCC | 0.03 (0.94–1.00) |
| 6 | UROS_IVS1 CAGTAAAGGGTGACTGTTGTTATTGCTGA | TACCAGAACACCATGACATAGAAGG | 0.03 (1.00–1.06) |
| 7 | UROS_2 CTGCCAGGCAATAATGAAGTTCTTTACTGAAG | GATGCGAAGGAAGATGACTGTGGCCAGGATCC | 0.05 (1.00–1.10) |
| 8 | UROS_3 CCAATCCAGAACTATATCTTTTCATTTCCAGGAATTAGGAT TATATGGACTT | GAAGCCACTTTGATCCCCTGTTTTATCGTTTGAGTTTTGTC | 0.09 (0.80–1.00) |
| 9 | UROS_4 CTTTCTATCCTGAAGATTACGGGGGACTCA TTTTTACCAGCCCCAG | AGCAGTGGGAAGCAGCAGAGTTATGTTTGGAGCAAAAC | 0.07 (0.86–1.00) |
| 10 | UROS_5 CAGTCTGGGAAAGGTCTCTGAAAGAAAATGGAATGC | CAAGTCAGTGTATGTGGTTGGAAATGCTACTGCTTCTCT AGGTAAGGAGTC | 0.03 (0.96–1.10) |
| 11 | UROS_6 CCAAGTGAAGTAAATTGGCCTGGATAC | AGAAGGAGAAAACCTGTGGAATGCAG | 0.08 (1.00–1.16) |
| 12 | UROS_IVS6 GGATTAGGACCCAGTTCTCTTGTTCT | CTGGGACTTTTTAGGAGTGTCTCTG | 0.03 (1.00–1.05) |
| 13 | UROS_7 CTCAGCACTGCCTCTTCTATTTCCCTGTG | GAAACCTCAAAGAGAAATCCTGCCAAAAGC | 0.07 (0.84–1.00) |
| 14 | UROS_8 GTAAGTCTTTCTGCTTTTGATTGTGCCAG | GGATTGCCATGGAAAGCATAACTGTGTATC | 0.05 (0.91–1.00) |
| 15 | UROS_9 CTCTGGCCTCACATACAGTCTCAAGCACATTCAG | GAGTTATCTGGTGACAATATCGATCAAATTAAGG | 0.07 (1.00–1.14) |
| 16 | UROS_IVS9 CATCATGTGAGCATTATTGACACAGG | AGTGAAGGCTCCTGTGAAAAAGCTGC | 0.08 (0.95–1.20) |
| 17 | UROS_10 CTCTGTGGAAGCCAGCTTAAACC | CTAGCCCTGTGAGAGCTTCTGTGC | 0.04 (0.97–1.05) |
| 18 | MMP21_IVS1 GGGACAGCCCTGCAAGGTTCCCAACACCGCAATAG TAAGGATC | CTAACAGCTGTCTGGGGTGGCATCCGTGGACTTCAC | 0.08 (1.00–1.17) |
| 19 | MMP21_5 CAGCTGGTACTGGCTTTATGAAAATCGAAACAATAGGACAC | GCTATGGGGACCCTATCCAAATCCTCACTGG | 0.06 (0.86–1.00) |
| 20 | MMP21_7 GAAAACCGGATTTTCAGTAGCTGAAGAAAATATGGCACTG TAAGTTAAAAC | CCAATGGGAAAAGCCTTAGTTGAAC | 0.02 (0.99–1.05) |
| UROD | | | |
| 1 | HECTD3_21 GAGGGAGGAACGGTAAACTGGGGCAGGGGTG TGCATAGCTCTAG | GAACAGGTTAAAGGCTAGGCTTTAGAGTTGGGAAAGGG | 0.03 (0.93–1.02) |
| 2 | HECTD3_11 CGAGTAAGTCCCTCACTCTCATGCCATACCTGTAGTC | CAGGGGCTTTTCATATTTGTGAGGGGCTTG | 0.02 (0.96–1.05) |
| 3 | HECTD3_5 CTGTCTGCCAGAGCCTACTTACTTGACAATGGTGC | CCTTCTCATAGTAAGCCGTACCCAGTGTGGCAC | 0.05 (0.90–1.10) |
| 4 | UROD_1 CAGATTCAAGTTAAATGTGGATTGAGCTCGCAG TTACAGACAG | CTGACCATGGAAGCGAATGGGTTGGG | 0.06 (0.91–1.10) |
| 5 | UROD_2 GAAACAGACTACACTCCCCTTTGGTGCATGCGCCAG | GCAGGCCGTTACTTACCAGGTAAGAGTC | 0.04 (0.95–1.06) |
| 6 | UROD_3 CAGGACTTTTTCAGCACGTGTCGCTCTC | CTGAGGCTGTGTGAACGACTCTG | 0.04 (0.92–1.05) |
| 7 | UROD_4 CTGCCACTAGCAACCTGTCTCCTG | TTTCTACAGCCACTGCGTCGCTC | 0.02 (0.96–1.05) |
| 8 | UROD_5 GGATCCAGAAGTGGTAGCCTCTGAG | CTAGGCTATGTGTTCCAAGCCATCACC | 0.03 (0.96–1.09) |
| 9 | UROD_6 CCATCTTTCTATCCTTCTCTAGTGGACCCT | GATGACATACATGGTTGAGGGTGGTG | 0.04 (0.93–1.09) |
| 10 | UROD_7 GCATTGCAGCTGTTGAGTCCCATGCAGG | GCATCTTGGCCACAGCTCTTCAACAAGTTTGC | 0.04 (0.92–1.14) |
| 11 | UROD_8 CGGTAAGCCATGGAAGGGTGAGG | CCTTGAGGTTGAGGTGGGGGTG | 0.06 (0.91–1.14) |
| 12 | UROD_9 GCTGGCTTTGCTTCCAGGGAGTGTGTG | GGGAAGACGGTGACATTGCAG | 0.07 (0.86–1.10) |
| 13 | UROD_10 GGAGGAGATCGGGCAGTTGGTGAAG | CAGATGCTGGATGACTTTGGACCACATCGCTAC | 0.05 (0.93–1.10) |
| 14 | ZSWIM5_14 GAATTGTCGGAGTCTCAAAGTGTCTCCTATAG | GTGTCAAGAGAAGGGGAGGGTCTATTTTAAAG | 0.05 (0.94–1.09) |
| 15 | ZSWIM5_13 GTTGGGAGGACAGCGGGTGTGCTGAGGCTTAAAG | GTCTATCTATGCTCACCTTTGGCAG | 0.03 (0.93–1.09) |
| 16 | ZSWIM5_IVS8 GCAAATCTTATTTGAGGTTGCTGTATCACAACCTG AGTATACATG | CAAATGGCTTAGGCTTACATAAATATCC | 0.05 (0.92–1.09) |
| 17 | ZSWIM5_7 GCACACCATAAAGCCCCTGGAAAATAAAGCAAC CCATATTC | TTAACATCTCTCCTTTGATGTGTGTGAAGCCTTAATG | 0.02 (0.93–1.03) |
| 18 | ZSWIM5_IVS5 GTCTGGTCAGCCTCTCCACTAGATTTCAAGTAT TCAGTTACAG | CTATGTCAAGTGAGCTAGGTATAATGCATGAAGCC | 0.02 (0.94–1.03) |
| CPOX | | | |
| 1 | UP_1 GAATACCTTTAAAGTTTACCACATGTGATGTAG | GTTCTTGATAAGGATCTTTATTAGGCTCAAGTCTC | 0.03 (0.94–1.02) |
| 2 | UP_2 CAGCACTGATCTAAGATATTCAAGACCTTTAGGTGAATACAAG | CACCTACTTTCAATGGGTATGAGTATTAGGTTGTC | 0.05 (0.87–1.02) |
| 3 | UP_3 GTAGCGTATCTCAGCTAAAACCTTATTACTTCAATGAATTAAC | CTTTAAACCAGCCTCCCTTTTCTAAGCCTTTAGTG | 0.02 (0.95–1.00) |
| 4 | UP_4 GAAAAGCGCCTTTGTGCTAACTTGTAAGACCTTAAATG | GATGTTTGATAATGCCAAAGATGATGGTACTAACAGCTGTTTG | 0.05 (0.96–1.06) |
| 5 | CPOX_1 CCAACTTTCTGTGGACCGGTGG | GAGAGGAAGGAAGGTAAGGAGGC | 0.04 (0.94–1.06) |
| 6 | CPOX_2 GTGTGTTTTCGAAAAGGCTGGGGTGAAGCTTTCTGTTGTTTCATG | GAAATCTTTCAGAGGAAGCTGCAAAACAATG | 0.05 (0.96–1.09) |

Table 1 Continued

| | Name | Upstream hybridizing sequence | Downstream hybridizing sequence | s.d. |
|------|-----------|--|---|-------------------|
| 7 | CPOX_3 | CATTTTGTGCTATGGGCGTGAGCTCTGTTATCCACC | CCAAGAATCCTCATGCTCCTACTATCCATTTTC | 0.03 (0.94–1.04) |
| 8 | CPOX_4 | CAAGCAGTGGTGGTTGGTGGTGGATGTGAC | CTCACTCCAACATACTTGAATCAAGAAGACG | 0.04 (0.94–1.05) |
| 9 | CPOX_5 | GTACAGAGCTGTGCCAGGGCTGTAGTTCTTCTTACATTCC | CCTTGTGAAAAAGCACTGTGATGACTCATTTC | 0.05 (0.93–1.07) |
| 10 | CPOX_6 | CACAAAGTTTGGCCTCTTCACTCCAGGATC | CAGAATTGAAAGTATCTTGATGCTTTACCTCTAAC | 0.04 (0.92–1.02) |
| 11 | CPOX_7 | CATCCAAGGGACTGGGTGCGTTGATGCAG | GCAGAATGGCTGTGCAGGGGTTTGGAG | 0.04 (0.93–1.06) |
| 12 | DOWN_1 | CCACTGTGTTTCATGTCTCACTTTATGATTG | GGAGATTCTGAAGTTGGCAATGTCTACAATTCC | 0.02 (0.96–1.01) |
| 13 | DOWN_2 | CAATACCTTGTCTCACAGTTTAGTGCCTC | CAGCCCCTAACATTATTTGCATTCAAG | 0.05 (0.92–1.08) |
| 14 | DOWN_3 | CCAAATCCTACCTGTTTTAGACCAATCCAGG | GATATTATGTATGGCAGCTGGTGGTTAC | 0.05 (0.94–1.06) |
| 15 | DOWN_4 | CTACACTTCACAGCTCCACCAG | GCATTACACTTTGAAACCTAAATGGAGGC | 0.03 (0.96–1.04) |
| 16 | GPR15_1 | CTCTTCTCTCTGGCAAATCTTCT | GCATGAATGAAGGTGGAGAGAGC | 0.05 (0.90–1.04) |
| PPOX | | | | |
| 1 | KARCA_2 | CCTATTGGTATGGCACCTCCGCCAAAACCTTTCAC TCTCATCCACACT | CTCGAAAAACAAAAGCCTAAGCAGATTTCTCA GGCAATTTATCC | 0.03 (0.92–1.00) |
| 2 | PFDN2_4 | CCTTTGCGTGAAGCTGTGTGTCAGTGTCTCAATGATCT TCTGTATCTAG | AGGACAAGTGACAGGGATTATATAAGGAATTCAGGACTCC | 0.03 (0.94–1.03) |
| 3 | PFDN2_1 | CCACAGGCTCCCCCTTCTCGCTAGTATTCCAGACCCAGG | TGGGTACCTGCTTCGCGTTAGCCACTCCTCACCTGC | 0.04 (0.90–1.04) |
| 4 | NIT_3 | CTGAGGCATTTGACTTCATTGCACGGGACCTGCAGA GACGCTACACC | TGCTGAACCACTGGGTGGGAACTTTTGGAGAATAAC | 0.03 (0.90–1.00) |
| 5 | DEDD_4 | CAGGTACGCAATGCTTTCCAGAATCCCTGCTCA GCAGCTGCAATCC | CCACCGTACTGAAAGGGCAGGAAAGAACCAGTGAAGAC | 0.04 (0.89–1.00) |
| 6 | UP_1 | CTCTGGGTCAACACATCATAGCCACATGTCCC TACTAAATATT | TTTTACAAGTCAGTGGTAGTACCAGTTAGAGTAAAG CAGATCTGTTC | 0.03 (0.93–1.00) |
| 7 | UFC1_1 | CGTGTGTGTCTGAGATCCCGGTGC | TGAAGACTAACGCCGACCCCGAGATCGTGAAGTTGTGGG | 0.04 (0.91–1.02) |
| 8 | USP21_3 | CACCTGTTAATATCCAGCCCCGA | GTGGGATCCAAGCTACCATTTC | 0.03 (0.93–1.04) |
| 9 | PPOX_Prom | CTAACATCAAGGAGCTGTTTATGGAGCCGCTCCCCGTG | GACTGGCCTTAAGTGTCCCTATCTATTCTCCCTAC | 0.04 (0.87–1.01) |
| 10 | PPOX_2 | CTAAGGTGAGTGTCCACTTGTGC | CAGAGGGAGCTTCATTTAATGCTCTTCC | 0.04 (0.93–1.08) |
| 11 | PPOX_3 | CTAGTGGAGAGCAGTGCAGTGTCTGGGAGGCT | GGATTCTGCTCCGTTCCAGGCCCCAATGGTGC | 0.05 (0.87–1.03) |
| 12 | PPOX_4 | CTGCATGCCCTACCCACTGGCCTCAGGTAA | CACCAGCACCTCCGCTCCTTTACTGTGCC | 0.02 (0.94–1.00) |
| 13 | PPOX_5 | CAAAGAGCCTGATGAGACTGTGC | ACAGTTTTGCCAGCGCCGCTTGGACCTGAGG TGACACTTGC | 0.03 (0.94–1.049) |
| 14 | PPOX_6 | CGCTCCTTAGTCTAGTCTCACCCCT | TAAGTGGCGTCTCTAGCCATGGACAGTCTC | 0.03 (0.93–1.01) |
| 15 | PPOX_7 | CTTTGCTTCTCTGCAGGGCGGA | CCCCACAGCCAGACTCAGCACTCATTTC | 0.03 (0.95–1.04) |
| 16 | PPOX_8 | CTCTCAAATGTTTTCATGCTCTCAGGTATC | TCTAAGGGACAGCAGTCTGGAGGC | 0.04 (0.90–1.05) |
| 17 | PPOX_9 | CTAGTGCATCACTGCAGTGTCTGTAGCTGTGG | TGAATCTGCAGTACCAAGGAGCCCTCTGCCTGTCC | 0.04 (0.89–1.03) |
| 18 | PPOX_10 | CTCCAGGATTTGGACATTTGGT | GCCATCTTCAGAAGATCCAGGAGTC | 0.03 (0.94–1.03) |
| 19 | PPOX_11 | CAGCTGTACACAATTAGGACTGAAGGAGATGCCGA | GCCACTGCTTGGTCCATCTACACAAGGTAAGTTGGGATAAAC | 0.03 (0.93–1.04) |
| 20 | PPOX_12 | CCTCTCCTCTTCTCAGAAGTGCATTTC | CCCAGTATACTAGGTCAGTGGCAAAAAC | 0.04 (0.92–1.03) |
| 21 | PPOX_13 | CTGTTAATGACTGTATAGAGAGTGGCGCCAGGCAGC | AGTCAGTGTCCGGCACAGAACCCTAACAGC | 0.05 (0.90–1.05) |
| 22 | B4GAL_3 | CTGAGGTTACTGTAGACATCACGAGGGTGAGAATAGTCAAAT | GTCGGTCCCTGATCTCGGCCAAATAGGGCACTGAGAC TTCGGAAGC | 0.04 (0.93–1.06) |
| 23 | ADAM_9 | CCACATTGTTGTATCCGTACCTGTGTGGAAGGAGTA GATGGGGAGCTCAG | GATCACCTGAATCCCCTCAGCCTTCTGAGC | 0.04 (0.88–1.01) |
| 24 | NDUF_1 | CTGAGGGGACTGAGTGTGCTGAGAAAA | GAGGGTAGGGAACAGGTAAGGAGGCATCAAGAG GGAAGAAC | 0.05 (0.92–1.10) |
| FECH | | | | |
| 1 | NARS_IVS2 | CTGAAGACACACGGTTAGTAAAAGGCAGAACC | CAGGTTTGTATTCCGAAGCCCTCGTTCTTTCCAC | 0.02(0.98–1.04) |
| 2 | NARS_5 | CAAATGAAGAGTGAATCCCGGAAAAGAAAGAGG TGAGTCTGTACC | TTGCCATCGTGAACGTTGTTAAAGTTAGAAGCTGGCAAC | 0.06 (0.97–1.15) |
| 3 | NARS_10 | CTTTTAAAAAATGCAGGCTCCAGGT | GGCCATGAGCTGAGTTGTGAC | 0.03 (0.97–1.07) |
| 4 | NARS_16 | CTTGAACGATTCTTAACGTGGATTCTGAATAGGTATCACATCC | GAGACGTGTGCTTATACCCTCGATTTGTCCAGCGTTGCAC | 0.07 (0.92–1.11) |
| 5 | UP_1 | CCAGTCTGAGCCCCCTAGTGAAT | CTGGATGGTTTTTGTAAATACGGATGGCCAGC | 0.03 (0.99–1.07) |
| 6 | FECH_PROM | CCTCAAAGAATGCATTGCCAGGCTGG | CCTTCACTTTTCTAATGCTCTTCACTGAGTTTCAC | 0.04 (0.92–1.04) |
| 7 | FECH_IVS1 | CGGTGCATATTTTATAAGTACTTTTGAAGTAATCCTCATCTCC | TATGTGATAGAAAAGTCCATTATAGATTTCAA TACTTGTGTATCTC | 0.04 (0.92–1.02) |
| 8 | FECH_2 | CCATGGAGGTGGAAGTCAGGTGCAGC | TGCAGCGCGCTCACCACAGAAAC | 0.04 (0.98–1.09) |
| 9 | FECH_IVS2 | CTTGACTAAAGCTTTCACTTACATTCTGGAATAGAGG | GGTGCAGACTGGGCTTACTAATGCGCTATTTCATCATGC | 0.03 (0.97–1.05) |
| 10 | FECH_3 | CACCCTGGAAGAAAATAAGAAGTGTGACAAATCAAC | CGTTGTATTTTATTTATATAGGAAGCCGAAAACCTG | 0.04 (0.94–1.06) |
| 11 | FECH_4 | CTGGCACCATTATCGCCAAACG | CCGAACCCCAAGATTCAAGAGCAGTACC | 0.03 (0.99–1.06) |

Table 1 Continued

| | Name | Upstream hybridizing sequence | Downstream hybridizing sequence | s.d. |
|------|------------|---|---|------------------|
| 12 | FECH_IVS4 | CATTGAACAGCTGCTCTGAGGGTACGTAAGCTAGGCCAGTG | AGTGATGTGTTTTGGGCTCACTCTGGC | 0.03 (0.97–1.05) |
| 13 | FECH_5 | CTAGAAAGGGCTATTGCTTTCACACAG | TATCCACAGTACAGCTGCTCCACCACAGG | 0.03 (0.97–1.06) |
| 14 | FECH_6 | CCTTTACGAGTCTGTTTTTATTTCCACATAATCAAATGCAT | CTACTAAGTGGTCTCTGTATATTTCCAGGCAGC | 0.02 (0.98–1.04) |
| 15 | FECH_7 | CTTTATCCTCTTTCTGTGTTACTCACTCAG | TGCTTTGAGATCATATTCTAAAGGAACG | 0.06 (0.98–1.12) |
| 16 | FECH_8 | CTCAGGAGGTAAGCGCCACTGTCCAAAAAGTCATG | GAAAGGCTGGAGTACTGCAACCCTAC | 0.06 (0.90–1.07) |
| 17 | FECH_9 | CTCAAACAGACGAATCTATCAAAGGGCTTTGTGAGAGG | GGGAGGAAGAATCTCTTGGTCCGATAGC | 0.02 (0.95–1.01) |
| 18 | FECH_10 | CATTGTTCTAAGGTATCTACAGTGTACAATCG | TTTTAGTAGAACATACCAGAGTAATCTCTGAGAAAC | 0.06(0.97–1.15) |
| 19 | FECH_11 | CTGTGTTCCAAGCAGCTGACCCTGAGC | TGTCGCTCTGTGCAATCCTGTCTGC | 0.01 (0.97–1.00) |
| 20 | DOWN_1 | CTTTGGATAATGATAAAGGTCTCTTGTGGCCGGG TCGAATTTCCAGG | GCCAGCCAAAACAAACATGCAAGGGAATGCAG | 0.02 (0.99–1.05) |
| 21 | DOWN_2 | CAAATGTTAATCAGATCTGGGGTGGTATACTCTTGCC TAACGCAATCTGAG | GTTTTTCTGGAATTTGGTCTGACTTTGGTTTG GACAGTGCC | 0.03 (0.96–1.05) |
| 22 | DOWN_3 | CACTATTGTCTCCATCGCAACGGGAGATATAGACAGGTAA | CTTTTCTCAAAGATGGGGCTCTCTCTGAGT CCAAGCTTGGCAAGGCC | 0.06 (0.90–1.04) |
| 23 | DOWN_4 | GACATAAAATGGCATAACAAAATATACCTGGGTTTC ATAAGACAAGTC | TTCTTGCAAAGTGAACCAGCCAGACCCATGCATTGCC | 0.03 (0.97–1.06) |
| 24 | ONE-CUT_2 | CGTTGGAGGTCAGTGAACACCAGG | CGGGACTTCTCTGGGAATTGTTTC | 0.03 (0.94–1.02) |
| CTRL | | | | |
| 1 | DEFB129_2F | GCCACCATCAGCACTATGACC | CCAGGACAGATCACATACACTGC | 0.01 (0.99–1.02) |
| 2 | DACH_9F | CATTTAAGACCCTGAGACTATCTGTTGAAGCTG CCTGTTTTAGCGT | CTGTTCTGCTTGTTCACGCCGTTTCTCTCAAATC | 0.05 (0.93–1.05) |
| 3 | ILKAP_7F | CTGGCTGGAAGCTGTTTAAGGAACCTTTCATCAG TATGCTTGAAGTGT | CCAAAAGGCATCTTTCACGGTTTTCTCTACAC TGATTACATCTCC | 0.05 (0.90–1.07) |

Abbreviation: s.d., standard deviation.

the genomic region was amplified. Each probe was designed at least corresponding to each coding exon of heme gene, and if a high GC percentage in exon 1 did not allow for positioning the probe, it was located in the upstream promoter region. In the presence of heme genes longer than 15 kb, the probes were also located in the longest introns. With regard to the flanking genes, the probes were designed corresponding to coding exons or intergenic regions, and at any rate spaced at 10 kb.

Each probe was designed as two different oligonucleotides, each containing an unique sequence annealing to the target DNA and a common tag corresponding to MAPH primers, which have no complementarities in the whole human genome (forward tag 5'-GGCCGCGGGAATTCGATT-3' reverse tag 5'-CACTAGTGAATTCGCGGC-3'). Unique sequences were identified using the BLAT program (<http://genome.ucsc.edu>), and care was taken to ensure that no known sequence variants were present in the probe-annealing sites. Using the RAW program (MRC-Holland, Amsterdam, the Netherlands), the hybridizing regions of each oligonucleotide were chosen such that the melting temperature was at least 67.5 °C with a GC percentage between 30 and 63%. The entire oligonucleotides (unique sequence plus tag) were checked in DNA folding form (<http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi>) to have ΔG (secondary structure) ≥ 0 with parameters set on $[Na^+] = 0.35$ M and $T = 60$ °C.

The ligation site was chosen to be flanked by no more than three guanine and/or cytosine bases to ensure efficient ligation, and A and T bases were never permitted to be adjacent to the universal primer tags to ensure efficient amplification. The probes within each set were designed to produce PCR products differing by 2 bp in length to allow separation in the size range of 80–132 bp using capillary electrophoresis on an ABI PRISM 310 sequencer (Applied Biosystems, Foster City, CA, USA). The probes corresponding to the three internal control genes (ILKAP, DACH and DEFB129) had a fixed length of 132 nucleotides (nt), 118 nt and 80 nt, respectively. Each probe was chemically synthesized by Eurofins MWG-Operon (Ebersberg, Germany) as two different unlabelled oligonucleotides. The downstream oligo of each probe was 5'-phosphorylated to allow ligation and purified by reversed-phase high performance liquid chromatography, whereas the upstream oligo was purified

by HPSF (high purity salt free) method. Probe mixes were prepared by combining each oligonucleotide so that all were present at a final concentration of 4 fmol μl^{-1} . The seven probe mixes are available on request.

MLPA reaction

Reagents for MLPA were purchased from MRC-Holland. The MLPA reactions were performed, as previously described.^{16,17} In brief, approximately 100 ng of genomic DNA (Puregene kit, Qiagen, Hilden, Germany) in a final volume of 5 μl was heated for 5 min at 98 °C. After cooling to room temperature, 1.5 μl of the probe mix and 1.5 μl of SALSA hybridization buffer (MRC-Holland) were added to each sample, followed by heat denaturation (1 min at 95 °C) and hybridization (16 h at 60 °C). Ligation was performed by adding 32 μl of ligation mix at 54 °C for 15 min and the reaction was stopped by incubating for 5 min at 98 °C. PCR amplification was carried out for 35 cycles in a final volume of 25 μl , adding dNTPs mix to a final concentration of 200 μM , and both the MAPH-F and MAPH-R primers to a final concentration of 200 nM, with MAPH-F being labelled with 6-FAM. A total of 0.75 μl of water, 13.5 μl of HiDi formamide (Applied Biosystems) and 0.5 μl of internal size standard (500 ROX; Applied Biosystems) were added to 0.75 μl of PCR reaction. The mixes were denatured for 2 min and cooled on ice before being separated by capillary electrophoresis on the ABI Prism 310 sequencer, with the following settings: filter set D; capillary length 47 cm; POP-6 polymer; injection time 10–30 s; injection voltage 3.0 kV; run temperature 60 °C; run time 25–30 min.

Data analysis

For quantitative analysis of probes, which were successfully amplified, the trace data produced by the capillary electrophoresis were retrieved using the accompanying software (GeneMapper, Applied Biosystems) and subsequently exported to Excel (Microsoft) for further analysis. The data of each probe set were analyzed separately. Three probes for unlinked loci were included in each probe set as a reference in each sample (internal control). The height of each gene-specific probe peak was divided by the sum of the heights of the three

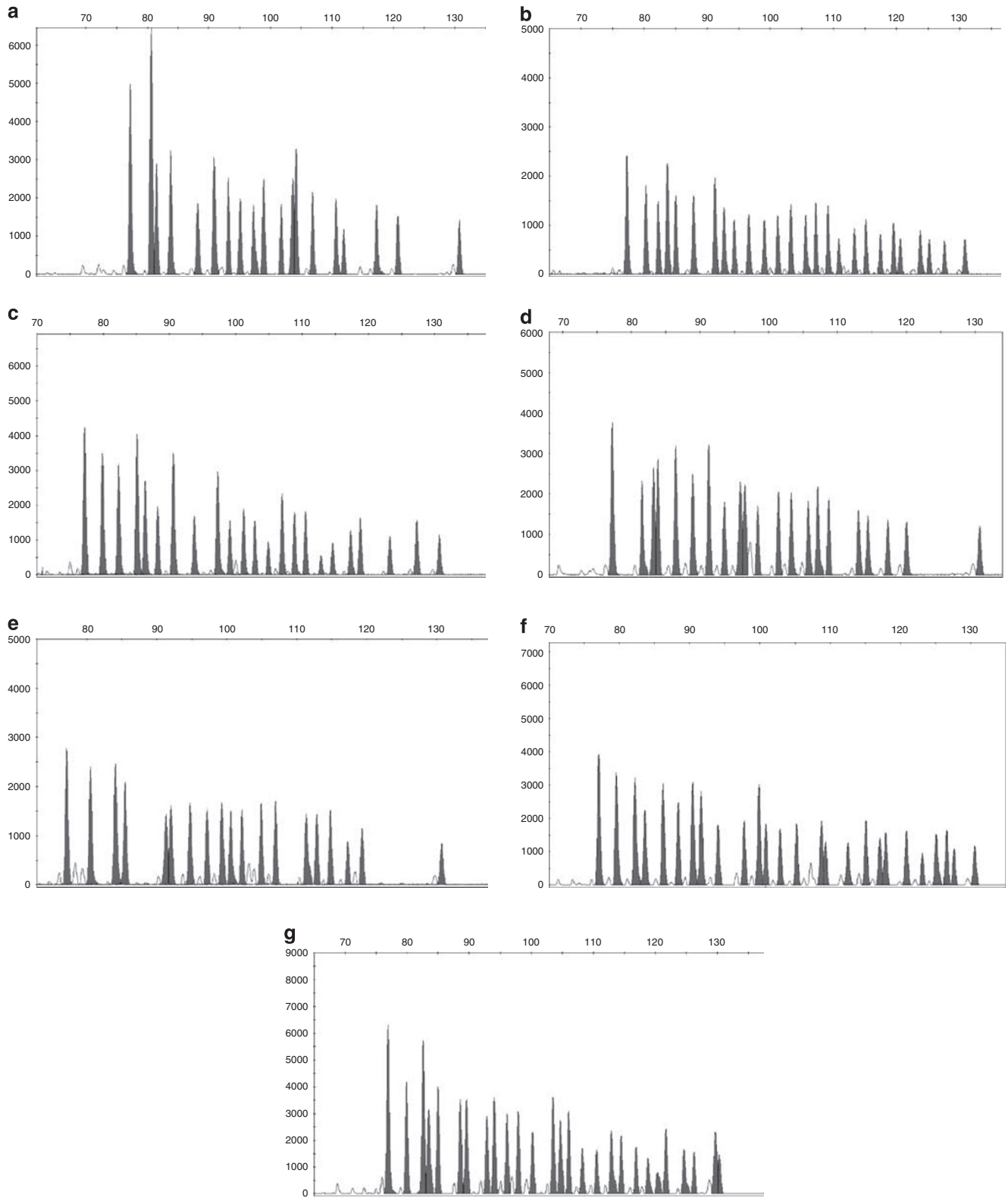


Figure 1 Peak patterns. (a) 19 probes in ALAD set; (b) 26 probes in HMBS set; (c) 23 probes in UROS set; (d) 21 probes in UROD set; (e) 27 probes in PPOX set; (f) 19 probes in CPOX set; and (g) 27 probes in FECH.

reference probe peaks to obtain a ratio. The median ratio for each probe across all wild-type samples (external control) was calculated and was used as the divisor to normalize each probe value to 1.0 dosage quotient (DQ),

corresponding to a copy number of two. The resulting data were then represented by a scatter plot, and the thresholds for deletions and duplications were set at 0.8 and 1.2, respectively.

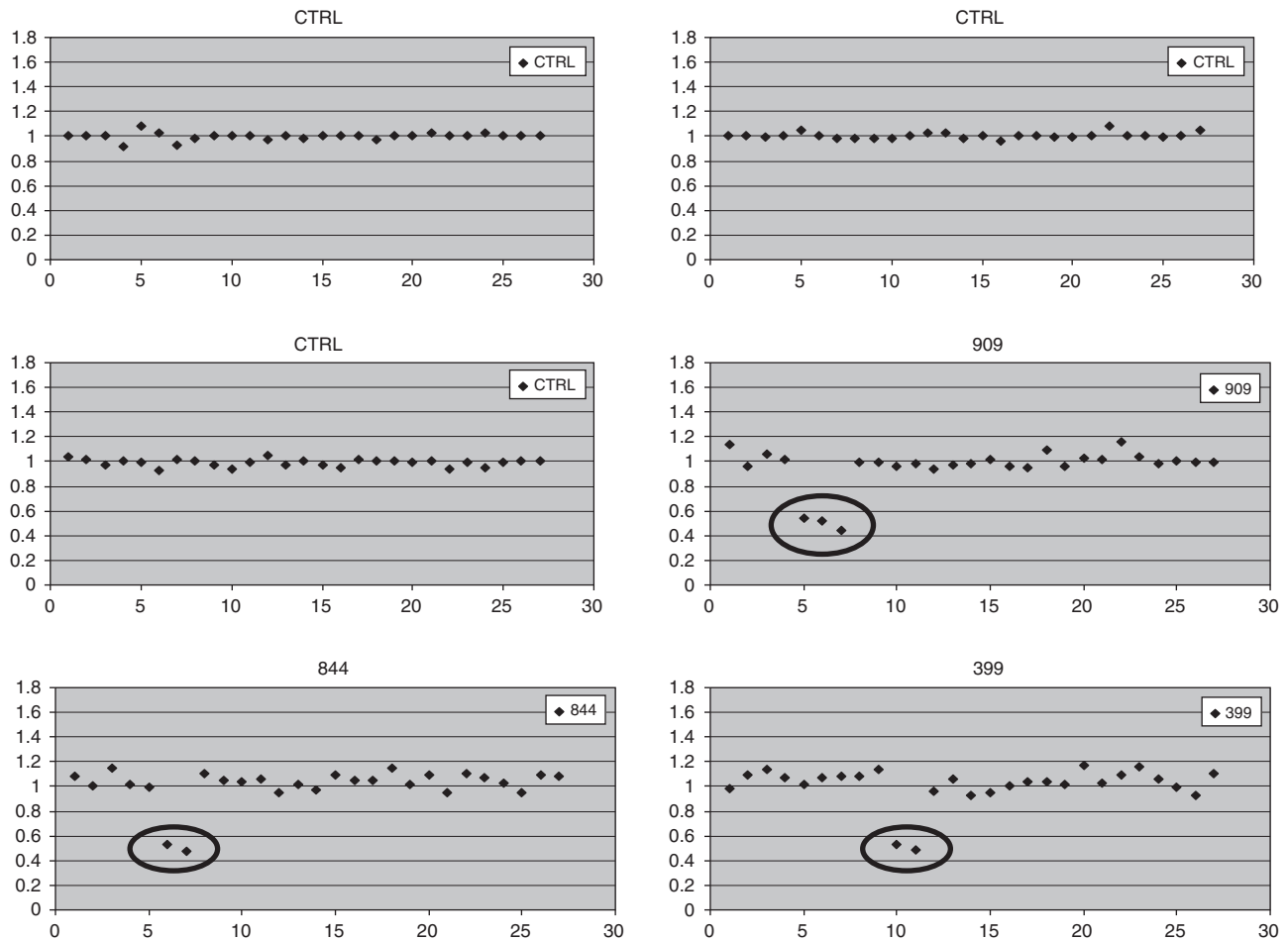


Figure 2 Scatter plots of FECH experiment. The first three plots marked as CTRL correspond to normal participants, plots 909 and 844 represent two known FECH promoter deletions of different lengths, the plot marked as 399 shows the deletion of exons 3 and 4.

RESULTS

The probes were tested on seven control samples to assess their reliability and consistency. Each set was tested at least thrice. All synthesized probes generated an amplification product with fluorescent peaks within the required intensity range (Figure 1) and, comparing independent samples, all showed standard deviation of lower than 10% (Table 1). Greater standard deviation values (up to 20%) were observed when comparisons were performed between samples from different sources (that is, methods of DNA extraction). Comparing both identical input samples and independent duplicate samples, the reproducibility of the methods was very satisfactory with standard deviation < 5%. Using three internal controls having lengths of 80 nt, 118 nt and 132 nt for normalization, we obtained a very small DQ variation of probe across the genes (DQ ranged between 0.9 and 1.1). The sizes of the control probes—small, medium and large—reduce the bias due to amplification of larger products. Greater DQ variations (from 0.8 up to 1.2) resulted in cases of very poor DNA quality. To prove the validity of the method, DNA from six patients carrying deletions, which had already been characterized by other techniques, was used as positive controls for HMBS and FECH. In all cases, the deletions were clearly confirmed (Figures 2 and 3). DNA of patients carrying point mutations was used to test the specificity of binding, except for the ALAD set. Under our experimental conditions,

a mismatch at the 3'-end of the oligonucleotides, and also at 2–3 nt before, completely prevented the appearance of probe amplification products. Mismatches at the middle of the oligonucleotides had no effects (Figure 3).

DISCUSSION

In this paper, we describe a novel approach for molecular diagnosis of porphyrias. The MLPA is an attractive method compared with more complicated and time-consuming techniques such as Southern blot and gene dosage. It is based on comparative quantification of specifically bound probes that are amplified by PCR using universal primers. These alternative techniques have advantages, in that multiplexing numerous targets becomes much easier. Moreover, the use of one fluorescent primer reduces the cost compared with using fluorescent probes for each target. In the original description, the probes were generated by cloning into specially developed M13 vectors, but a simpler method, which relies on chemical synthesis of oligonucleotides, has also been described.^{18,19} In this case, the synthesis length, limited to a maximum of 70–75 nt, reduces the number of probes that could be used in a single probe set, even though the use of multiple fluorophores avoids this problem.¹⁷ We chose the chemical synthesis of probes because it allows for cheaper and more rapid probe production, and their use in these assays was robust and reliable.

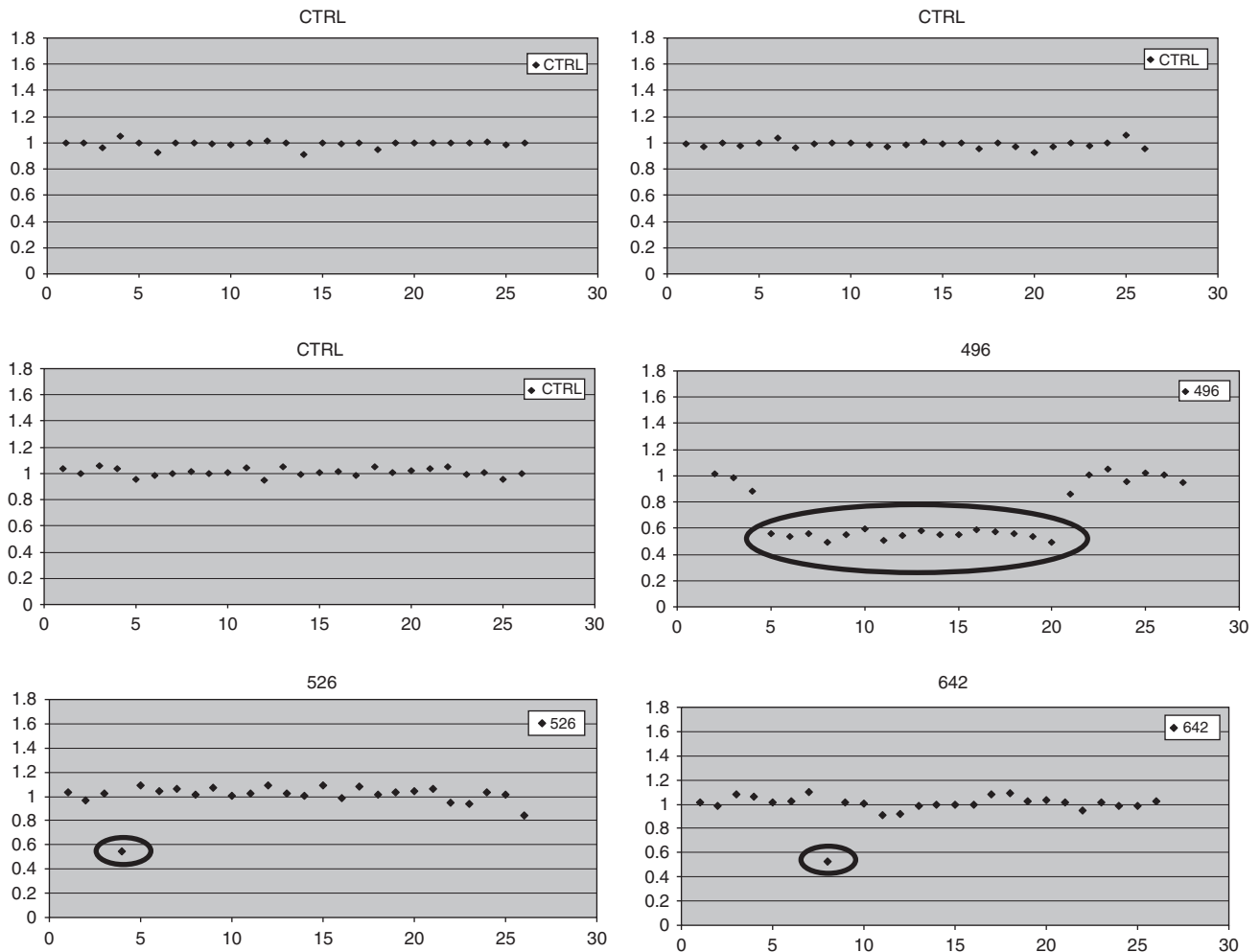


Figure 3 Scatter plots of HMBS experiment. The first three plots marked as CTRL correspond to normal participants, plot 496 represents a deletion of entire HMBS gene and two flanking downstream genes, the plots marked as 526 and 642 show the absence of probe amplification products due to mismatch at 3'-end of the oligonucleotides.

The majority of probes validated on seven wild-type controls showed standard deviation between 0.03 and 0.06 compared with those of commercially available cloned probes.¹⁶ The size range of 80–132 bp was used for the different probes of each set, with up to 27 probes being combined within a single probe mix, confirming that individual oligonucleotides of up to 70 bp in length can be used effectively. The main limitation of this technique is the need for good quality and accurately quantified DNA, a factor that affects cloned and synthetic probes alike. Point substitutions in the genome could interfere with probe annealing and ligation, causing a loss of probe signal and a false positive MLPA result. Even though we accurately excluded annealing sites containing known sequence variants (SNPs) in the probe design, the presence of point mutation in DNA of patients cannot be prevented. For this reason, it is mandatory to perform standard mutational analysis before MLPA. In conclusion, the MLPA assay was shown to be an efficient method for ascertaining copy number changes at multiple loci of heme genes, as all the known tested deletions were confirmed. In the recent years, deletions of single genes or parts of them have been reported as responsible for different forms of Porphyria.^{11,13,14} Chromosome deletions of megabases have also been reported in late-onset porphyria caused by clonal expansion in the myelodysplastic process,²⁰ suggesting that heme genes are prone

to this type of molecular abnormality. Considering the high molecular heterogeneity and the technical difficulties in determining insertions, deletions or complex rearrangements of a few kilobase pairs, it is quite possible that patients with clinical symptoms of porphyria remain undiagnosed even in specialized centers, because of undetected mutations. Undiagnosed patients with porphyrias are often exposed to useless treatments, including surgery, that may be fatal (many drugs, commonly used in clinical practice or anesthesia, have porphyrinogenic effects). Late diagnosis may be responsible for increased morbidity, high health-care costs and poor quality of life. Considering that acute porphyric attacks can be prevented by avoiding triggering factors, to prevent clinical manifestations, it is mandatory to make available all the procedures that can increase the possibility of a correct and early diagnosis. MLPA properly designed for porphyria genes was shown to be a reliable and reproducible method for detecting deletions that so far have been difficult to identify.

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