VALIDATION

Pelobatus fuscus qPCR detection kit

SYL103

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1. *In silico* validation

Table 1. *In silico* validation of primers and probe

Parameter	Forward primer	Reverse primer	Probe*
Length (bp)	20	24	25
GC %	60	45	52
Stability	2.3	1.7	-
T_m (°C)	66	63	72
Target region	CytB (mtDNA)	CytB (mtDNA)	CytB (mtDNA)
Dimer formation	No	No	No
Nucleotide runs	Yes (CCC)	No	No

*Probe labelled with FAM.

Table 2. *In silico* validation primers and probe combination

PCR product size (bp)	87
Combined dimer formation	No
***In silico* PCR on Genbank**	*Pelobatus fuscus*
Date of in silico PCR	March 2022

Conclusion:

The *in silico* analysis identified the target organism exclusively, with no predicted amplification of non-target taxa.

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2. Experimental validation

All validation experiments were conducted using the Sylphium qPCR mix and analyzed with the Bio-Rad CFX96 Touch Real-Time PCR Detection System.

2.1. Analytical sensitivity, fluorescence output signal and efficiency

Standard solutions containing nominal target concentrations of 10^6, 10^5, 10^4, 10^3, 10^2, 10, and 0 target copies per reaction were analyzed at the optimal primer and probe concentrations. Fluorescence output was compared with the background signal, with a minimum 100-fold increase in fluorescence considered acceptable. Reaction conditions were optimized when fluorescence signals were insufficiently distinguishable from background noise.

The amplification efficiency of the primer and probe set was calculated from the slope of the standard curve and was considered acceptable when between 90% and 110%.

Results

Positive amplification was observed down to the lowest tested nominal concentration of **10 target copies per reaction** under the tested conditions (**Figure 1; Table 3**). The fluorescence output of positive reactions exceeded the predefined criterion of a 100-fold increase relative to the background signal.

The amplification efficiency of the assay was **100%**, indicating that the primer and probe set performed within the predefined acceptable efficiency range of 90-110% (**Figure 2; Table 4**).

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Figure 1. Amplification curves obtained at the optimal primer and probe concentrations and an annealing temperature of 55°C.

Table 3. Detection results and Cq values obtained at the optimal primer and probe concentrations and an annealing temperature of 55°C

Nominal target copies per reaction	Target detected	Mean Cq ± SD
0	No	n.a.
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

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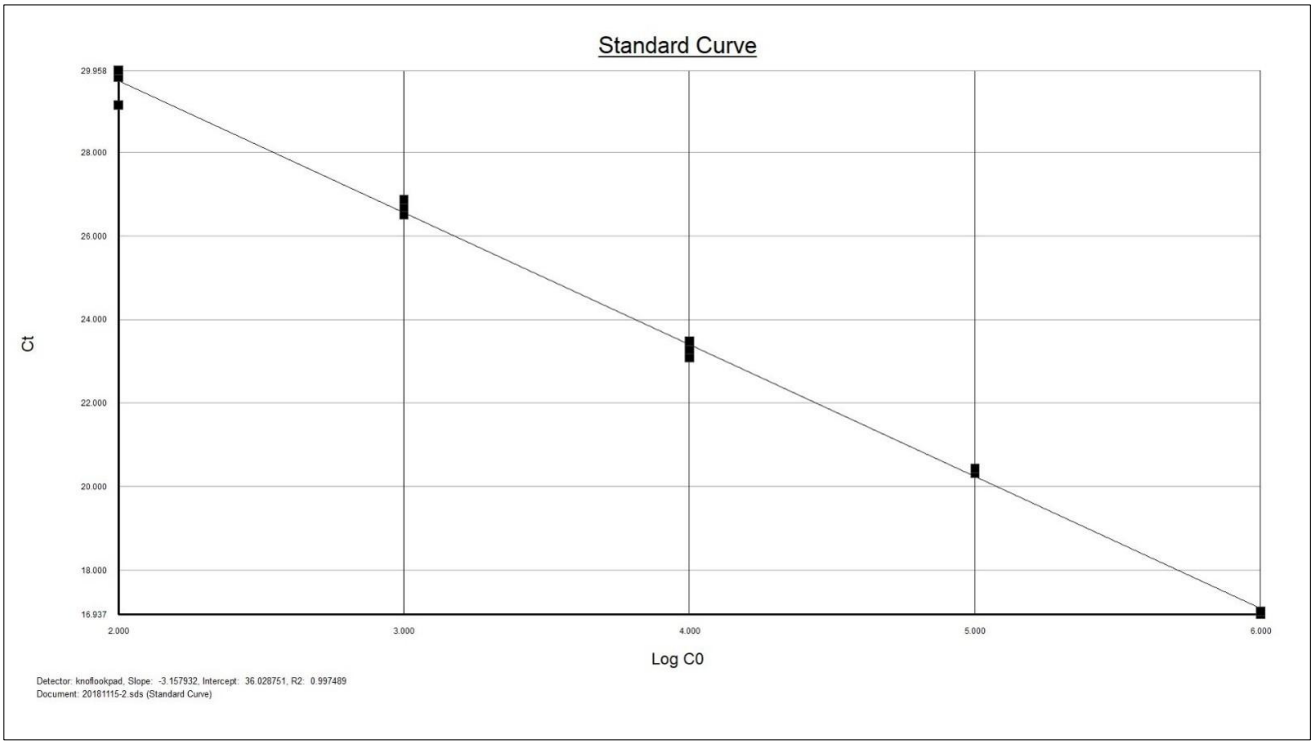


Figure 2. Standard curve generated from nominal target concentrations of 10^2, 10^3, 10^4, and 10^5 copies per reaction.

Table 4. Standard curve parameter obtained with the *Pelobatus fuscus* qPCR detection kit

Parameter	Result
Slope	-3.2
Amplification efficiency (%)	100
R^2	0.997

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2.2. Detection confirmation of *Pelobatus fuscus* in environmental samples

The amount of eDNA released by a target organism into the environment depends on the target species, the environmental conditions, and season (CEN / TC230-water analysis, proposal documented in N 1229). Samples were collected with the **SYL009 Complete eDNA sampling set** and eDNA was isolated using the **SYL002 Environmental DNA isolation kit**.

Results:

The kit SYL103 - *Pelobatus fuscus* detection kit successfully detected *Pelobatus fuscus* DNA in environmental samples from different locations in the Netherlands and Belgium when 100-200 mL water was filtrated.

PCR products obtained from positive environmental samples were analyzed by Sanger sequencing. The resulting sequences were identical, and BLAST analysis confirmed that the amplified products originated from *Pelobatus fuscus*. No amplification was observed in the tested target-organism-free environmental samples.

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