Validation report:

Misgurnus fossilis qPCR detection kit

with eTaq qPCR master mix

#SYL159

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1. Validation report *Misgurnus fossilis* detection kit

1.1 In silico validation

Table 4: Forward primer *in silico* validation

Length	25
GC %	48
Stability	1.2
T_M (°C)	65
Target region	CytB (mtDNA)
Dimer	No
Run	No
Database hit	*Misgurnus fossilis*

Table 5: Reverse primer *in silico* validation

Length (bp)	26
GC %	35
Stability	2.7
T_M (°C)	58
Target region	CytB (mtDNA)
Dimer	No
Run	No
Database hit	*Misgurnus fossilis*

Table 6: Probe *in silico* validation

Length	37
GC %	37
T_M (°C)	70
Target region	CytB (mtDNA)
Dimer	No
Run	No
Fluorescence label	FAM

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

PCR product size (bp)	107
Combined dimer formation	No
***In silico* PCR on Genbank**	*Misgurnus fossilis*
Date of *In silico* PCR	*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°C to 70°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 70.0°C. Nonspecific additional fragments were formed at 51.9°C and 56.4°C. No primer dimers were formed at any tested temperature. The optimal annealing temperature was between 58.8.0°C and 70.0°C (See Table 7).

Table. 7: Temperature gradient PCR on genomic DNA of *Misgurnus fossilis.*

Annealing temp.	**50.0°C**	**51.9°C**	**56.4°C**	**58.8°C**	**61.2°C**	**63.6°C**	**68.1°C**	**70.0°C**
Expected fragment	Strong	Strong	Strong	Strong	Strong	Strong	Strong	Strong
Primer dimer	No	No	No	No	No	No	No	No
Additional fragments	No	weak	weak	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 10 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)

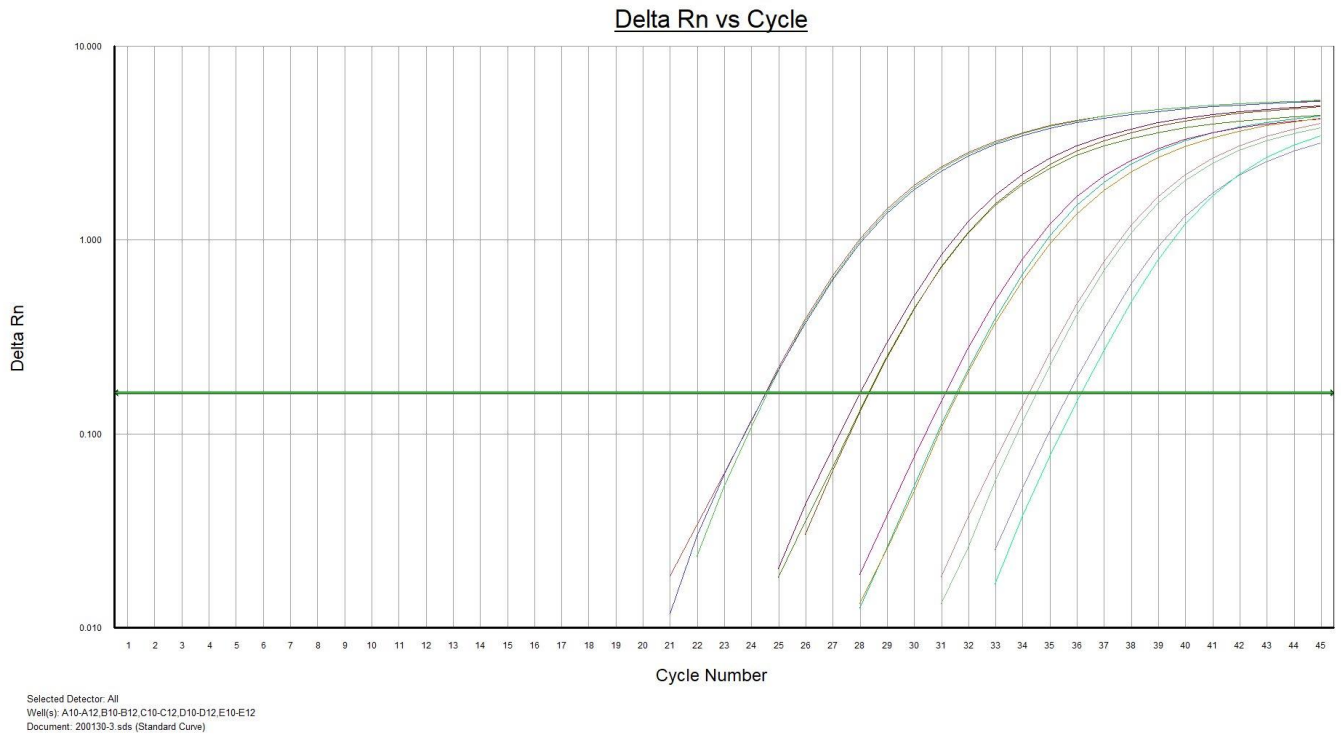

Figure. 1: Obtained fluorescent signal at optimal primer/probe concentration at a annealing temperature of 60°C.

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

target DNA[1] copy	Target detected	CT value	Standard deviation
0	No	-	-
1	Yes	36.1	0.8
10	Yes	34.8	0.8
10^2	Yes	31.4	0.2
10^3	Yes	28.2	0.2
10^4	Yes	24.5	0.0

[1] Estimated by gel electrophoresis

Fig. 2: Standard curve of SYL159 based on 10^4, 10^3, 10^2 and 10 DNA target copies

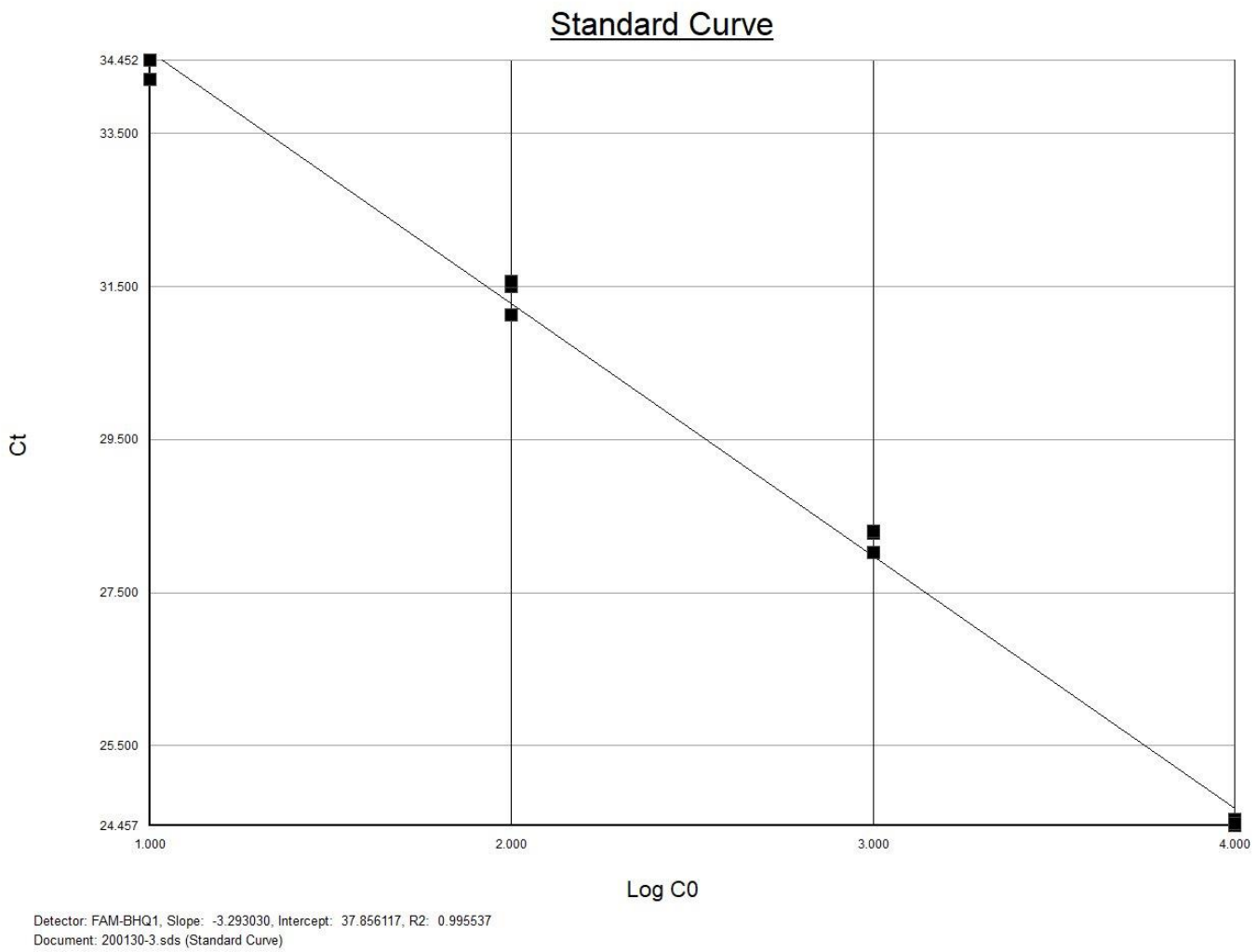

Table 9: Values obtained from the standard curve

Slope	-3.29
Efficiency	101.5%
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 0.01 within these samples with an average of 27.1

Sample	CT value	Standard deviation	Difference to spike
Sandy	-	-	-
Clayey	-	-	-
Peaty	-	-	-
Sandy + spike	27.1	0.1	0%
Clayey + spike	27.1	0.0	0%
Peaty + spike	27.1	0.1	0 %
Spike only	27.1	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 *Xxx* was suspected. Samples were taken using SYL001 - Environmental sampling kit and eDNA was isolated using SYL002 - Environmental DNA isolation kit.

Results:
The SYL159: *Misgurnus fossilis* detection kit was able to detect *Misgurnus fossilis* in environmental samples from different locations in the Netherlands. On average, a positive sample gave 1 to 10 molecules *Misgurnus fossilis* DNA when 150 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°C – 63.3°C (section 1.2.1, section 1.2.5, table 7, table 8)
- Statistical differences (p>0.05) due to influence of chemical and biological factors present in environmental samples on the performance of the test were not found. (section 1.2.4, table 10)
- Fluorescent output signals of positive samples is at least 100 fold stronger than the background

1.3.2 Detection limit

"Limits (lower and upper limit) within which the analysis can be reliably applied"

The detection limits (lower and upper limit) for qualitative analysis was determined between 10^4 and 1 target DNA copies per reaction. The detection limits for quantitative analyses were determined between 10^4 and 10 target 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 101.5 %, this means that the primer/probe mixture can be regarded as optimal. (section 1.2.3, 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 statistical differences (p>0.05) between different kinds of environmental water samples (sandy, clayey or peaty bottoms) spiked with 1000 target DNA copies. (section 1.2.4, table 10)

1.3.6 Correctness

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

- The test was able to detect *Misgurnus fossilis* DNA in environmental samples from locations were the presence of *Misgurnus fossilis* was suspected. (section 1.2.6)
- The method did not give any other combined BLAST hit than the target organism *Misgurnus fossilis*
- The method was able to detect all spiked target DNA in target organism free environmental samples (section 1.2.4, table 10)
- The method did not give any signal in target organism free environmental samples (section 1.2.4, table 10)
- The standard error of the mean based on the measured CT values from the spiked experiments (1000 target copies) was 0.07 on an average of 30.4.