Validation report:

Lota lota qPCR detection kit

with eDNA qPCR master mix

#SYL115

Document date: 03 March 2022

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1. Validation report *Lota lota* detection kit

1.1 In silico validation

Table 4: Forward primer *in silico* validation

Length	21
GC %	47
Stability	1.7
T_M (°C)	61
Target region	CytB (mtDNA)
Dimer	No
Run	No
Database hit	*Lota lota*

Table 5: Reverse primer *in silico* validation

Length (bp)	20
GC %	55
Stability	3.6
T_M (°C)	61
Target region	CytB (mtDNA)
Dimer	No
Run	No
Database hit	*Lota lota*

Table 6: Probe *in silico* validation

Length	27
GC %	48
T_M (°C)	72
Target region	CytB (mtDNA)
Dimer	No
Run	No
Fluorescence label	FAM

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

PCR product size (bp)	116
Combined dimer formation	No
***In silico* PCR on Genbank**	*Lota lota*, low affinity with *Phycis phycis, Pseudogastromyzon lianjiangensis, Pseudogastromyzon myersi, Bhavania australis, Anguilla dieffenbachia, Gastromyzon ctenocephalus*
Date of BLAST® analyses	*March 2022*

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. No nonspecific additional fragments were formed. No primer dimer were formed at any tested temperature. The optimal annealing temperature was between 50.0°C and 65.0 (table 8).

Table. 8: Temperature gradient PCR on genomic DNA of *Lota lota*.

Annealing temp.	**50.0°C**	**52.7°C**	**54.4°C**	**57.0°C**	**59.7°C**	**62.3°C**	**65.0°C**	**72.0°C**
Expected fragment	Strong	Strong	Strong	Strong	Strong	Strong	Strong	No
Primer dimer	No	No	No	No	No	No	No	No
Additional fragments	No	No	No	No	No	No	No	No

1.2.2 detection limits, fluorescence output signal and efficiency

Standard solutions with 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 (LDqPCR) for this kit was determined at 1 copy per reaction. Limit of quantification (LQqPCR) was determined at 1 copies per reaction. (Fig. 2, table 9).

The fluorescence output signal was at least 100 (5 for positive sample, 0,01 for a negative sample) times stronger than the background signal. (fig. 1, table 8)

Selected Detector: All
Well(s): A7-A9,B7-B9,C7-C9,D7-D9,E7-E9,F7-F9,G7-G9
Document: 200305-2.sds (Standard Curve)

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[1] copy	Target detected	CT value	Standard deviation
0	No	-	-
1	Yes	33.5	0.6
10	Yes	30.1	0.2
10^2	Yes	26.7	0.2
10^3	Yes	23.1	0.2
10^4	Yes	19.8	0.3

[1] Estimated by gel electrophoresis

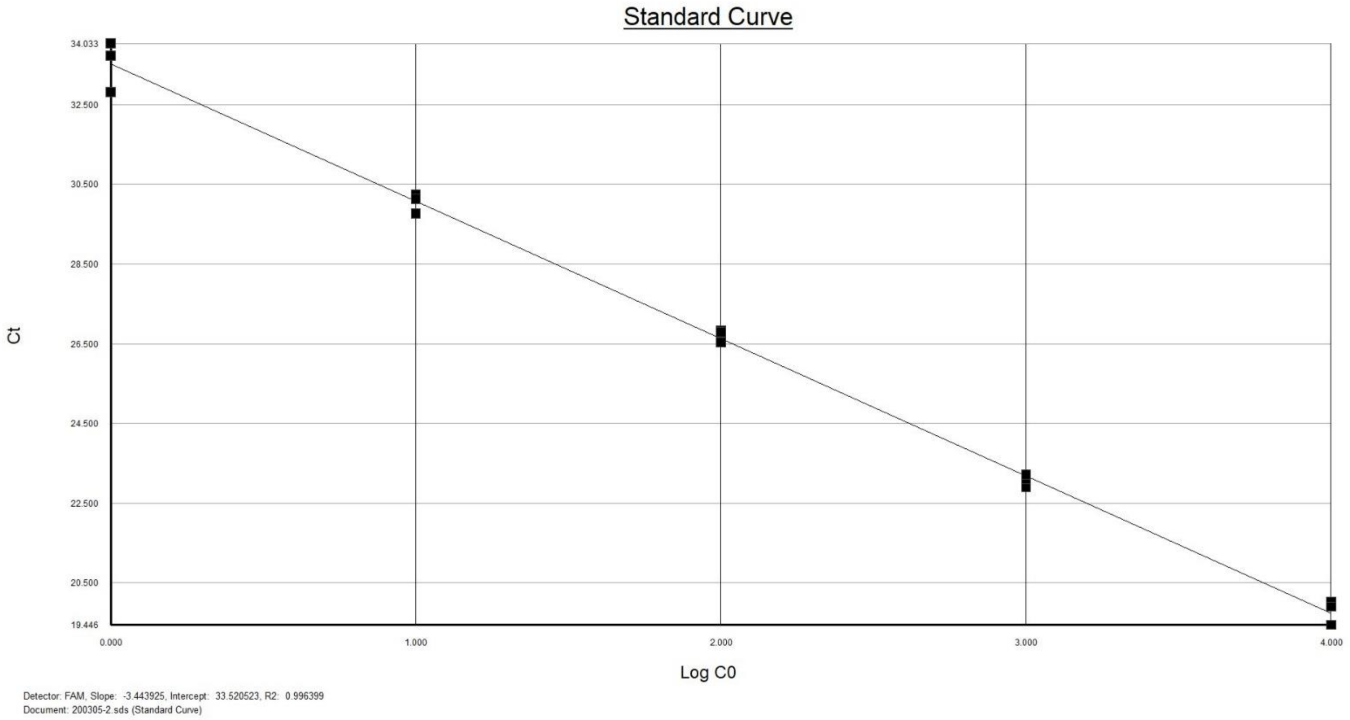

Fig. 2: Standard curve of SYL115 based on 1. 10, 10^2, 10^3 and 10^4 DNA target copies

Table 9: Values obtained from the standard curve

Slope	-3.44
Efficiency	95.3
R^2	0.996

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 24.4

Sample	CT value	Standard deviation	Difference to spike
Sandy	-	-	-
Clayey	-	-	-
Peaty	-	-	-
Sandy + spike	24.3	0.0	0.5 %
Clayey + spike	24.3	0.0	0.6 %
Peaty + spike	24.6	0.0	1.5 %
Spike only	24.2	0.0	-

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 *Lota lota* was suspected. Samples were taken using SYL001 - Environmental sampling kit and eDNA was isolated using SYL002 - Environmental DNA isolation kit.

Results:

The kit SYL115 – *Lota lota* detection kit was able to detect *Lota lota* in environmental samples from different locations in the Netherlands. On average, a positive sample gave 1 to 10 molecules *Lota lota* 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 1.2.1, section 1.2.2, table 8, table 9)
- Differences due to influence of chemical and biological factors present in environmental samples on the performance of the test was below 3%. (section 1.2.2, 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 copies per reaction. Limit of quantification (LQqPCR) was also determined on 1 copies per reaction. (section 1.2.2, 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 95.3%, this means that the primer/probe mixture can be regarded as optimal. (section 1.2.2, 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 differences (<3%) between different kinds of environmental water samples (sandy, clayey or peaty bottoms) spiked with 1000 target DNA copies. (section 1.2.3, table 11)

1.3.6 Correctness

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

- The test was able to detect *Lota lota* DNA in environmental samples from locations were the presence of *Lota lota* was suspected. (section 1.2.6)
- The method did not give any other combined BLAST hit than the target organism *Lota lota*
- The method was able to detect all spiked target DNA in target organism free environmental samples (section 1.2.3, table 10)
- The method did not give any signal in target organism free environmental samples (section 1.2.3, 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 24.4.