Original Research

Sanger sequencing of MMR genes in a one-plate system

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Abstract

Both incidence and mortality of colorectal cancer (CRC) in Romania have shown a continuous increase during the last decades. Hereditary Non-Polyposic Colorectal Cancer (HNPCC), also known as Lynch syndrome, is mainly attributable to mismatch repair (MMR) genes MSH2, MSH6, and MLH1. Individuals carrying germ-line mutations of these genes present high lifetime risk of colorectal and other cancers, compared to non-carriers. Oncogenetics is developed worldwide nowadays, for identifying hereditary predisposition to cancer and offering appropriate clinical follow-up to patients and mutation carriers in Lynch families. Molecular oncogenetic diagnosis in Lynch syndrome is based on complete Sanger sequencing of entire MMR genes, which is time and resources consuming, therefore needing an appropriate and adapted optimization. Conventional sequencing requires a sufficient number of available samples to be processed simultaneously, which increases the waiting time for diagnostic results. Complete analysis for only one patient meets difficult technical problems due to the complex co-amplification of all gene regions of interest within the same conditions, therefore increasing the costs and reducing the cost-effectiveness of the test. Here we present an original and robust technical protocol for sequencing the entire MSH2, MSH6, and MLH1 coding sequence for one patient in a single PCR plate. Our optimized and verified system overcomes all technical problems and offers a quick, robust, and cost-effective possibility to personalize molecular oncogenetic diagnosis in Lynch syndrome.

Keywords: *Lynch syndrome; MMR genes; Sanger sequencing; molecular diagnostic; cost-effectiveness.*

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Introduction

Colorectal cancer (CRC) is the third most common cancer and cause of cancer death worldwide (1-3). Lately, an alarming CRC incidence increase (75% fold) was reported for Romania (4), one of the few countries worldwide where increases in mortality rates by CRC are still occurring (2,9% per year). Up to 30% of CRCs have evidence of a familial component (5) and about 5% are thought to be due to inherited mutations in known genes (2), the rest being due to low-penetrance alleles (6). Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is characterized by early-onset CCR occurring in many members of the same family line, increased frequency of multiplicity for CRC, and is mainly attributable to germline mutations in the mismatch repair (MMR) genes *MSH2* (OMIM

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609309, 2p21), *MLH1* (OMIM 120436, 3p22.2) and *MSH6* (OMIM 600678, 2p16.3), and to a lesser extent to PMS2 or EPCAM genes. The more appropriate name of Lynch syndrome (LS) – named after Dr. Henry Lynch (7,8) – has been proposed (9,10) because the syndrome also involves predisposition to other sites. MMR genes cause LS with a penetrance of approximately 80% for CRC, 60% for endometrial cancer, and well below 20% for the other cancers (stomach, ovaries, small bowel, hepatobiliary epithelium, urogenital epithelium, and brain) (11). The overall lifetime risk of CRC for patients with LS is estimated to be 80% (12), while only 5.5% in the general population (13).

MSH2 mutations are responsible for about 35% of LS families, while *MLH1* and *MSH6* contribute to 25 and 15% respectively (11). Mutations in *MLH1* and *MSH2* are located in all regions of these genes, without obvious hot spots (14). Mutation-detection strategies must therefore cover the entirety of these genes. The identification of a germline mutation is the gold standard for the diagnosis of Lynch syndrome (15) and is performed by complete Sanger sequencing of *MSH2*, *MLH1*, and *MSH6* genes (16), exons and exon-intron boundaries. This is a huge work to do, as the genes are very large (*MLH1*-19 exons on 100 kb genomic DNA, *MSH2*-16 exons on 73 kb, *MSH6*-10 exons on 24 kb) (17). Patients with LS are included in molecular testing according to inclusion criteria such as Amsterdam I and II (18), or Bethesda (19). However, MMR mutations are detected in only 60% of Amsterdam criteria fulfilling families, while up to 20% of families not fulfilling these criteria carry deleterious mutations and are therefore excluded from genetic counselling (20).

The main problems associated with molecular oncogenetic diagnosis are raised by elevated costs of human and material resources involved for entire gene sequencing and interpretation, as well as by the long period of time the patients have to wait for the result (21). This period of time (6-8 months for LS diagnostic) is due on the one hand to the laborious interpretation step of the many sequence variants identified by sequencing (22), and, on the other hand, to the limited number of samples available for analysis at one time (23). Also, recurrent MMR mutations, detectable by simple procedures and facilitating diagnostic approaches (24,25), have rarely been observed worldwide for MMR genes. While classical Sanger sequencing is performed in 96-well plated and would demand 95 available samples for maximum efficiency, performing the entire sequencing on one only sample is a challenging work, due to the difficulty of co-amplifying a great number of gene regions within the same PCR conditions. Such a system would, once implemented, allows a rapid MMR screening for a limited number of patients (down to 1), with reasonable cost-effectiveness, and especially in a very short period of time. We present the development of our optimized protocol, with its 2 variants adapted to 1 or 2 patients simultaneously.

Patients and methods

Patients

We identified and recruited HNPCC families at Sf. Spiridon University Emergency Hospital and the Oncology Institute of Iaşi, Romania. Family inclusion on Lynch syndrome was based on Amsterdam II criteria (18), i.e. at least 3 CRC or Lynch-related cancers in the same family line affecting at least 2 generations, with at least one case diagnosed before age 40. All patients agreed by written informed consent. Personal and family cancer histories were obtained from patients and participating relatives. This study was approved by the local Ethical Committee, UMF Iasi.

Molecular analysis

We performed genomic DNA extraction using the Wizard™ Genomic DNA purification

kit (Promega™ Inc, Madison, WI, USA). Spectrophotometric evaluation of DNA quantity and purity was used.

Sanger dideoxy sequencing was performed on 56 amplicons covering the whole coding sequence of *MSH2, MSH6, and MLH1*, including junctions with introns. Primers were designed using Primer Express™ Software v3.0.1 (Life technologies) and Primer3 web version 4.1.0 (26). PCR was performed in 20 µl reaction, containing one unit ApliTaq® Polymerase with appropriate Buffer (Applied Biosystems™ Inc, Foster City, CA, USA), 0.4 mM each dNTP, 0.4 µM of each primer, 100 ng genomic DNA. We generally performed PCR reaction on a GeneAmp® Dual PCR System (Applied Biosystems™ Inc, Foster City, CA, USA) using a common PCR program for all amplicons (94ºC/5min, 35 cycles of 94ºC/20 sec – 54ºC/20 – 72ºC/30 sec, 7 min/72ºC).

After gel electrophoresis evaluation, amplicons were purified by ExoSAP-IT™ (Applied Biosystems), following the manufacturer's instructions. Amplicons ware sequenced both in forward and reverse reactions using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems). Sequencing reaction was performed on a 96-Well GeneAmp® PCR System 9700 (Applied Biosystems), 94ºC/1 min, 25 cycles of 94ºC/10 sec – 52ºC/5sec – 60ºC/3min. Sequence products were purified using BigDye XTerminator™ Purification Kit (Applied Biosystems), and migrated by capillary electrophoresis on a Life Technologies 3500 Series Genetic Analyzer (Applied Biosystems). We performed analysis of raw data using Seqman® (DNA Star™ Inc, Madison, WI, USA) and Variant Reporter™ Software v2.0 (ThermoScientific). Mutations were systematically confirmed on an independent different DNA sample.

Data interpretation and analysis

All mutations and sequence variants are described according to the recommendations from the Human Genome Variation Society (HGVS), with first nucleotide of DNA numbering being the A from initiator translated ATG (27). We used reference sequences NM_000251.2 (coding), NG_007110.2 (genomic) and NP_000242.1 (proteic) for *MSH2*, NM_000179.2 (coding), NG_007111.1 (genomic) and NP_000170.1 (proteic) for *MSH6*, NM_000249.3 (coding), NG_007109.2 (genomic) and NP_000240.1 (proteic) for *MLH1* gene respectively. For bioinformatic prediction of variants, we used Alamut® (Interactive Software™) (28).

Results

Although the whole genomic region covered together by MMR genes represents almost 200 kb DNA, the coding region represents overall 9166 nucleotides to sequence. However, sequencing for diagnosis should equally cover exon/intron boundaries, as well as promoter and other regulating regions situated 5' or 3' outside the exons, as mutations in regulatory regions may be deleterious and affect the overall final protein (22). We calculated 18000 nucleotides to decrypt for a molecular diagnostic test to be validated for MMR genes in Lynch syndrome. All the regions of interest need to be double-strand sequenced and lectured, while the reading sequences need to be accurate enough to avoid any false positive or false negative ones possibly interfering with the final diagnosis.

We defined amplicons covering the coding regions of each exon, as well as exon/intron boundaries. The final coverage area is represented by 16 exons in *MSH2* gene (about 5,7 kb), 10 exons in *MSH6* gene (about 6,5 kb), and 19 exons in *MLH1* gene (about 5,4 kb). This total of 45 exons does not exactly correspond to the actual number of amplicons. The length of concerned exons varies between 200 and 600 bp, the efficiency of the PCR and subsequent sequencing reactions being optimum between 250 and

400 bp. While the majority of exons can generate amplicons within this range, there still are a few examples that need particular adaptation. Exon 4 of *MSH6* is exceptionally large (2543 bp), several contiguous amplification fragments being necessary for the entire coding region to be covered. We calculated a total of 8 contiguous amplicons (named 4-1, 4-2, etc.), ranging between 370-580 bp and representing together over 3,6 kb sequence. Exon 12 of *MLH1* (371 bp) is particularly complex to amplify and requires 2 contiguous amplicons measuring together 579 bp. Exons 8, 9 and 10 of *MSH6* are particularly close to each other on genomic DNA, which hardens primer definition within the region, and brings the necessity of covering the sequence with 4 different amplicons. Nonetheless, some exons prove to be difficult to amplify or to sequence on both strands, due to repetitive regions causing enzyme slippage. In order to ensure an accurate lecture on the entire exon region, we imagined for those cases at least 2 independent amplicon variants.

Developing a one-plate PCR system for each patient

According to the description above, an overall amount of 56 amplicons is needed for covering the entire MMR sequence of interest (Figure 1C). As each amplicon has theoretically different PCR amplification conditions, efficiency and limitations, the classical and most efficient system consists of amplifying each region independently, for a given number of available DNA samples, with a maximum efficiency for 95 samples (in this case, an entire plate of 96 wells, including a no template control, would be used for each amplicon – Figure 1A). Amplifying each exon separately allows using different annealing temperatures, PCR program, and even different amounts of template DNA or primers for each reaction, which is beneficial for PCR and sequencing accuracy, and for overall cost-effectiveness.

Unfortunately, 95 samples are rarely available in molecular oncogenetic diagnosis for common laboratories covering limited regions or populations. Also, the period of time necessary for 95 samples to be available considerably delays the duration of each diagnosis. For those reasons, an intermediate amplification system can be imagined, with two or more exons amplifiable in one same plate (Figure 1B) for an adequate number of samples (i.e. 47 samples for 2 amplicons, 31 samples for 3 amplicons, or 23 samples for 4 amplicons in the same plate). However, the choice of the amplicons to be co-amplified in the same plate should take into account the different amplification and sequencing conditions.

As such, an extreme challenge is represented by the co-amplification of all MMR exons in a single plate when only one DNA sample is available (Figure 1C). Of course, such a system would answer the question of duration, but would raise problems linked to efficiency, cost-effectiveness, or even accuracy of the final result. In such a system, all exons are co-amplified in a single PCR reaction, which means the amplification conditions for each amplicon should be similar. We defined PCR primers for the amplification of each region of interest, taking into account the particularity of each amplicon in terms of repetitive or GC-rich regions, but we also tried to design primers with appropriate Tms, in order to ensure a common annealing temperature of 54ºC for each amplicon, as well as the possibility of being amplified in a common 35-step PCR reaction. Obviously, these requirements are almost impossible to meet entirely for all 56 amplicons, but for each amplicon we tried to be close enough to the common reaction conditions. Since the same PCR primers were also used for forward and reverse sequencing, we also took into account the correspondence of each primer to a common sequencing reaction.

After multiple steps of optimization regarding primer sequences and relative concentra-

H	G	F	E	D	C	B	A	
S8 E1	S7 E1	S6 E1	S5 E1	S4 E1	S3 E1	S2 E1	S1 E1	**1**
S16 E1	S15 E1	S14 E1	S13 E1	S12 E1	S11 E1	S10 E1	S9 E1	**2**
S24 E1	S23 E1	S22 E1	S21 E1	S20 E1	S19 E1	S18 E1	S17 E1	**3**
S32 E1	S31 E1	S30 E1	S29 E1	S28 E1	S27 E1	S26 E1	S25 E1	**4**
S40 E1	S39 E1	S38 E1	S37 E1	S36 E1	S35 E1	S34 E1	S33 E1	**5**
S48 E1	S47 E1	S46 E1	S45 E1	S44 E1	S43 E1	S42 E1	S41 E1	**6**
S56 E1	S55 E1	S54 E1	S53 E1	S52 E1	S51 E1	S50 E1	S49 E1	**7**
S64 E1	S63 E1	S62 E1	S61 E1	S60 E1	S59 E1	S58 E1	S57 E1	**8**
S72 E1	S71 E1	S70 E1	S69 E1	S68 E1	S67 E1	S66 E1	S65 E1	**9**
S80 E1	S79 E1	S78 E1	S77 E1	S76 E1	S75 E1	S74 E1	S73 E1	**10**
S88 E1	S87 E1	S86 E1	S85 E1	S84 E1	S83 E1	S82 E1	S81 E1	**11**
NTC E1	S95 E1	S94 E1	S93 E1	S92 E1	S91 E1	S90 E1	S89 E1	**12**

Figure 1A. Amplification of 95 DNA samples for a single PCR amplicon (S=sample, E=amplified exon, NTC=no template control)

H	G	F	E	D	C	B	A	
S8 E1	S7 E1	S6 E1	S5 E1	S4 E1	S3 E1	S2 E1	S1 E1	**1**
S16 E1	S15 E1	S14 E1	S13 E1	S12 E1	S11 E1	S10 E1	S9 E1	**2**
S24 E1	S23 E1	S22 E1	S21 E1	S20 E1	S19 E1	S18 E1	S17 E1	**3**
NTC E1	S31 E1	S30 E1	S29 E1	S28 E1	S27 E1	S26 E1	S25 E1	**4**
S40 E2	S39 E2	S38 E2	S37 E2	S36 E2	S35 E2	S34 E2	S33 E2	**5**
S48 E2	S47 E2	S46 E2	S45 E2	S44 E2	S43 E2	S42 E2	S41 E2	**6**
S56 E2	S55 E2	S54 E2	S53 E2	S52 E2	S51 E2	S50 E2	S49 E2	**7**
NTC E2	S63 E2	S62 E2	S61 E2	S60 E2	S59 E2	S58 E2	S57 E2	**8**
S72 E3	S71 E3	S70 E3	S69 E3	S68 E3	S67 E3	S66 E3	S65 E3	**9**
S80 E3	S79 E3	S78 E3	S77 E3	S76 E3	S75 E3	S74 E3	S73 E3	**10**
S88 E3	S87 E3	S86 E3	S85 E3	S84 E3	S83 E3	S82 E3	S81 E3	**11**
NTC E3	S95 E3	S94 E3	S93 E3	S92 E3	S91 E3	S90 E3	S89 E3	**12**

Figure 1B. Example of amplification of 31 DNA samples for three PCR amplicons (S=sample, E=amplified exon, NTC=no template control)

H	G	F	E	D	C	B	A	
S1-M2 e8	S1-M2 e7	S1-M2 e6	S1-M2 e5	S1-M2 e4	S1-M2 e3	S1-M2 e2	S1-M2 e1	**1**
S1-M2 e16	S1-M2 e15	S1-M2 e14	S1-M2 e13	S1-M2 e12	S1-M2 e11	S1-M2 e10	S1-M2 e9	**2**
S1-M6 e4-5	S1-M6 e4-4	S1-M6 e4-3	S1-M6 e4-2	S1-M6 e4-1	S1-M6 e3	S1-M6 e2	S1-M6 e1	**3**
S1-M6 e8-var1	S1-M6 e7-var2	S1-M6 e7-var1	S1-M6 e6	S1-M6 e5	S1-M6 e4-8	S1-M6 e4-7	S1-M6 e4-6	**4**
S1-M1 e8	S1-M1 e7	S1-M1 e6	S1-M1 e5	S1-M1 e4	S1-M1 e3	S1-M1 e2	S1-M1 e1	**5**
S1-M1 e15	S1-M1 e14	S1-M1 e13	S1-M1 e12-2	S1-M1 e12-1	S1-M1 e11	S1-M1 e10	S1-M1 e9	**6**
S1-M6 e10	S1-M6 e9-var2	S1-M6 e9-var1	S1-M6 e8-var2	S1-M1 e19	S1-M1 e18	S1-M1 e17	S1-M1 e16	**7**
								8
								9
								10
								11
								12

Figure 1C. Amplification of one only DNA samples for all PCR amplicons (S=sample, E=amplified exon)
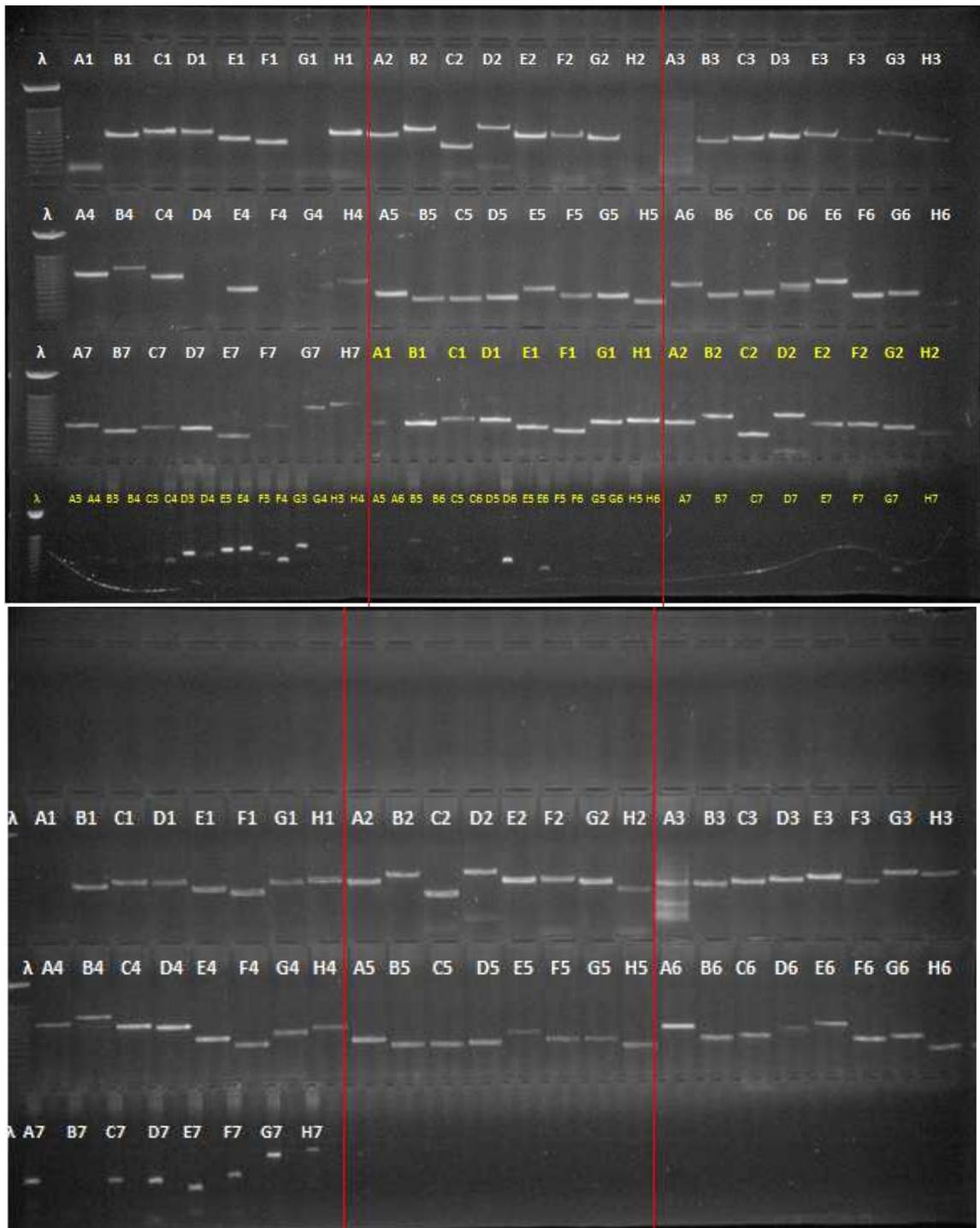
Figure 2A (up). Different attempts to optimize co-amplification of all 56 exons (in this figure, for 2 patients). Figure 2B (down). Efficient co-amplification of all 56 exons (in this figure, for 1 patient)

tions, we obtained an acceptable result presented in figure 2B. A comparison between figures 2A and 2B demonstrates the importance of template DNA quality on the overall amplification efficiency.

In figure 2B one can observe most of the exons being correctly and specifically co-amplified in one reaction, in the presence of a single patient DNA template. We can observe lack of amplification for exon M2-e1 (difficult amplification proven in several different reactions) or for exon M1-e17 (accidental random loss, not reproducible). Also, exons like M6-e1 (special conditions) or M2-e16 (accidental) present additional non-specific bands, while exons M1-e6, M1-e7 or M1-e12 show poor amplification with less intense PCR bands. Some of these disadvantages disappeared because they were compensated for in efficient sequencing reactions, while some others directly influenced the accuracy of the final sequence lectured. After testing at least 10 DNA samples in this system, we concluded to the problems commonly appearing in every reaction. Here are some of our main conclusions:

Me-e1 is a very particular exon and its amplification demands a separate touch-down PCR reaction. However, we observed its possible amplification in our common conditions in 20% of the reactions.

Exons M2-e5, M2-e2, M1-e1, M1-e4, M1-e9 can only be sequenced on one strand (either forward or reverse), due to complex repetitive regions 5' or 3' of the exon

Exons M6-e1, M6-e7, M6-e8, M1-e5 are the most common to be missed in efficient amplification. However, each one of them appears correctly amplified in more than 50% of the samples. Using several different amplicon variants for those exons generally overcomes the problem.

Up to 5 exons (<10% of the total) are randomly missed from amplification due to general random pipetting or other laboratory environ-

ment errors. The missed exons are different for each sample.

The general efficiency widely varies with the quality and quantity of starting DNA. Extracted DNA to be used should always be double-measured and correctly diluted before being used for PCR reactions.

We can conclude that with our system, only 5-10% of the amplicons should be re-amplified in complement reactions, which is comparable with the amount of complements needed in all laboratory sequencing reactions.

Developing a one-plate PCR system for two patients

As one can observe in figure 2A, PCR products from 2 different plates can be simultaneously migrated in the same electrophoresis system. This aspect raised the question whether 2 DNA samples could be entirely co-amplified in the same plate. The system described above uses for a patient 7 out of 12 columns of a PCR plate, while some of the exons need re-amplification due to PCR loss. Also, some of the exons are amplified in several variants, which may become obsolete (e.g. M1-e12 can easily be covered by the first amplicon variant). Therefore, we decided to keep the amplicons "working" together in the same plate, while excluding difficult or iterative amplicons from the plate, which can be amplified separately. The novel system we managed to develop is presented in Figure 3A, while the electrophoretic profile from the whole plate (2 patients) can be seen in Figure 3B.

Practically, we pulled out from the plate 8 out of 56 amplicons, and covered 6 columns with the remaining amplicons from Sample 1, the rest of the plate being filled by corresponding amplicons from Sample 2. The eliminated amplicons were M1-e1, M6-e1, M6-e7, M6-e8, M1-e5, M1-e11, M1-e12 (variant 2). These amplicons can be amplified together in a separate system or even in separate reactions each (Figure 1A and

| H	G	F	E	D	C	B	A	
S1-M2 e8	S1-M2 e7	S1-M2 e6	S1-M2 e5	S1-M2 e4	S1-M2 e3	S1-M2 e2	S1-M2 e1	**1**
S1-M2 e16	S1-M2 e15	S1-M2 e14	S1-M2 e13	S1-M2 e12	S1-M2 e11	S1-M2 e10	S1-M2 e9	**2**
S1-M6 e4-6	S1-M6 e4-5	S1-M6 e4-4	S1-M6 e4-3	S1-M6 e4-2	S1-M6 e4-1	S1-M6 e3	S1-M6 e2	**3**
S1-M6 e10	S1-M6 e9-var2	S1-M6 e9-var1	S1-M6 e8-var2	S1-M6 e6	S1-M6 e5	S1-M6 e4-8	S1-M6 e4-7	**4**
S1-M1 e10	S1-M1 e9	S1-M1 e8	S1-M1 e7	S1-M1 e6	S1-M1 e4	S1-M1 e3	S1-M1 e2	**5**
S1-M1 e19	S1-M1 e18	S1-M1 e17	S1-M1 e16	S1-M1 e15	S1-M1 e14	S1-M1 e13	S1-M1 e12-1	**6**
S2-M2 e8	S2-M2 e7	S2-M2 e6	S2-M2 e5	S2-M2 e4	S2-M2 e3	S2-M2 e2	S2-M2 e1	**7**
S2-M2 e16	S2-M2 e15	S2-M2 e14	S2-M2 e13	S2-M2 e12	S2-M2 e11	S2-M2 e10	S2-M2 e9	**8**
S2-M6 e4-6	S2-M6 e4-5	S2-M6 e4-4	S2-M6 e4-3	S2-M6 e4-2	S2-M6 e4-1	S2-M6 e3	S2-M6 e2	**9**
S2-M6 e10	S2-M6 e9-var2	S2-M6 e9-var1	S2-M6 e8-var2	S2-M6 e6	S2-M6 e5	S2-M6 e4-8	S2-M6 e4-7	**10**
S2-M1 e10	S2-M1 e9	S2-M1 e8	S2-M1 e7	S2-M1 e6	S2-M1 e4	S2-M1 e3	S2-M1 e2	**11**
S2-M1 e19	S2-M1 e18	S2-M1 e17	S2-M1 e16	S2-M1 e15	S2-M1 e14	S2-M1 e13	S2-M1 e12-1	**12**
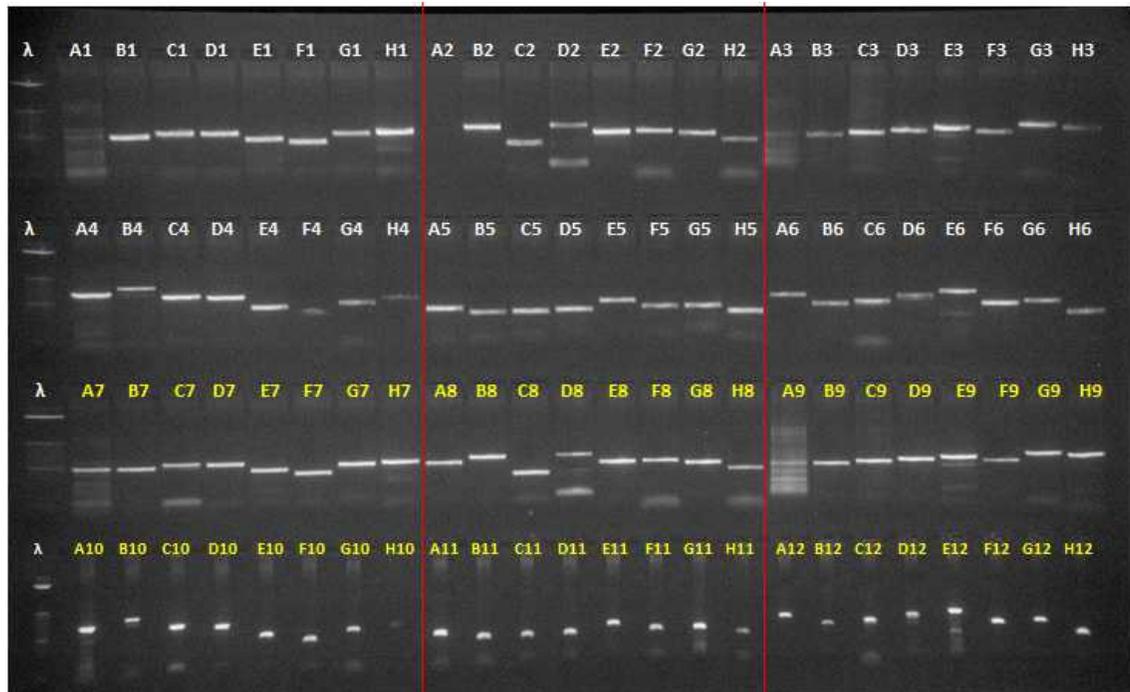
Figure 3A (up). Co-amplification of the most efficient 48 exons for 2 patients in the same PCR plate (S=sample, E=amplified exon). Figure 3B (down). Co-amplification of the most efficient 48 exons for 2 patients in the same PCR plate
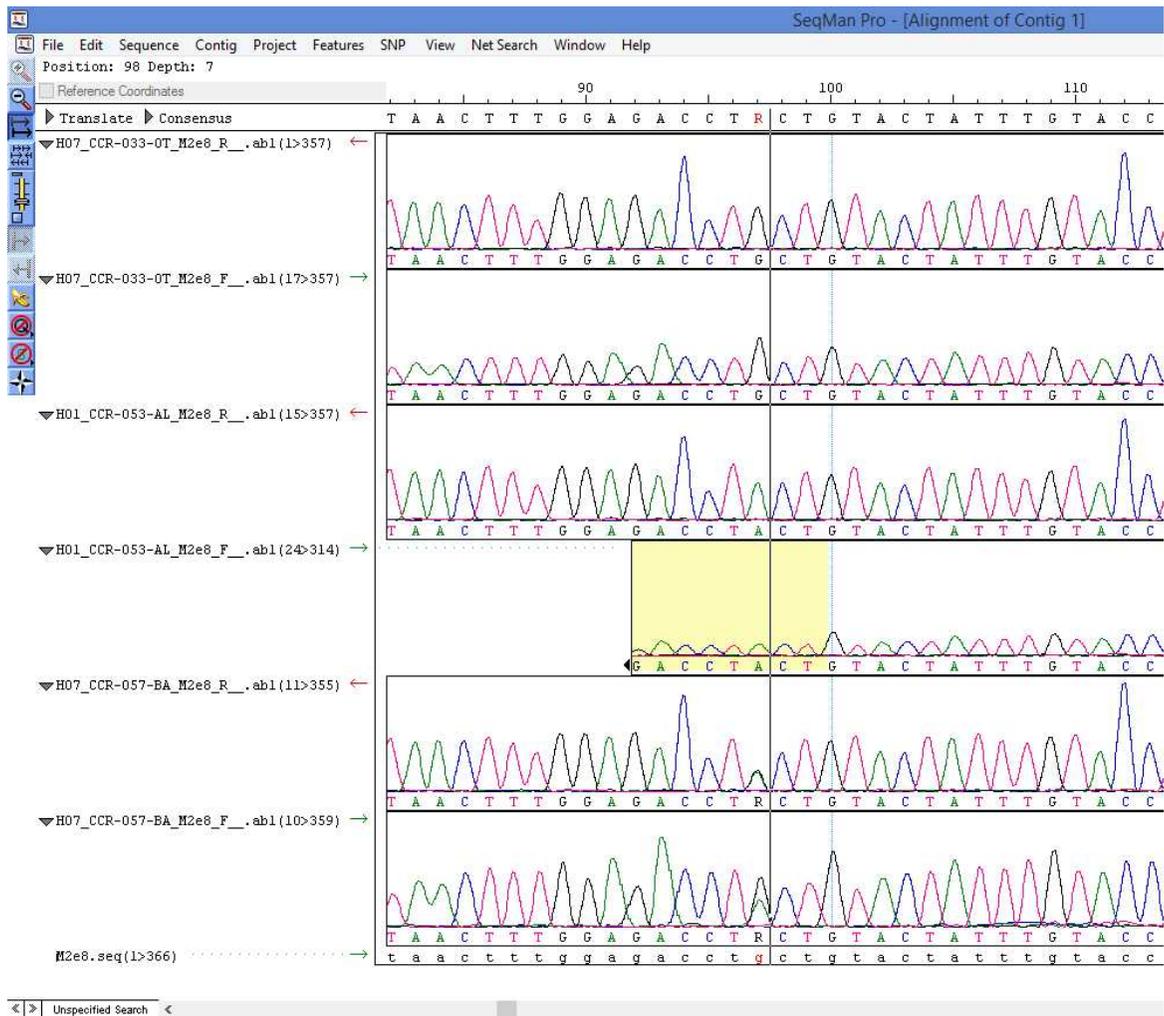
Figure 4. Example of detection of the MSH2 c.1277-118G>A variant, in forward and reverse sequencing, in heterozygous state (bottom), homozygous (middle) or wild-type (upper)

1B), depending on the number of available samples. However, one should notice that the main plate we imagined covered more than 85% of the entire region of interest, and the probability of finding deleterious mutations in MMR genes is accordingly inside this plate. In a diagnostic system, when identifying a deleterious pathogenic mutation, one has not to consider continuing the sequence of the other exons.

The accuracy of our implemented system can be easily observed in figure 4. We chose a dis-

tant intronic variant upstream exon 8 of *MSH2* (c.1277-118G>A), detectable either in homozygous or heterozygous state, both on forward and reverse strands.

Discussion

Improving molecular diagnosis for Lynch syndrome in Romania is essential. Both incidence and mortality are increasing in our country, so early diagnosis and prevention should

become national health priorities. The observed epidemiologic trends are similar with those recorded in Western Europe in the early 90s, where the increase awareness about CRC and methods of screening and early diagnosis determined a stabilization of incidence and mortality due to CRC in the late 90s (29). This moment also corresponds to the emergence and development of Oncogenetics as the medical and diagnostic follow-up of patients and their families presenting a hereditary monogenic risk to cancer. The prognosis for patients with CRC heavily depends on the stage on diagnosis (6): 5-year survival is over 90% for early stages, but only 5% for late ones, which emphasizes early diagnosis.

Although overall diagnosis in LS includes germ-line and somatic analysis, the oncogenetic approach is mainly based on germ-line mutation detection by entire MMR gene sequencing. While promising progress has been recently reported by next-generation sequencing, the gold standard in oncogenetic molecular diagnosi remains Sanger sequencing (30). As shown earlier, this methodology is not only expensive and complex, but also time consuming, therefore a patient should wait a minimum of 8 months for a diagnosis, a period which increases with the limited number of available samples. The challenge we took in this context is to entirely sequence the MMR genes, for a single patient, on a single PCR plate, without losing any of the accuracy of the general sequencing procedure.

We developed an integrated system able to rapidly cover the entire coding region of *MSH2*, *MSH6* and *MLH1* by Sanger sequencing. This can be performed for absolutely all 56 needed amplicons in a single PCR amplification plate, followed by separate forward and reverse corresponding sequencing plates. Alternatively, this can be performed for 2 DNA samples simultaneously, in the same plate, for a total of 48/56 amplicons, the remaining ones being amplified separately. In both systems presented, the over-

all efficiency achieves 95% (maximum we observed – i.e. the number of amplicons not needing re-amplification after the final sequence lecture). However, we have to notice that this efficiency is directly correlated with the quality of template DNA, as well as with the overall accuracy of laboratory workflow. Nonetheless, the systems described are optimized to function on local conditions and equipment, each reproduction in different conditions needing separate optimization.

Acknowledgements

Conflicts of interest

The authors declare no conflict of interest

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