Sylphium
molecular ecology

Scyliorhinus canicular qPCR detection kit

With eDNA qPCR hot start mix

#SYL175 validation report

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For general laboratory and research use only.

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1. Validation report *Scyliorhinus canicula* detection kit

1.1. In silico validation COI

Forward primer	Forward primer	Reverse primer	Probe
Length (bp)	25	20	26
GC %	52	59	57
Stability	2.8	3.0	-
T_M (°C)	65	68	74
Target region	COI (mtDNA)	COI (mtDNA)	COI (mtDNA)
Dimer	No	No	No
Run	No	No	No

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

PCR product size (bp)	117
Combined dimer formation	No
In silico PCR on Genbank	*Scyliorhinus canicula*
Date of In silico PCR	April 2024

Table 2. Combined primers and probe *in silico* validation.

1.2. 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.

1.2.1. Optimal annealing temperature primer/probe set

The annealing temperature significantly influences the performance, robustness, and specificity of the primer/probe set. To determine the optimal annealing temperature, a temperature gradient PCR was conducted, spanning a range from 41°C to 65°C in eight incremental steps. The template concentration was approximately 100 molecules per reaction.

Results:

A fluorescent signal (RFU) was detected at all temperatures. The highest signal was observed from 50.2 to 65.0°C. At these temperatures the CT value was also the lowest. (**Table 3**, **Figure 1**).

Annealing temp.	41°C	42.5°C	45.6°C	50.2°C	56.0°C	60.8°C	63.5°C	65.0°C
RFU	1500	1200	1500	1600	1600	1400	1400	1500
CT	31.3	31.7	31.4	30.8	29.7	29.5	29.6	29.6

Table 3. Temperature gradient PCR on DNA of *Scyliorhinus canicula.*

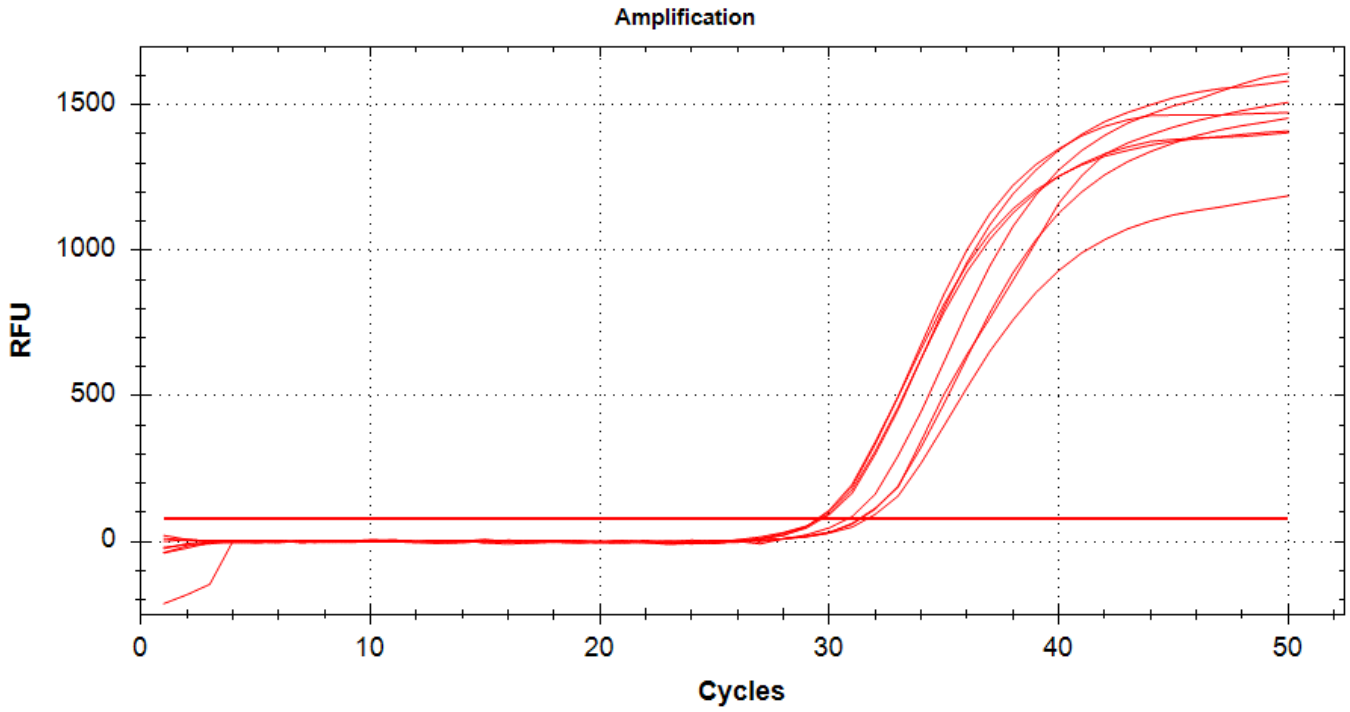

Figure 1. Temperature gradient PCR with ± 100 copies template per reaction.

1.2.2. Detection limit, fluorescence output signal and efficiency

Standard solutions with 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 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:

The detection limits (low and high) for qualitative detection was determined between 1-10 and >10^5 target copies per reaction. The detection limits (low and high) for quantitative detection was determined between 1-10 and >10^5 target copies per reaction. The fluorescence output signal was at least 100 (8 for positive sample, 0,01 for a negative sample) times stronger than the background signal (**Figure 2**). The efficiency of the test was above 90%. Which means that the efficiency of the primer/probe mixture can be regarded optimal (**Figure 3**, **Table 5**).

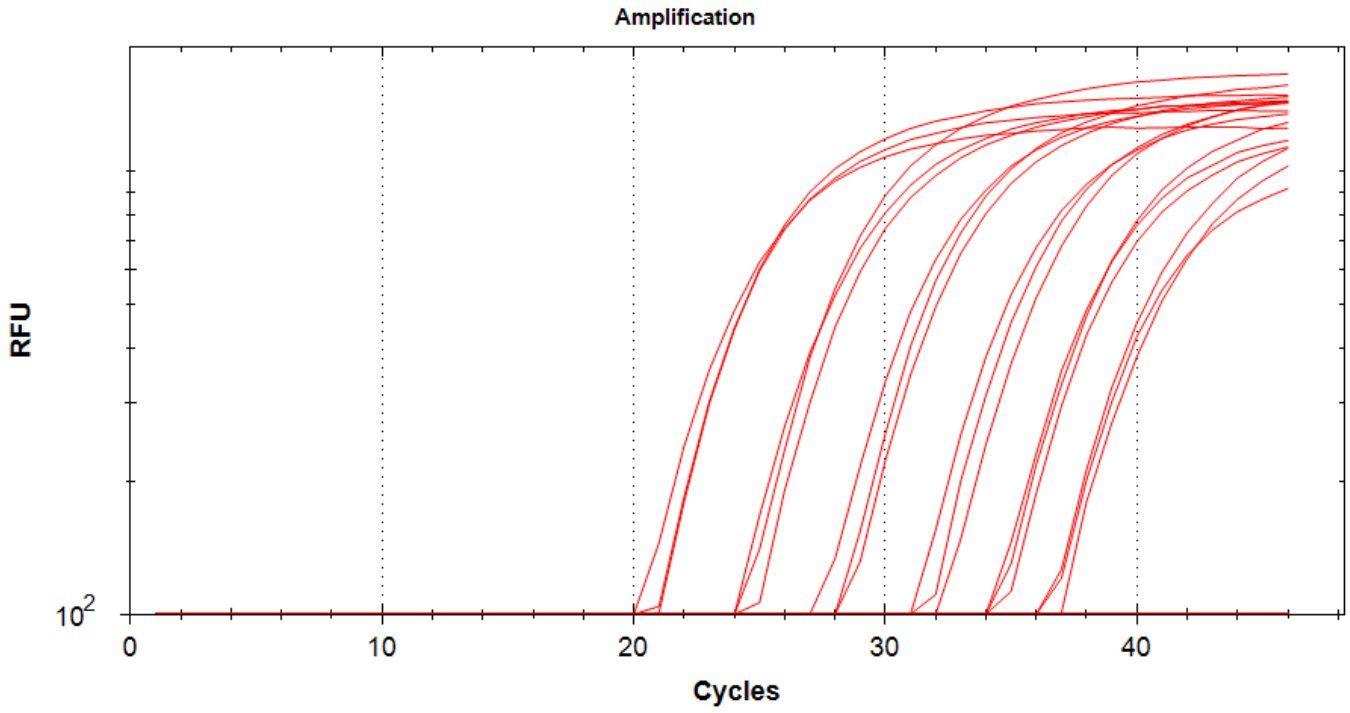

Figure 2. Obtained fluorescent signal at optimal primer/probe concentration at a annealing temperature of 65°C.

target DNA copy	Target detected	CT value
0	Yes	n.a.
1	Yes	35.9
10	Yes	31
10^2	Yes	28.0
10^3	Yes	28.0
10^4	Yes	24.2
10^5	Yes	20.2

Table 4. CT values obtained at optimal primer/probe concentration.

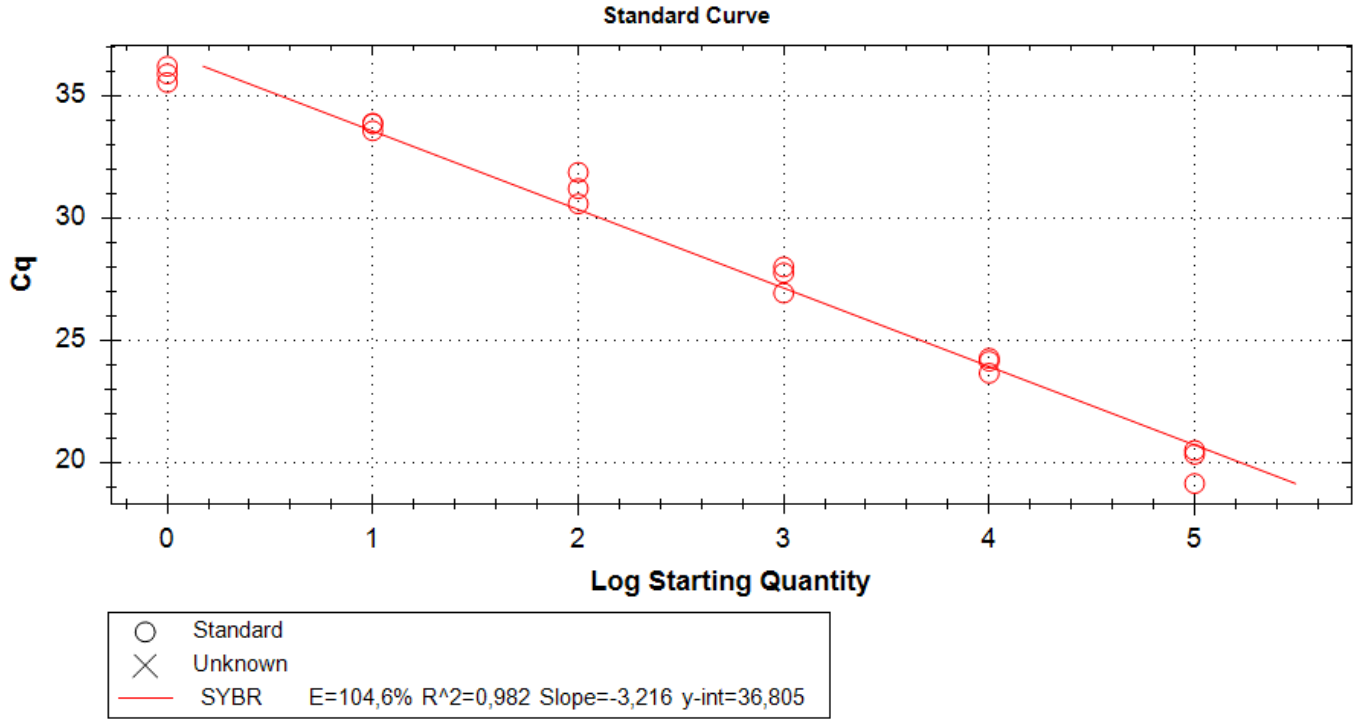

Figure 3. Standard curve of SYL175 based on 1, 10, 10^2, 10^3, 10^4 and 10^5 DNA target copies.

Slope	-3.216
Efficiency	104.6
R^2	0.982

Table 5. Values obtained from the standard curve.

1.2.3. Influence of inhibiting factors present in environmental samples and repeatability

The essay was evaluated for its ability to perform in the presence of potential inhibitors in three types of environmental water samples (with sandy, clayey, and peaty substrates). Each sample type was tested in triplicate and spiked with 1000 copies of the target DNA. The ΔCT between spiked samples and spike-only was determined and was required to be less than 2. Standard deviations and standard errors of the mean were calculated to assess robustness, repeatability, and measurement uncertainty.

Results:

No fluorescent output signal was detected in the environmental samples without the addition of target organism DNA (spike). All spiked samples and spike-only analyses consistently produced positive signals across all replicates (**Table 6**). In all cases, the ΔCT was less than 2.

Sample	CT value	Standard deviation	ΔCT
Sandy	-	-	-
Clayey	-	-	-
Peaty	-	-	-
Sandy + spike	27.0	0.1	0.1
Clayey + spike	26,8	0.1	0.3
Peaty + spike	27,1	0.2	0.2
Spike-only	26.9	0.0	-

Table 6. CT values obtained with target organisms free environmental samples spiked with 1000 target copies.

1.2.4. Detection conformation of *Scyliorhinus canicula* in environmental samples

The amount of eDNA that a target organism leaves behind in the environment depends on the type of target organism, environmental factors, and the season (CEN / TC230-water analysis, proposal documented in N 1229). To determine the minimal filtration volume, samples were taken at locations where Scyliorhinus canicula may occur during the optimal seasons (spring, summer and, autumn). Samples were taken with the SYL009 - Environmental Sampling Kit and eDNA was isolated with the SYL002 - Environmental DNA Isolation Kit.

Results:

The kit SYL175 - Scyliorhinus canicula detection kit was able to detect Scyliorhinus canicula in samples from three different fish tanks.

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: 50.2°C to 65.0°C (**Section 1.2.1**, **Table 3**).
- Influence of chemical and biological factors present in environmental samples on the performance of the test were not found (**Section 1.2.3**, **Table 6**).
- 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"

- Limit of detection (LOD) for this kit was determined on 1 copy per reaction. Limit of quantification (LOQ) was also determined on 10 copies per reaction (**Section 1.2.2**, **Table 4**).

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 104.6 %, this means that the primer/probe mixture can be regarded as optimal (**Section 1.2.2**, **Table 5**).

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 (ΔCT <2) between different kinds of environmental water samples (sandy, clayey or peaty bottoms) spiked with 1000 target DNA copies (**Section 1.2.3**, **Table 6**).

1.3.6. Correctness

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

- The test was able to detect *Scyliorhinus canicula* DNA in environmental samples from fish tanks were the presence of *Scyliorhinus canicula* was present (**Section 1.2.5**).
- The method did not give any other combined BLAST hit than the target organism *Scyliorhinus canicula*
- The method was able to detect all spiked target DNA in target organism free environmental samples (**Section 1.2.3**, **Table 6**).
- The method did not give any signal in target organism free environmental samples (**Section 1.2.3**, **Table 6**).

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