Validation report

Cottus perifretum/rhenanus qPCR detection kit

#SYL143

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1. Validation report *Cottus perifretum/rhenanus* detection kit

1.1 In silico validation

Table 4: Forward primer *in silico* validation

Length	20
GC %	55
Stability	2.0
T_M (°C)	60
Target region	CO1 (mtDNA)
Dimer	No
Run	No
Database hit	*Cottus perifretum/rhenanus*

Table 5: Reverse primer *in silico* validation

Length (bp)	21
GC %	47
Stability	1.3
T_M (°C)	60
Target region	CO1 (mtDNA)
Dimer	No
Run	No
Database hit	*Cottus perifretum/rhenanus*

Table 6: Probe *in silico* validation

Length	37
GC %	32
T_M (°C)	66
Target region	CO1 (mtDNA)
Dimer	No
Run	No
Fluorescence label	FAM

Table 7: Combined primers and probe *in silico* validation

PCR product size (bp)	99
Combined dimer formation	No
Combined 1000 BLAST® analyses of both primers	*Cottus perifretum/rhenanus, Cottus Gobio, Cottus microstomus, Cottus rhenanus,*
Date of BLAST® analyses	*November 2020*

1.2 Experimental validation

All validation experiments were performed with the Sylphium qPCR mix and performed on Applied biosystems 7300 qPCR machines and Biometra gradient PCR machines.

1.2.1 Gradient PCR

Influence of the annealing temperature on the performance and specificity of the primer set was tested with a temperature gradient between 50.0°C to 72.0°C in eight steps. Optimal temperature and the temperature range in which the test can perform was determined.

Results:

The expected product was formed between 50.0°C and 65.0°C. Nonspecific additional fragments were formed at 47.5°C and 49.7°C. No primer dimer were formed at any tested temperature. The optimal annealing temperature was between 55.5°C and 64.4°C (table 8).

Annealing temp.	**50.0°C**	**52.1°C**	**54.4°C**	**57.0°C**	**59.7°C**	**62.3°C**	**65°C**	**67.6°C**	**69.9°C**	**72.0°C**
Expected fragment	Strong	Strong	Strong	Strong	Strong	Strong	Strong	No	No	No
Primer dimer	No	No	No	No	No	No	No	No	No	No
Additional fragments	No	No	No	No	No	No	No	No	No	No

Table. 8: Temperature gradient PCR on genomic DNA of *Cottus perifretum/rhenanus.*

1.2.2 detection limits, fluorescence output signal and efficiency

Standard solutions with 10^6, 10^5, 10^4, 10^3, 10^2, 10, 1 and 0 target copies per 2 µl were analyzed to determine the detection limit at optimal primer/probe concentrations. Fluorescent output signals were compared with the background to have at least a 100 fold increase in fluorescence signal. Reaction condition were adjusted if the fluorescence signal was too low in contrast to the background noise. Based on the slope of the standard curve the efficiency of the primer/probe set was determined. The efficiency should be between 90% and 110%. Limits of quantification and detection were determined on basis of this efficiency limitations.

Results:

Limit of detection (LODqPCR) for this kit was determined at 1 copy per reaction. Limit of quantification (LOQqPCR) was determined at 100 copies per reaction. (Fig. 2, table 9).

The fluorescence output signal was at least 100 (5 for positive sample, 0,05 for a negative sample) times stronger than the background signal. (fig. 1, table 8)

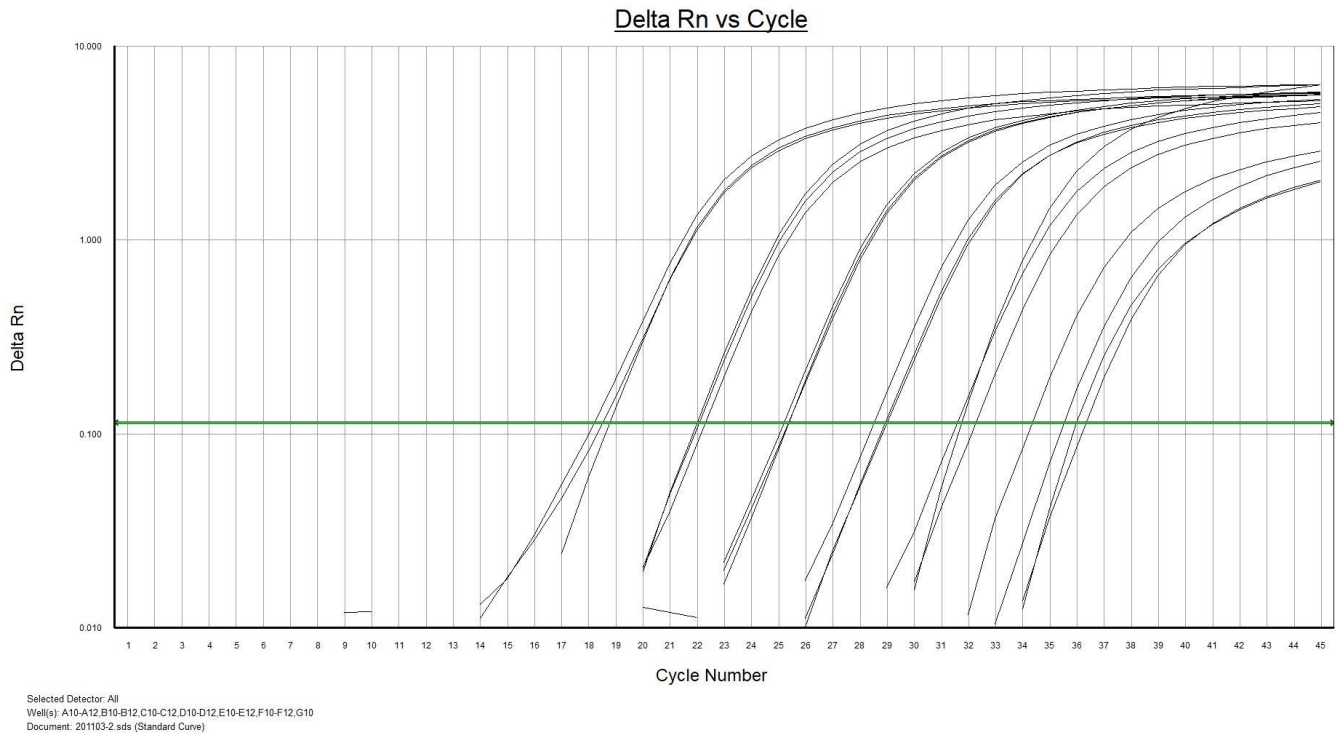

Figure. 1: Obtained fluorescent signal at optimal primer/probe concentration at a annealing temperature of 58°C.

Table 8: CT values obtained at optimal primer/probe concentration.

target DNA copy	Target detected	CT value	Standard deviation
0	No	-	-
1	Yes	35.5	-
10	Yes	35.5	1.1
10^2	Yes	31.8	0.4
10^3	Yes	28.8	0.3
10^4	Yes	25.3	0.1
10^5	Yes	22.1	0.1
10^6	Yes	18.5	0.3

Fig. 2: Standard curve of SYL143 based on 10, 10^2, 10^3 , 10^4, 10^5 and 10^6 DNA target copies

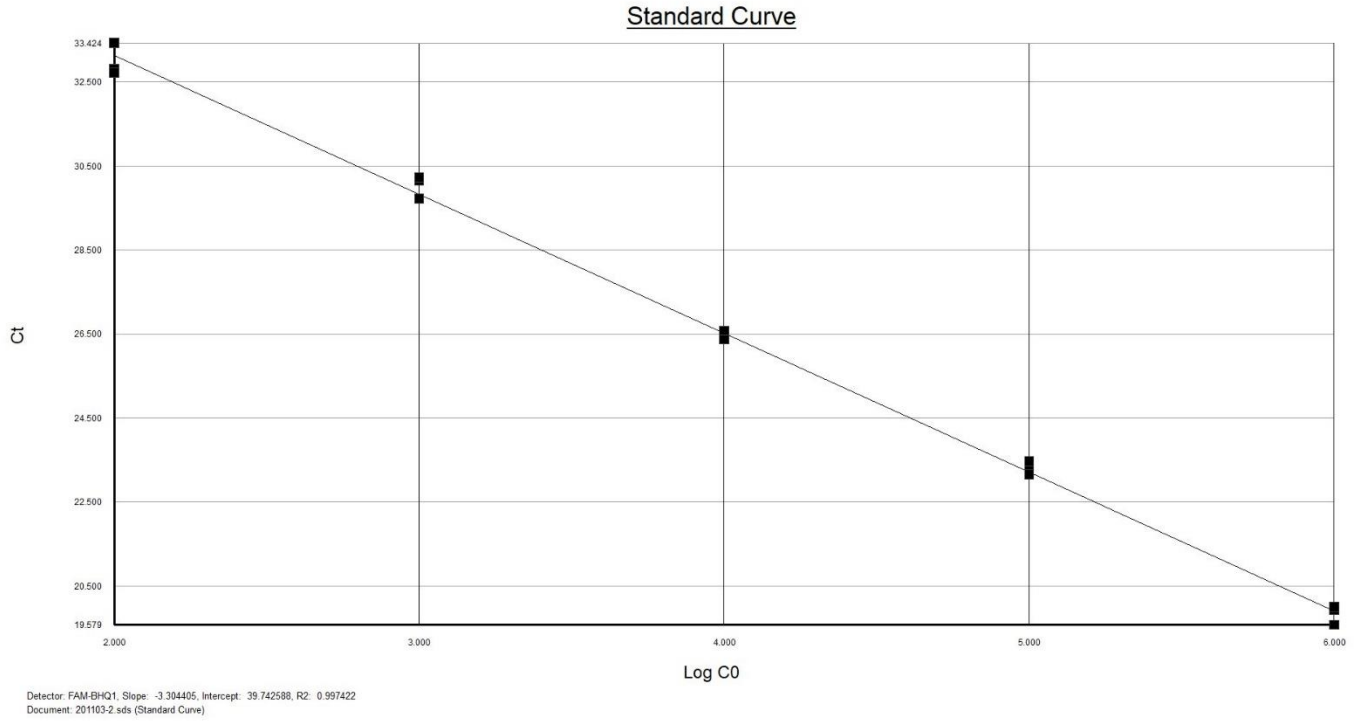

Table 9: Values obtained from the standard curve

Slope	-3.30
Efficiency	99.5%
R^2	0.997

1.2.3 Influence of inhibiting factors present in environmental samples and repeatability

Performance of the test was tested on the influence of inhibiting factors present in three different kinds of environmental water samples (sandy, clayey or peaty bottoms) in three fold spiked with 1000 target DNA copies. Standard deviations, difference to spike and the standard error of mean were calculated to determine robustness, repeatability and measurement uncertainty.

Results:
No fluorescent output signal was detected in the environmental samples without added target organism DNA (spike). Spiked samples and the spike only analyses gave in all replicates a positive signal. (see table 11). The difference between environmental samples and the spike only sample was less than 3%. Influence of chemical and biological factors present in environmental samples was not detected. The standard error of mean was 0.03 within these samples with an average of 28.1

Sample	CT value	Standard deviation	Difference to spike
Sandy	-	-	-
Clayey	-	-	-
Peaty	-	-	-
Sandy + spike	28.1	0.1	- 0.18%
Clayey + spike	28.2	0.0	- 0.36%
Peaty + spike	28.0	0.1	0.14%
Spike only	28.1	0.1	-

Table 11: CT values obtained with target organisms free environmental samples spiked with 1000 target copies.

1.2.4 Detection conformation in environmental samples

Conformation of detection with the test was performed on samples form locations where the presence of *Cottus perifretum/rhenanus* was suspected. Samples were taken using SYL001 - Environmental sampling kit and eDNA was isolated using SYL002 -Environmental DNA isolation kit.

Results:
The kit SYL143 – *Cottus perifretum/rhenanus* detection kit was able to detect *Cottus perifretum/rhenanus* in environmental samples from different locations in the Netherlands. On average, a positive sample gave 1 to 10 molecules *Cottus perifretum/rhenanus* DNA when 100 - 200 ml water was filtrated.

1.2.5 Sequence conformation of specificity.

PCR products obtained from environmental samples were sequenced for conformation of identity of the formed product. If any product was formed with target organism free environmental samples, than these products were also be sequenced.

Results:
Target organism free environmental samples did not give any PCR product. PCR products obtained from environmental samples were sequenced in triplo via the Sanger sequencing method. The obtained sequences were identical to each other and Blast ® hits confirmed identity of the target organism.

1.3 Summary of validation

1.3.1 Robustness

"Influence of temperature variations and inhibiting factors present in environmental samples on the performance and specificity of the primer set"

- Primers specific at temperature range: 50.0°C – 65.0°C (section 4.2.1, section 4.2.2, table 8, table 9)
- Differences (± 3%) due to influence of chemical and biological factors present in environmental samples on the performance of the test were not found. (section 4.2.2, table 11)
- Fluorescent output signals of positive samples is at least 100 fold stronger than the background

1.3.2 Detection limits

"Lowest limits in which the analysis can be reliably applied"

Limit of detection (LDqPCR) for this kit was determined on 1 copy per reaction. Limit of quantification (LQqPCR) was also determined on 10 copies per reaction. (section 4.2.2, table 8)

1.3.4 Efficiency

"The comparison of what is actually produced with what can be achieved with the same consumption of resources"

The efficiency of the primer set is 99.5%, this means that the primer/probe mixture can be regarded as optimal. (section 4.2.2, table 9)

1.3.5 Repeatability

"The degree of similarity between the results of measurements of the same measured quantity that were performed under different measurement conditions"

There was no difference (± 3%) between different kinds of environmental water samples (sandy, clayey or peaty bottoms) spiked with 1000 target DNA copies. (section 4.2.3, table 11)

1.3.6 Correctness

"The ability of the method to do what it 'says' to do"

- The test was able to detect *Cottus perifretum/rhenanus* DNA in environmental samples from locations were the presence of *Cottus perifretum/rhenanus* was suspected. (section 4.2.6)
- The method did not give any other combined BLAST hit than the target organism *Cottus perifretum/rhenanus*
- The method was able to detect all spiked target DNA in target organism free environmental samples (section 4.2.3, table 10)
- The method did not give any signal in target organism free environmental samples (section 4.2.3, table 11)
- The standard error of the mean based on the measured CT values from the spiked experiments (1000 target copies) was 0.03 on an average of 28.3.