Validation report

Pelobatus fuscus qPCR detection kit

with eDNA qPCR master mix

#SYL103

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1. Validation report *Pelobatus fuscus* detection kit

1.1 In silico validation

Table 4: Forward primer *in silico* validation

Length	20
GC %	60
Stability	2.3
T_M (°C)	66
Target region	CytB (mtDNA)
Dimer	No
Run	CCC
Database hit	*Pelobates fuscus*

Table 5: Reverse primer *in silico* validation

Length (bp)	24
GC %	45
Stability	1.7
T_M (°C)	63
Target region	CytB (mtDNA)
Dimer	No
Run	No
Database hit	*Pelobates fuscus*

Table 6: Probe *in silico* validation

Length	25
GC %	52
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)	87
Combined dimer formation	No
***In silico* PCR op Genbank**	*Pelobates fuscus*
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. The optimal annealing temperature range in which the test can perform was determined.

Results:

The expected product was formed at all tested temperatures (50°C to 70°C). The Optimal annealing temperature was between 50 °C and 63,6°C. (See fig. 3)

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	Weak	No	No
Primer dimer	No	No	No	No	No	No	No	No
Additional fragments	Weak	No	No	No	No	No	No	No

Table. 8: Temperature gradient PCR on genomic DNA of *Pelobatus fuscus.*

1.2.2 detection limit and fluorescence output signal

Standard solutions with 10^6, 10^5, 10^4, 10^3, 10^2, 10 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 to low in contrast to the background noise.

Results:

Limit of detection (LDqPCR) for this kit was determined on 1 copies per reaction. Limit of quantification (LQqPCR) was determined on 10 copies per reaction. The fluorescence output signal was at least 100 (7 for positive sample, 0,01 for a negative sample) times stronger than the background signal. (fig. 4, table 9)

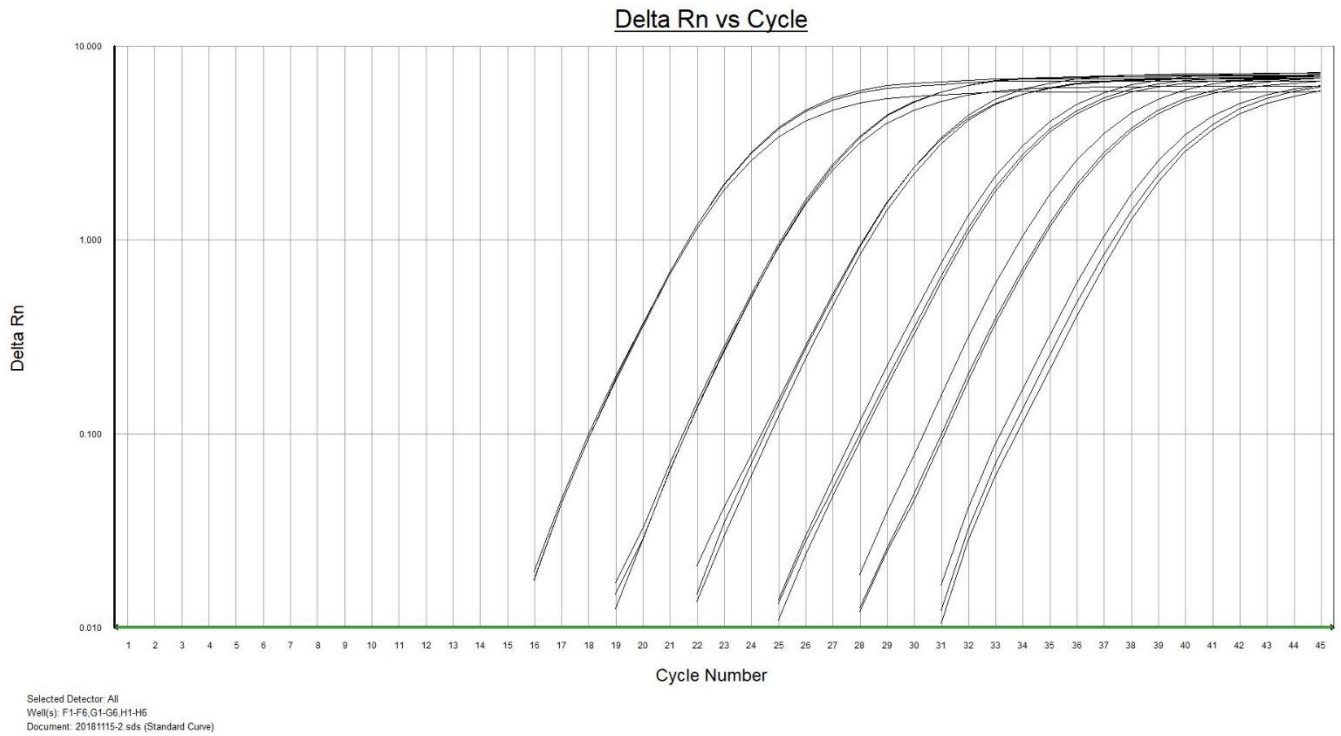

Figure. 4: Obtained fluorescent signal at optimal primer/probe concentration at a annealing temperature of 55°C.

Table 9: CT values obtained at optimal primer/probe concentration.

target DNA[1] copy	Target detected	CT value	Standard deviation
0	No	-	-
10	Yes	32.7	0.3
10^2	Yes	30.0	0.4
10^3	Yes	27.2	0.1
10^4	Yes	23.8	0.2
10^5	Yes	20.8	0.1
10^6	Yes	17.3	0.0

[1] Estimated by gel electrophoresis
[2] Not determined as very low amounts of DNA cannot reliable quantitatively detected by the qPCR technique

1.2.3 Efficiency

Based on the slope of the standard curve the efficiency of the primer/probe set was determined.

Results:

The efficiency of the test was above 90%. Which means that the efficiency of the primer/probe mixture can be regarded optimal (Fig. 5, table 10).

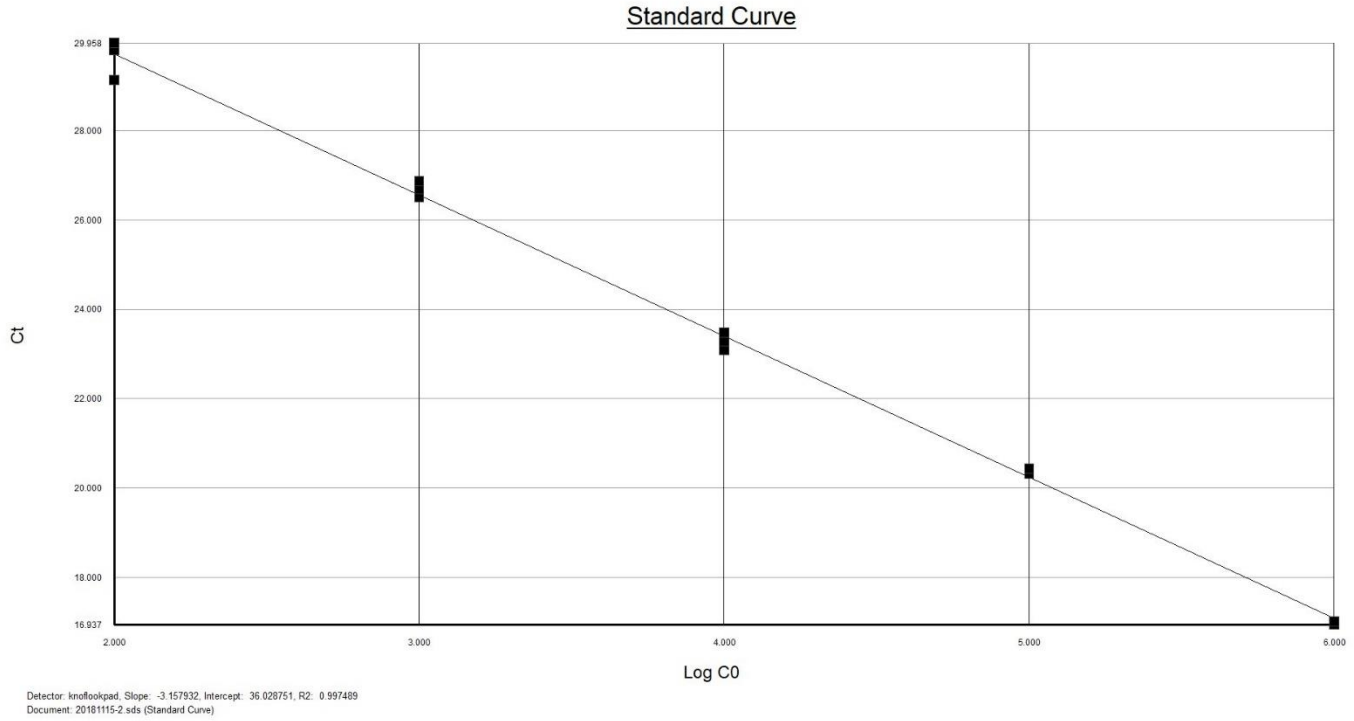

Fig. 5: Standard curve of SYL106 based on 10^2, 10^3, 10^4, 10^5 and 10^6 DNA target copies

Table 10: Values obtained from the standard curve

Slope	-3.2
Efficiency	100%
R^2	0.997

1.2.4 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, one way ANOVA 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). There were no statistically significant differences between group means as determined by one-way ANOVA ($F(3,8) = 1.49$, $p = .28$)

Influence of chemical and biological factors present in environmental samples was not detected. The standard error of mean was 0.17 within these samples with an average of 27.0

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

Sample	CT value	Standard deviation
Sandy	-	-
Clayey	-	-
Peaty	-	-
Sandy + spike	27.2	0.6
Clayey + spike	27.2	0.1
Peaty + spike	27.1	0.3
Spike only	26.7	0.2

1.2.5 Detection conformation in environmental samples

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

Results:
The kit SYL103 – *Pelobates fuscus* detection kit was able to detect *Pelobates fuscus* in environmental samples from different locations in the Netherlands and Belgium. On average, a positive sample gave 1 to 10 molecules *Pelobates fuscus* DNA when 100 - 200 ml water was filtrated.

1.2.6 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 – 63.6°C (section 1.2.1, section 1.2.2, table 8, table 9)
- 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 11)
- 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 10^6 and 10 target DNA copies per reaction. The detection limits for quantitative analyses were determined between 10^6 and 10 target copies per reaction, below this concentration the results become unreliable quantitative analyses, but still useful for qualitative analysis. (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 100 %, 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 11)

1.3.6 Correctness

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

- The test was able to detect *Pelobates fuscus* DNA in environmental samples from locations were the presence of *Pelobates fuscus* was suspected. (section 1.2.6)
- The method did not give any other combined BLAST hit than the target organism *Pelobatus fuscus*
- 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 11)
- The standard error of the mean based on the measured CT values from the spiked experiments (1000 target copies) was 0,17 on an average of 27.0.