QUALITY CONTROL AND INTERPRETATION OF MOLECULAR BIOLOGY RESULTS IN LABORATORY MEDICINE

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Abstract

Management of quality control and keeping of good laboratory praxis principles in molecular diagnostics is crucial for getting correct results. Standard PCR-RFLP methods still remain backup alternative to real-time PCR techniques in routine laboratory medicine diagnostics. In our paper we summarized basic and most frequent sources of errors and miss-interpretation of their results. We specified basic steps and algorithms of the proper solution of the most frequent problems with accent to the minimization of time, costs and reputation losses.

Key words
Quality control, PCR-RFLP, errors, electrophoreogram

Introduction

In recent years internal and external quality control became an important factor not only in the management process and good laboratory practice, but also for the expansion strategy of laboratory in the competitive environment of health care providers. In the early development of molecular - biological methods there was performed mainly qualitative determination of risk alleles or genetic information of pathogens [1]. Results of these tests represent solely qualitative data that could not be evaluated by conventional schemes, such as Westgard rules, Levey-Jennings charts and Youden plots. With time, however, even in this area have appeared quantitative analyses, the outcome of which is quantified by the numerical values. Typical examples are quantitative examinations of virion copies in the patient's body (hepatitis C virus, hepatitis B virus) or quantification of gene expression in some cancers [2]. Today dominate new generation of techniques. With the use of the real-time approach it is possible to monitor the progress of the polymerase chain reaction (PCR) directly in real time during the process. This approach brought elimination of laboratory procedures, following the classic PCR, above all DNA cleavage with restriction endonucleases and electrophoretical separation of the DNA fragments. It accelerated the analytical process and eliminated critical points of the manually performed steps. Certain disadvantage of these methodologies is the "virtuality" of the analytical process. Laboratory staff follows the data on the screen only: melting curves and graphs, as well as data of the number of cycles, temperature and time.

This was the reason for many laboratories that have still retained their technical equipment for classical molecular biological analyzes. Unfortunately, even this strategy of laboratory management does not necessarily mean a guarantee of successful verification of DNA analysis results. This is due to the low frequency of using these techniques. When using any laboratory equipment, the higher is the frequency of its performance, the better is the staff trained in diagnostical skills. Doubly that applies to classic manual techniques, such as PCR-RFLP [3-5], intended for using in the case of problems with real-time PCR techniques. Therefore at low training and skills of staff there is the possibility of alternative using classic

PCR-RFLP technology only a matter of psychology, but it is not really feasible alternative. For an inexperienced analyst, may therefore be using of PCR-RFLP methodology even greater puzzle than those of real-time PCR. Therefore re-testing has been relative frequent even in the case of published methods [6,7].

Aim

Our goal was to demonstrate the possible causes of erroneous results of PCR-RFLP analyses and their possible impact on the accuracy and interpretation of results.

Material and methods

All used PCR-RFLP methods are optimized and integrated with the management of internal/external quality control. Methodologies of single nucleotide polymorphisms examinations PCR-RFLP are based on the valid standard operating procedures, which followed the Operating manual of the laboratory and all other relevant laboratory documentation. More detailed information about quality control, used enzymes and solutions can be obtained from the authors. Parameters of particular analytical steps are given in table 1.

Within internal quality management there were used two types of samples in each tested series of samples. The positive control contained the template DNA with known genotype. The purpose of this control was to verify the effectiveness and functionality of DNA-amplification and restriction endonucleases. The negative control contained all the reaction components except the template DNA. It was used to verify the absence of contamination of samples and their cross-contamination (also called as "carry-over").

Table 1 PCR-RFLP parameters of tested alleles

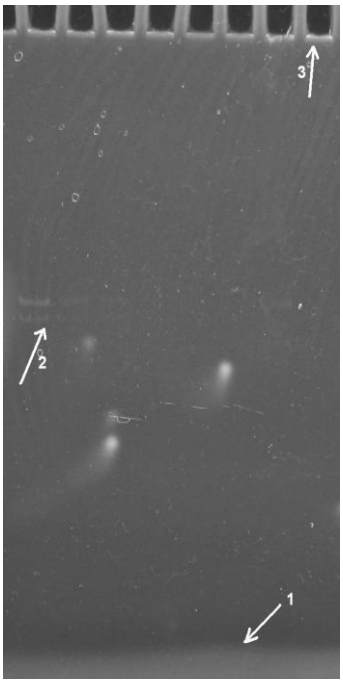
Tested alleles	PCR parameters						RFLP	Electrophoresis		Visualization
	Start	D	A	E	N	End		PAGE	Buffer	
FV-Leiden G1691A FII G20210A*	95°C/ 5min	95°C / 1min	57°C / 1min.	72/ 1:40 min.	39	72°C / 10 min.	*HindIII*; 37°C / 4hours	250V/ 100mA/ 210 min.	TBE	EtBr
MTHFR C677T	95°C / 3min.	95°C / 1min.	55°C / 2min.	72/ 2min.	29	72°C / 5min.	*MboII*; 37°C / 3hours	250V/ 100mA/ 210 min.	TBE	EtBr / GW
GPIIIa T1565C („Pl^{A2}“)	95°C / 3min.	95°C / 1min.	58°C / 1min.	72/ 1min.	35	72°C / 10min.	*MspI*; 37°C / 4hours	250V/ 100mA/ 210 min.	TBE	EtBr
ACE ins/del	95°C / 3min.	95°C / 1min.	56°C / 0:45min.	72/ 1:20min.	25	72°C / 10min.	-	250V/ 100mA/ 50 min.	TBE	GW
HFE G845A (C282Y)/ HFE C187G (H63D)*	96°C / 2min.	94°C / 0:30min.	55°C / 0:15min.	72/ 0:30min.	35	72°C / 10min.	*BbrPI* / *Eco72I*; 37°C / 4hours	250V/ 100mA/ 120 min.	TBE	GW

Legend: * multiplex PCR, PAGE – 12% polyacrylamide gel, TBE – Tris-borate EDTA buffer, EtBr – ethidiumbromid, GW – gold view. PCR-RFLP analysis was based on the former published procedures [3-9]. PCR parameters: Start – first starting step, D – denaturing step of cycle, A – annealing step of cycle, E – extension step of cycle, N – number of cycles repeating, End – terminal step of PCR; RFLP – parameters of restricted fragments length polymorphism analysis

Results and discussion

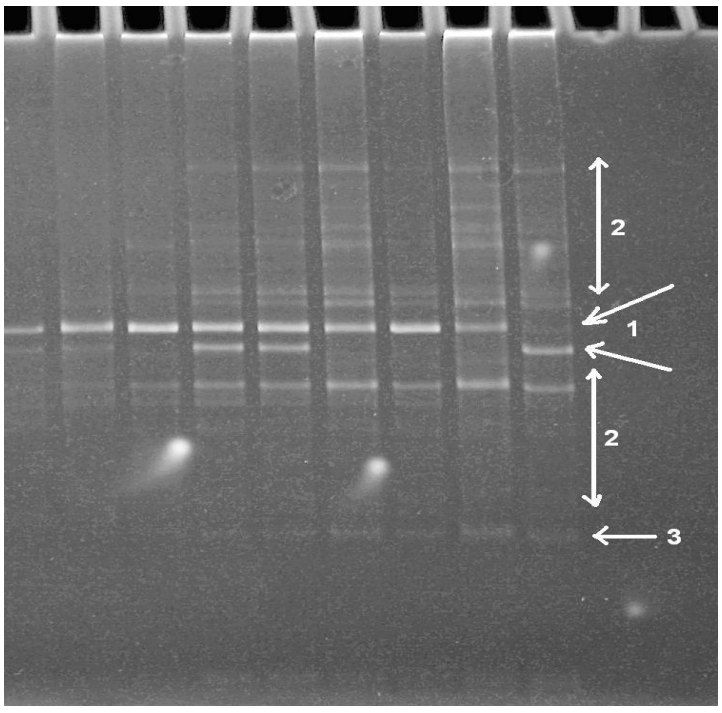
Errors in PCR/restriction analysis: Assuming successful isolation of DNA, the first critical step of PCR-RFLP analysis is the PCR itself. Figure 1 illustrates the electrophoreogram of samples with almost no amplification in PCR. At the bottom of the electrophoreogram is visible band of primers (fig. 1, arrow 1). The target DNA is amplified only in low quantity (fig. 1, arrow 2). At the start we can see remnants of the template DNA (fig. 1, arrow 3). Possible cause of is the wrong concentration of PCR reaction components and / or errors in adjustable physical parameters of thermal cycler (temperature, time, number

of cycles). The solving of this problem is checking and re-setting of all parameters of the PCR reaction.



Legend: Results of the PCR-RFLP analysis of the MTHFR C677T polymorphism. Weak signal indicates failures in PCR parameters (concentration of components, time and/or temperature parameters)

Fig. 1 *Failed step – weak amplification of DNA*

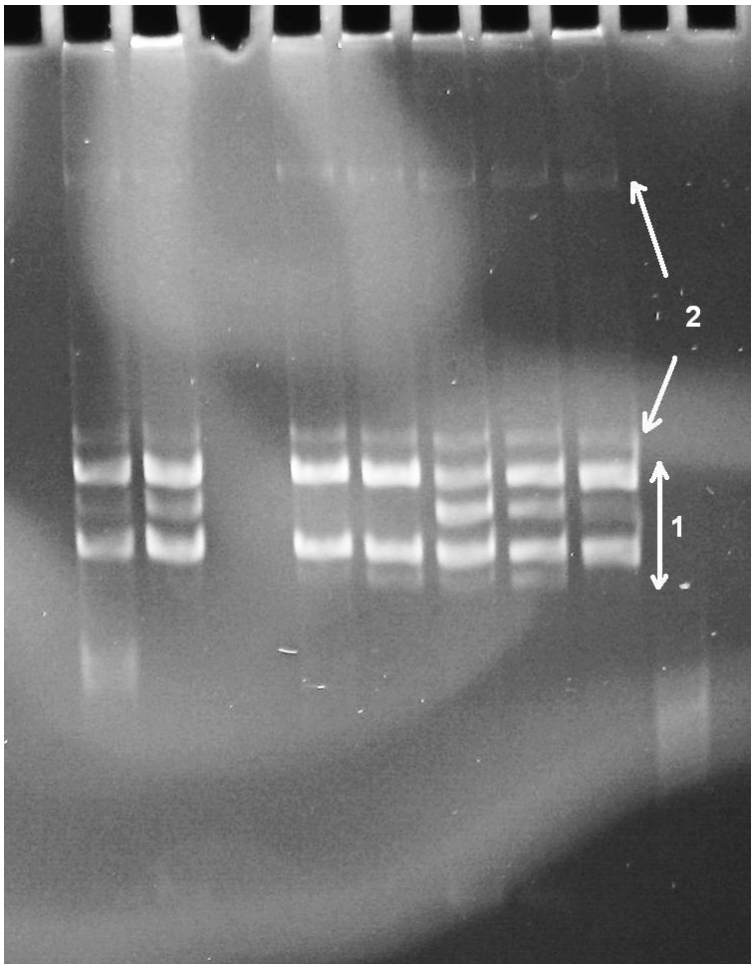


Legend: Results of the PCR-RFLP analysis of the MTHFR C677T polymorphism. Too many bands indicate non –specific condition of PCR including time/temperature setting of thermal cycler and primers/dNTP/buffers concentrations

Fig. 2 *Failed step – redundant fragments*

Another problem arising from the erroneous PCR reaction is excessing amount of DNA fragments (figure 2). In addition to the desired DNA fragments with exact defined size (fig. 2, arrow 1) contains the electrophoreogram significant number of redundant lanes (fig. 2, arrow 2), spreading along the whole field length of the sample path from the start line to the bottom line of primers (fig. 2, arrow 3). The most common cause of it is an inappropriate setting of physical parameters of the PCR reaction. Lower temperatures and / or longer time of particular steps are usually responsible for the non-specific binding of primers and the creation of redundant DNA fragments. The solving of the problem is optimizing of the settings of particular steps on the thermal cycler. It is important to remember that each device requires its own optimization process and we cannot simply copy and set-in data taken from the literature guides.

Too high concentrations of particular reagents in PCR master mix are cause of electrophoreograms shown in figure 3. We can see over-amplified DNA fragments (fig. 3, arrow 1) together with artificial bands (fig. 3, arrows 2), which are frequent accompaniment. The problem can be solved by lowering concentrations of particular reagents in the PCR master mix.

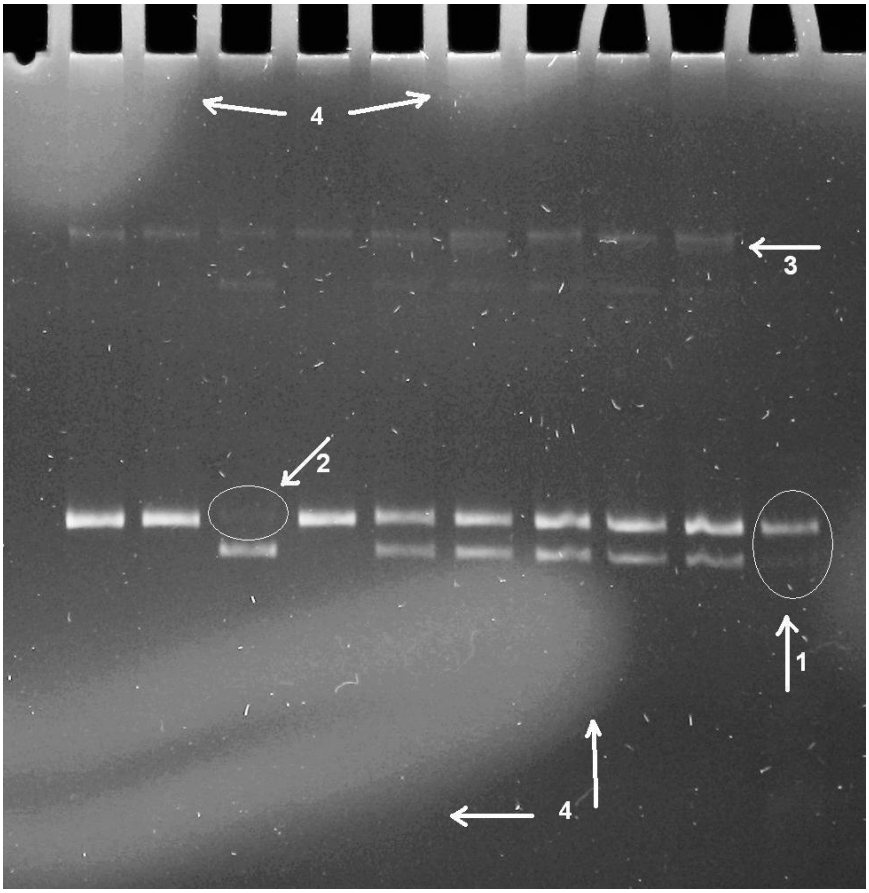


Legend: Results of the PCR-RFLP analysis of the HFE G845A (C282Y)/ HFE C187G (H63D).

Fig. 3 *Failed step – over-amplified fragments*

Much more critical is the problem of sample contamination. In Figure 4, we can see that the PCR was also activated in negative control without added template DNA (arrow 1). Moreover, there are noticeable signs of incomplete cleavage with restriction endonuclease (arrow 2). There are also redundant DNA fragments (arrow 3). The solution of the problem is

ensuring clean handling of samples under good laboratory practice and proper storage of all reagents.



Legend: Results of the PCR-RFLP analysis of the MTHFR C677T polymorphism.

Fig. 4 *Failed step – cross-contamination, non-homogenous staining*

Errors in electrophoresis:

Inhomogeneous staining of the gel can be caused by different exposure to staining solution. This most often happens by non-homogeneous adherence of the gel to the bottom of the staining container followed by partial underflow of the staining solution beneath the gel. As we can see on the figure 5, the consequence is typical electrophoreogram looking like heterogenous bright waves (arrows 4).

Serious problem to the outcome of the analysis is an error in the preparation of the separating gel for DNA electrophoresis. In Figure 5, we can see the consequences of inhomogeneous polymerization of the polyacrylamide gel. There is total loss of the samples caused by the deformation and self-destruction of the gel with inhomogeneous density.

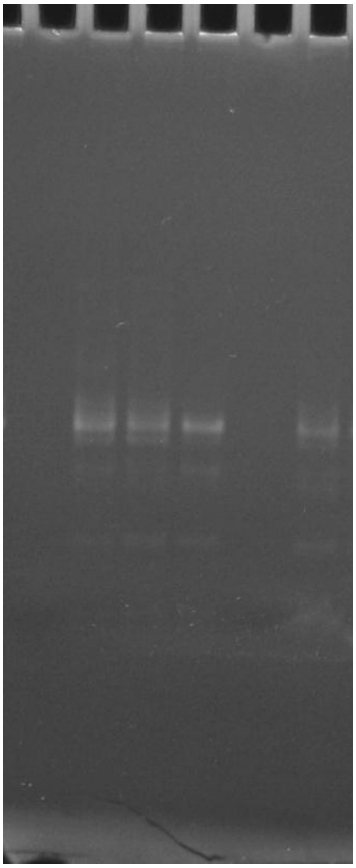
Legend: Results of the PCR-RFLP analysis of the MTHFR C677T polymorphism (left side) and FV-Leiden G1691A/FII G20210A (right side of the gel). Note: Soft wavy lines on gel surface are caused by overlap of the protective cellophane foil and not a fault of the gel.

Fig. 5 *Failed step – gel polymerization*

Blurring of the DNA strips on the electrophoreogram may occur due to non-standard state of electrophoresis buffer (e g. after repeated use of TBE buffer; figure 6). Therefore the buffer concentration and condition is very crucial for successful result of electrophoresis.

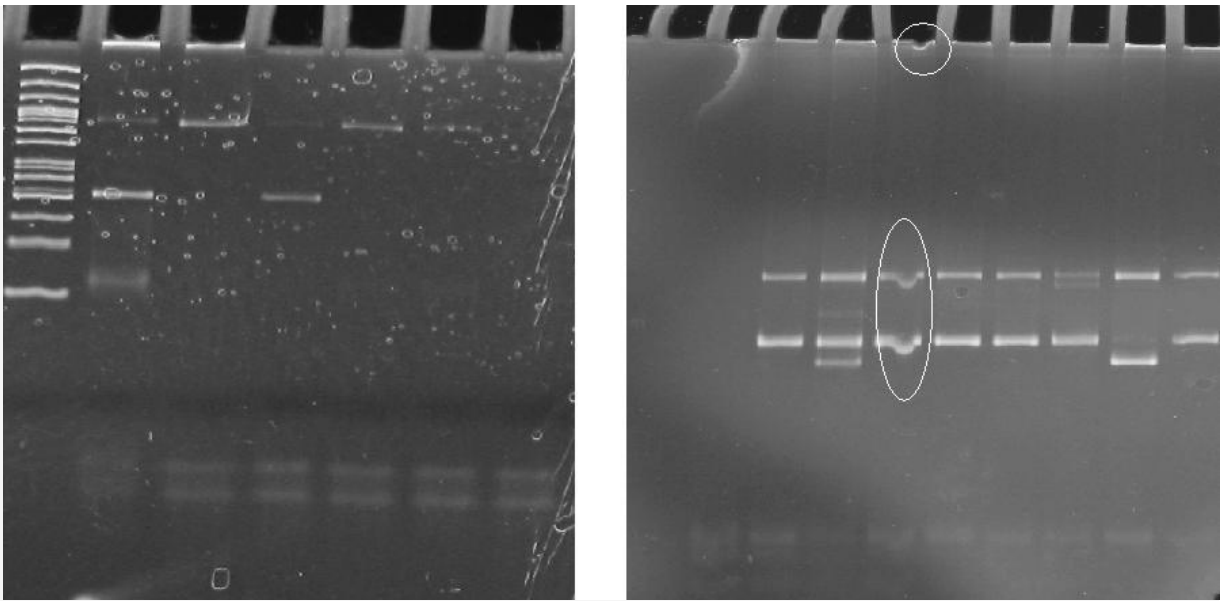
Another significant phenomenon that makes the evaluation of the electrophoreogram more difficult is formation of bubbles. In the figure 7 we can see, that bubbles are unwanted whenever they can deform the pattern of the migrating DNA.

The most serious situation is, the combination of more sources of errors. Such a situation we can see on the figure 8. The electrophoreogram shows samples with errors in PCR (template DNA at the starts), together with the "smears" of nonspecific fragments and electrophoreogram deformations due to different buffer concentrations between samples and size marker. On the left side is visible distinctive over staining due to underflow of the staining solution. All above mentioned errors of electrophoresis lead to repeating of the electrophoresis.



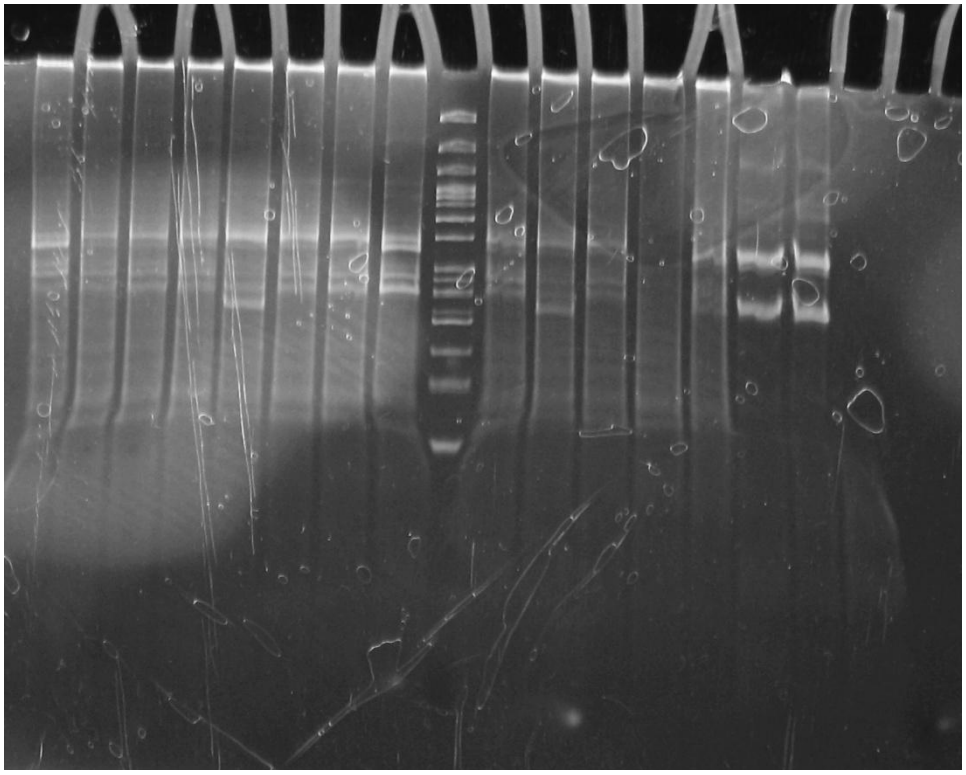
Legend: Results of the PCR-RFLP analysis of the FV-Leiden G1691A/FII G20210A. DNA strips are blurred due to inappropriate parameters of TBE buffer.

Fig. 6 *Failed step – blurring of the sample DNA*



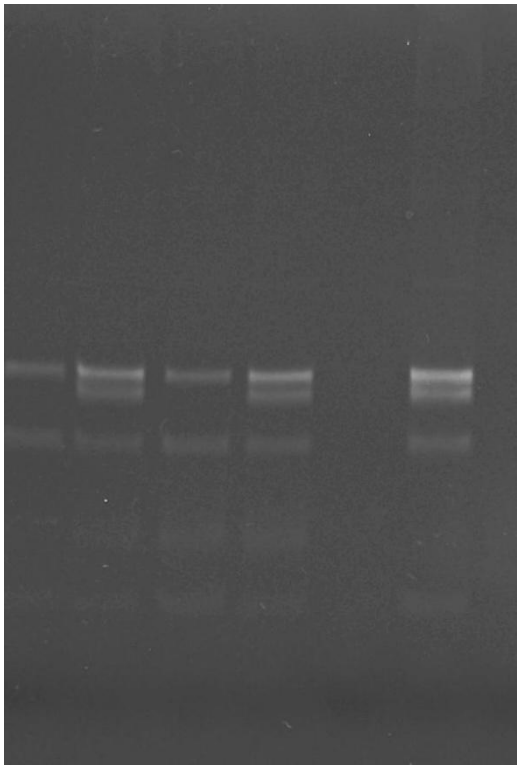
Legend: Results of the PCR-RFLP analysis of the multiplex PCR for FV-Leiden G1691A / FII G20210A (right) and ACE ins/del (left).

Fig. 7 *Failed step – bubbles in the separation gel*



Legend: Results of the PCR-RFLP analysis of the multiplex PCR for FV-Leiden G1691A / FII G20210A with the electrophoresis size marker at the middle line.

Fig. 8 *Failed step – cumulating of several errors*



Legend: Results of the PCR-RFLP analysis of the PCR for FV-Leiden G1691A.

Fig. 9 *Example of standard electrophoreogram*

Finally, we present the image of ideal electrophoreogram (figure 9) containing sharp DNA strips, both standard controls (positive and negative controls, from the right to the left side) and all patients with clear genotype.

Conclusion

All errors during PCR-RFLP increase costs of anaylses. It is desirable to keep rules of the good laboratory praxis along with the written manuals. In the case of non-successful result of PCR-RFLP analysis without known source of errors the best way what to do is to repeat the analysis without any changes, because an untentional rough error could arised. If the laboratory method gives erroneous results continuously, the checking of solutions and enzymes takes place and then also re-optimizing of physical parameters (time and temperature of PCR, current, voltage, time and cooling of electrophoresis).

In case of no improvement, the most appropriate alternative is to consult the problem with other independent laboratory or product advisor. Problems with apparatures and devices (thermal cyclers, electrophoresis power suppliers and electrophoretical apparatures) have to be first consulted with another laboratory, using the same machine and only then we should contact the manufacturer.

The validation can be done only in the case of the correct results of the both internal controls, used for the genotyping of the sample series. The positive control must clearly show expected known genotype and negative control must show no genotype and any DNA strip but primers. Any heterozygote sample must have the intensity of the strongest strip higher than the weakest strip of heterozygote control sample. This rule significantly reduces probability of false interpretation of electrophoreograms in the presence of redundant artificial strips. Another rule is the "two eyes pairs rule", requiring sample validation performed independently with two competent staf members.

All this remedies can reduce significantly the probability of PCR-RFLP results misinterpretation due to rough and random errors, but they will never be completely eliminated. The more samples are genotyped in the laboratory, the higher is the probability of a false validation case. It is therefore important to eliminate the error sources systematically. Unfortunately, DNA is too stabile for ignoring such a mistakes, which can in the future damage the reputation of the laboratory.

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