Method validation in cystic fibrosis DNA diagnostics: a basic tool for quality assurance

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Introduction

Validation of molecular genetic analytical methods has to be part of basic practice in clinical-diagnostic laboratories that are working in compliance with the ISO15189 guidelines (www.iso.org). In this regard, the laboratories should demonstrate that a given method is reliable, precise and robust. However, the ISO norm does not stipulate which validation parameters and to which extent these should be evaluated. Thus, it is up to individual laboratories to establish an acceptable validation process in order to comply with general principles of the ISO norm. Here we report practical examples of validation procedures utilized for a „in house" technique versus approaches applied to a commercially produced assay (ASR). For CF DNA diagnostics quality assurance it is crucial to establish such a procedure for the major *CFTR* allele F508del. Validation of the SALSA P091 MLPA assay for the detection of large rearrangements in the *CFTR* gene serves as another example.

Materials and Methods

For the examination of major *CFTR* mutation F508del our laboratory uses an „in-house" method based on PCR-related fragment analysis with fluorescently labeled primers using the 3130/3130xl Genetic Analyzer (Applied Biosystems, USA) for capillary electrophoresis.
Large rearrangements in *CFTR* gene (deletions/duplications of exons of *CFTR* gene and adjacent regions) are detected by commercially available assay SALSA P091 CFTR MLPA kit (MRC Holland) in our laboratory. The protocol recommended by manufacturer have been followed. The standard deviations and relative standard deviations (RSD) were calculated for the average dosage quotients of particular MLPA probes of WT samples.
DNA was extracted from peripheral blood lymphocytes using the Puregene Genomic DNA Purification Kit.
In both instances we assessed basic validation parameters comprising specificity, sensitivity, repeatability, reproducibility 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" F508del method validation we completed 347 analyses, while for the validation of the commercial *CFTR* MLPA kit we ran a total of 95 analyses.

Sensitivity and Specificity

In the case of our **„in-house" F508del** method validation we used 23 known F508del heterozygous and 2 F508del homozygous samples for **sensitivity** - and 22 negative samples for **specificity** evaluation. Analysed PCR products on Genetic Analyzer produced exceptionaly precise allele size calling. Average relative standard deviation of alleles size did not exceed 0.067%.

In the case of **commercial *CFTR* MLPA kit** validation we used 10 known negative samples without any deletion/duplication in *CFTR* gene for **specificity** evaluation. The RSDs for the samples were very low, and did not exceed 0.059 %. RSD for control probe in 13q14 was only one exception - 0.190%.
For **sensitivity** evaluation we used 5 samples with heterozygous deletions/duplication of exons of *CFTR* gene. Each sample was tested 5 times. The RSDs for the samples were very low, and did not exceed 0,066 %. RSD value for control probe in 13q14 in first case (0.138%) and control probe 17q21 in second case (0.1036%) were only exceptions.

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 was evaluated via two validation parameters: **repeatability** and **reproducibility**
In a case of the **„in-house" F508del method** for respective evaluation of both validation parameters, we used the same 3 known samples (known F508del heterozygous, homozygous and negative samples) each with ten 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.
Evaluation of **precision** of the **commercial *CFTR* MLPA kit** were tested with negative/mutated samples - one sample with heterozygous deletion of two exons of *CFTR* gene, and one negative sample without any deletion/duplication in *CFTR* gene, each with five preparations by the same operator. The samples for **repeatability** evaluation were prepared in a single series within a single day. The RSD for the dosage quotients for all tested samples was lower than 0,08%.
Samples for **reproducibility** evaluation were processed in two different days. The RSD for dosage quotients for tested samples for two days was lower than 0.062%.

Both methods produced consistent results during precision evaluation. All examined samples were correctly genotyped. In total, we performed 90 analyses for the „in-house" F508del method and 20 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, DNA concentration, PCR cycle number, different master mixes, polymerase).
To evaluate **robustness** of the **„in-house" F508del** method we used two different thermocyclers, three operators, different PCR annealing temperatures, PCR cycle number, variation in DNA concentration, different master mixes and polymerase.
For evalution different **thermocyclers** and **operators** we used 10 samples (five F508del heterozygous samples and five negative samples). 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.
Three **different operators** analysed exactly the same samples. **Results obtained from all operators were consistent and all samples were correctly genotyped.**
We used 2 samples (one heterozygous F508del sample and one negative sample) prepared in triplicate for PCR annealing temperature, PCR cycle number and different DNA concentration testing.
Examined **PCR annealing temperature** varied within the range of ± 1°C from the 50°C „baseline" In the case of **PCR cycle number** variation, we tested 28, 30 and 32 cycles. **DNA concentration** varied from 30ng/ul to 120 ng/ul.
All three annealing temperatures, PCR cycles and different DNA concentration assured good amplicons and precise allele size calling on Genetic Analyzer.
For the „in-house" F508del method we also evaluated other types of **PCR master mixes** and **polymerase**. Reliability of various PCR master mixes and polymerase was assessed by evaluation of specificity and sensitivity in 94 samples. We did not generate any false negative - or false positive genotypes, respectively.

For robustness evaluation of the **commercial *CFTR* MLPA kit** were tested 5 different **DNA concentrations** (50 ng/μl, 100 ng/μl , 200 ng/μl, 300 ng/ul and 400 ng/μl) with two samples in triplicate and two different **operators**. Each operator ran five times sample with heterozygous deletion of two exons of CFTR gene. There was not a significant difference in MLPA analyses between operators. Both operators produced accurate genotyping.
This method is appropriate for a range of DNA concentrations: 300 – 400ng/μl without any variations.

Robustness of „in-house" F508del assay and commercial *CFTR* MLPA kit assay was evaluated with 210 and 40 samples in total, respectively.

Conclusions

Method for detection of the *CFTR* F508del mutation was recently introduced by our laboratory. Thus, unlike in the commercial SALSA P091 CFTR MLPA kit assay we performed a „full validation procedure. SALSA P091 CFTR MLPA kit is an example of commercially produced diagnostic kit. This assay was evaluated without any changes to the manufactures instructions. Therefore, shortened validation procedure was performed. We have demonstrated, that both assays performed as expected by testing following validation parameters: specificity, sensitivity, repeatability, reproducibility and robustness.

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