Thrombophilic gene mutations: two different approaches to the validation of commercial and "in-house" DNA diagnostic assays

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Introduction

Validation of molecular genetic analytical methods is a basic tool for quality assurance and has to be part of routine practice in clinical-diagnostic laboratories that are working in compliance with the ISO15189 guidelines (www.iso.org).
Moreover, last year OECD issued the "Guidelines for Quality Assurance in Molecular Genetic Testing (www.oecd.org\sti\biotechnology)" which stipulate that laboratories providing clinical services should be working within a "Quality Assurance System". Via method validation laboratories assess assay performance and demonstrate its suitability for the „intended purpose".
Hereby, we report practical examples of validation procedures utilized for an 1/„in-house" test that we developed for the detection of the sequence variant PAI-1 4G/5G, versus approaches applied to a commercially produced, CE/IVD marked, RHA Kit Thrombo (Labo Bio:Medical Products; www.labobiomedicalproducts.com).

Materials and Methods

For the examination of sequence variant PAI-1 4G/5G our laboratory uses an "in-house" method based on fragment analysis of restriction endonuclease- digested amplicons (RFLP). As an alternative serves the „RHA Kit Thrombo" commercial assay. It is based on reverse blot hybridization (RHA) for simultaneous detection and identification of mutations in genes coding for FII (20210G>A), FV-Leiden (Arg506Gln), FV- Cambridge (Arg306Thr), including the MTHFR 677C>T variant. The entire procedure complies with the CE/IVD – related guidelines.
In both instances we assessed basic validation parameters comprising specificity, sensitivity, precision and robustness.

Results and Discussion

Validation is an evidence-based assessment as to how a diagnostic test performs in the laboratory and demonstrates its „suitability for the intended purpose". This process involves results of experiments to determine test **specificity**, **sensitivity**, **precision** and **robustness**. The extent of validation depends on the respective method assessed. For „in-house" method validation we completed 227 analyses, while for the validation of the commercial RHA Kit Thrombo assay, which has been already in routine use in our laboratory and has been involved in respective annual Czech External Quality assessment schemes, we only ran a total of 62 samples.

Specificity and Sensitivity

In the case of the **commercial RHA Kit Thrombo** validation, specificity and sensitivity were assessed retrospectively. We used results from annual (2000-2007) Czech EQA (External Quality Assessment) schemes. In total, 78 EQA samples were examined: 34 of them were negative („wild type"), while 44 were positive (heterozygous or homozygous) for the FV Leiden, FII and MTHFR alleles (Tab. 1.)

Genotype	N of samples
FV Leiden negative	13
FV Leiden heterozygote	10
FV Leiden homozygote	9
FII 20210G>A negative	16
FII 20210G>A heterozygote	12
FII 20210G>A homozygote	4
MTHFR 677C>T negative	5
MTHFR 677C>T heterozygote	5
MTHFR 677C>T homozygote	4
Total	78

Tab.1. EQA sample genotypes that were utilised for the assessment of specificity and sensitivity of the RHA Kit Thrombo assay.

In the case of our **„in-house"** method we used 12 known PAI-1 4G/5G heterozygous and 12 PAI-1 4G/4G homozygous samples for specificity- and 12 negative samples for sensitivity evaluation (Tab. 2). Figs. 1.+2 show RFLP products on agarose gels of heterozygous and homozygous PAI-1 4G/5G samples, while „K2" - represents an internal quality control heterozygous for the PAI-1 4G/5G sequence variant.

Genotype	N of samples
PAI-1 5G/5G, negative	12
PAI-1 4G/5G, heterozygote	12
PAI-1 4G/4G, homozygote	12
Total	36

Table 2. Sample genotypes that were utilised for specificity and sensitivity assessement of our „in house" method for the detection of the PAI-14G/5G sequence variant.

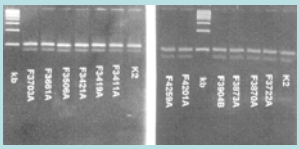

Fig. 1. Heterozygeus PAI-1 4G/5G restriction products on 3% agarose gels

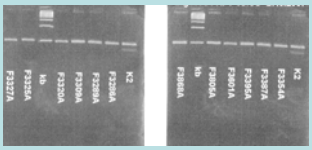

Fig. 2. Homozygous PAI-1 4G/4G restriction products on 3% agarose gels

All samples examined for specificity and sensitivity by both methods were correctly genotyped. We did not reveal any false positive or false negative results.

Precision: Repeatability and Reproducibility

Precision of both assessed methods was evaluated via two validation parameters: **repeatability** and **reproducibility**

Evaluation of **reproducibility** of the **RHA Kit Thrombo** assay was performed by analysis of 5 known samples (two known negative and three compound heterozygous mutated samples) prepared within three different days by the same operator. Samples genotypes are listed in a Tab. 3.

Genotype	I/2007	II1/2007	F554A	F4871A	F4879A
FV Leiden negative		x			
FV Leiden heterozygote	x				
FV Leiden homozygote			x		
FII G20210A negative		x			
FII G20210A heterozygote			x		
FII G20210A homozygote	x				
MTHFR C677T negative					
MTHFR C677T heterozygote	x				
MTHFR C677T homozygote		x			

Table 3 Sample genotypes for commercial RHA Kit Thrombo validation

The **precision** of the **„in-house" method** for detection of the PAI-1 4G/5G variant was evaluated , as described above. For respective evaluation of both validation parameters, we used the same 3 known samples (known PAI-14G/5G heterozygous, homozygous and negative samples) each with five replicates by the same operator. The samples for **repeatability** evaluation were prepared in a single series and performed within a single day. Samples for **reproducibility** evaluation were processed on three different days.

Both methods produced consistent results during precision evaluation. All examined samples were correctly genotyped. In total, we performed 45 analyses for the „in-house" method and 15 analyses for evaluation of the commercial assay.

Robustness

Robustness evaluation was performed in order to examine the assay sensitivity to small deviations, (different thermocyclers, operators, variation in annealing temperature or restriction temperature.)

For robustness evaluation of the **commercial RHA Kit Thrombo assay,** we used 5 known samples prepared by three different operators. Respective sample genotypes are listed in Tab. 3.

To evaluate robustness of the **„in-house"** PAI-1 4G/5G method we used two different thermocyclers, three operators, different PCR annealing and digestion temperatures. We used 10 samples (six PAI-1 4G/5G homozygous and heterozygous samples and three wild type samples and one heterozygous PAI-14G/5G internal quality control sample) for each variable being tested. Three **different operators** analysed exactly the same samples.
Results obtained from all operators were consistent and all samples were correctly genotyped.

The PCR reaction was ran on two **different thermocyclers from various manufacturers**. Samples were amplified according to our standard „SOP" PCR protocol. We generated same size and good quality of PCR products with each thermocycler tested.
Examined **PCR annealing temperature** varied within the range of ±1°C from the 60°C „baseline" .
All three annealing temperatures assured good amplicons, of the same intensity and size.
In the case of **restriction digest temperature** variation, we tested temperatures ± 3°C from the recommended digestion temperature for the respective restriction enzyme. Digestion with three different digestion temperatures produced correct fragments and proper genotypes for each sample analysed. Thus, the method is robust for digestion temperatures within the range spanning 52°C to 58°C.

Robustness of **commercial RHA Kit Thrombo** assay and **„in-house"**PAI-1 4G/5G assay was evaluated with 15 and 110 samples in total, respectively.

For the **„in-house"**PAI-1 4G/5G method we also evaluated other types of PCR master mixes. Reliability of various PCR master mixes was assessed by evaluation of specificity and sensitivity in 36 samples (Tab. 2.). We did not generate any false negative - or false positive genotypes, respectively.

Validation Parameter	Sample	Repetition	N of Analysis
Specificity	12	1	12
Sensitivity	24	1	24
Repeatability	3	5	15
Reproducibility			
1st day **(repeatability)**			
2nd day	3	5	15
3rd day	3	5	15
Variation of Annealing temperature			
59°C	10	1	10
60°C	10	1	10
61°C	10	1	10
Variation of Incubation temperature			
52°C	10	1	10
55°C	10	1	10
58°C	10	1	10
Different Thermal Cyclers			
Touchgene Techne	10	1	10
PTC 220	10	1	10
Different Operators			
VN	10	1	10
IA	10	1	10
HK	10	1	10
Different Master Mixes			
PyroStart Fast PCR Master Mix (2X), **specificity**	12	1	12
PyroStart Fast PCR Master Mix (2X), **sensitivity**	24	1	24
Total N of Analysis			**227**

Tab. 5. Validation parameters, number of samples and analyses utilised in evaluation of the „in-house" PAI-1 4G/5G method.

Validation Parameter	Sample	Repetition	N of Analysis
Specificity (EQA results)	34	1	34
Sensitivity (EQA results)	44	1	44
Reproducibility			
1st day	5	1	5
2nd day	5	1	5
3rd day	5	1	5
Robustness, different operators			
VN	5	1	5
IA	5	1	5
HK	5	1	5
Total N of Analysis			**108**

Tab. 4. Validation parameters, number of samples and analyses utilised in evaluation of the commercial RHA Kit Thrombo assay.

RHA Kit Thrombo assay is a typical example of standardized commercially produced, „IVD-CE labeled", diagnostic kit. This assay was evaluated without any changes to the manufacturer´s instructions. This assay has already been in routine use in our laboratory for several years and was involved in annual Czech EQA schemes. Therefore, we **shortened the validation procedure** by examining merely 30 samples for reproducibility and robustness, while for specificity and sensitivity testing we retrospectively evaluated previously performed EQA-related data (Tab 4.).

Summary

Tab. 4 and 5 illustrate different approaches and scope of validation for evaluated methods, **which both produced reliable results**.

Method for detection of the **PAI-1 4G/5G sequence variant** was recently introduced by our laboratory. Thus, unlike in the commercial RHA Kit Thrombo assay we performed a „full validation" procedure. The specificity, sensitivity, precision and robustness was assesed in a total of 227 analyses (Tab. 5.)

Acknowledgment

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