Sylphium
molecular ecology

Lampetra fluviatilis & *Lampetra planeri* qPCR detection kit

With eDNA qPCR hot start mix

SYL144

Validation report

Document date: November 10, 2025

For general laboratory and research only.

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1. In silico validation

Forward primer	Forward primer	Reverse primer	Probe
Length (bp)	25	27	34
GC %	48	37	48
Stability	1.6	1.8	-
T_M (°C)	64	63	72
Target region	CytB (mtDNA)	CytB (mtDNA)	CytB (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)	93
Combined dimer formation	No
In silico PCR on Genbank	*Lampetra fluviatilis, lampetra planeri*
Date of In silico PCR	November 2023

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

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. Optimal annealing temperature primer/probe set

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The annealing temperature strongly influences the performance, robustness, and specificity of the primer/probe set. To determine the optimal annealing temperature, a temperature-gradient PCR was performed, ranging from 55°C to 72°C in sixteen incremental steps. The template concentration was approximately 100 molecules per reaction.

Results:
A fluorescent signal (RFU) was detected at all temperatures. The highest signal intensity—and the lowest CT values—were observed between 55.0°C and 62.5°C. The temperature of 62.5°C was selected as optimal, as it provides the most stringent annealing conditions while still yielding the highest fluorescence signal (Table 3, Figure 1).

Annealing temp.	RFU	CT
72.0°C	-	-
71.7°C	-	-
71.1°C	-	-
70.4°C	-	-
69.2°C	80	41.6
68.0°C	550	31.3
66.3°C	850	28.2
64.6°C	850	28.1
62.5°C	900	28.1
60.8°C	900	28.1
59.3°C	900	28.3
58.0°C	900	28.2
56.8°C	950	28.1
56.0°C	1000	27.4
55.3°C	900	28.3
55.0°C	1000	28.1

Table 3. Temperature gradient PCR on DNA of *Lampetra fluviatilis & lampetra planeri.*

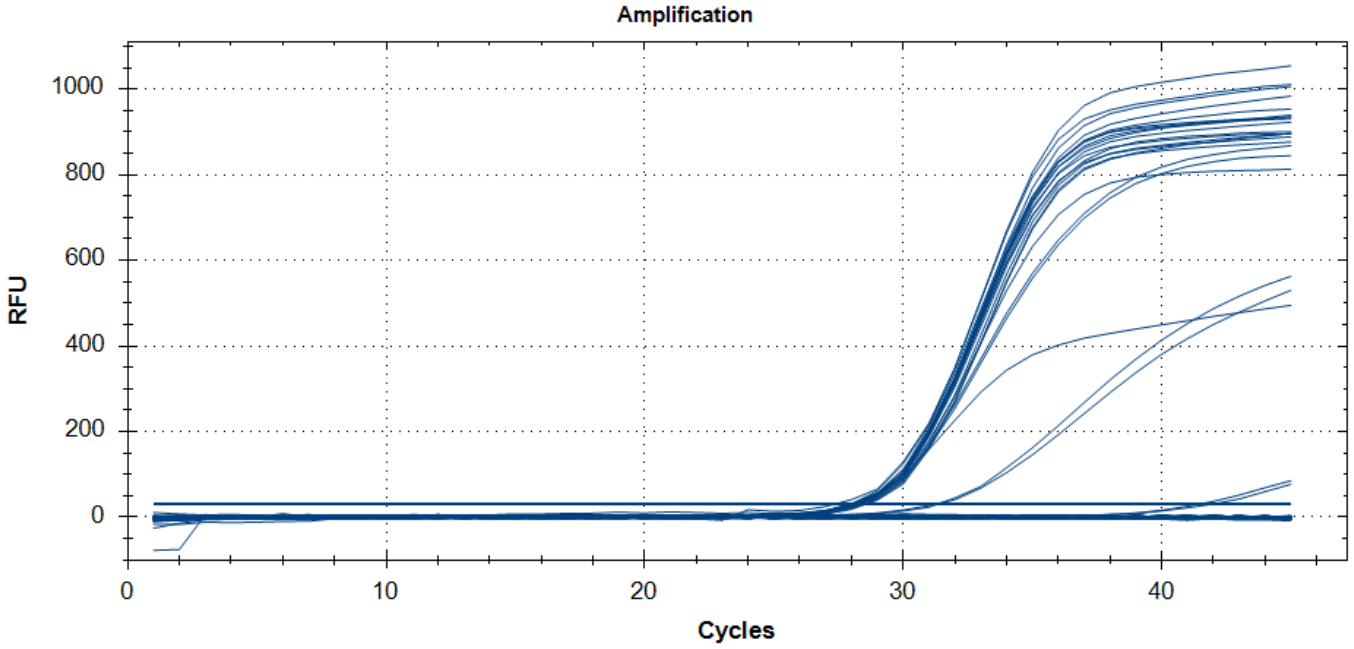

Figure 1. Temperature gradient PCR with ± 100 copies template per reaction.

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 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**).

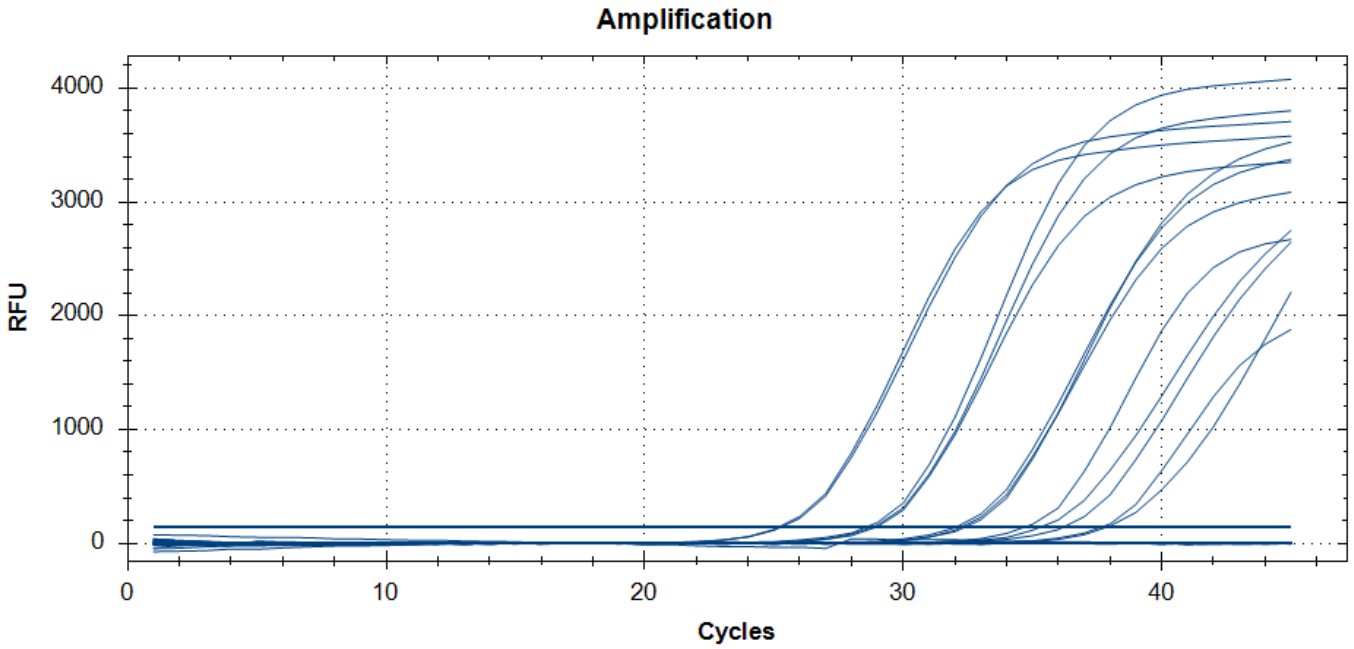

Figure 2. Obtained fluorescent signal at optimal primer/probe concentration at a annealing temperature of 62.5°C. (251104-8)

target DNA copy	Target detected	CT value
0	No	-
1	Yes	37.6
10	Yes	35.0
10^2	Yes	32.1
10^3	Yes	28.6
10^4	Yes	25.1

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

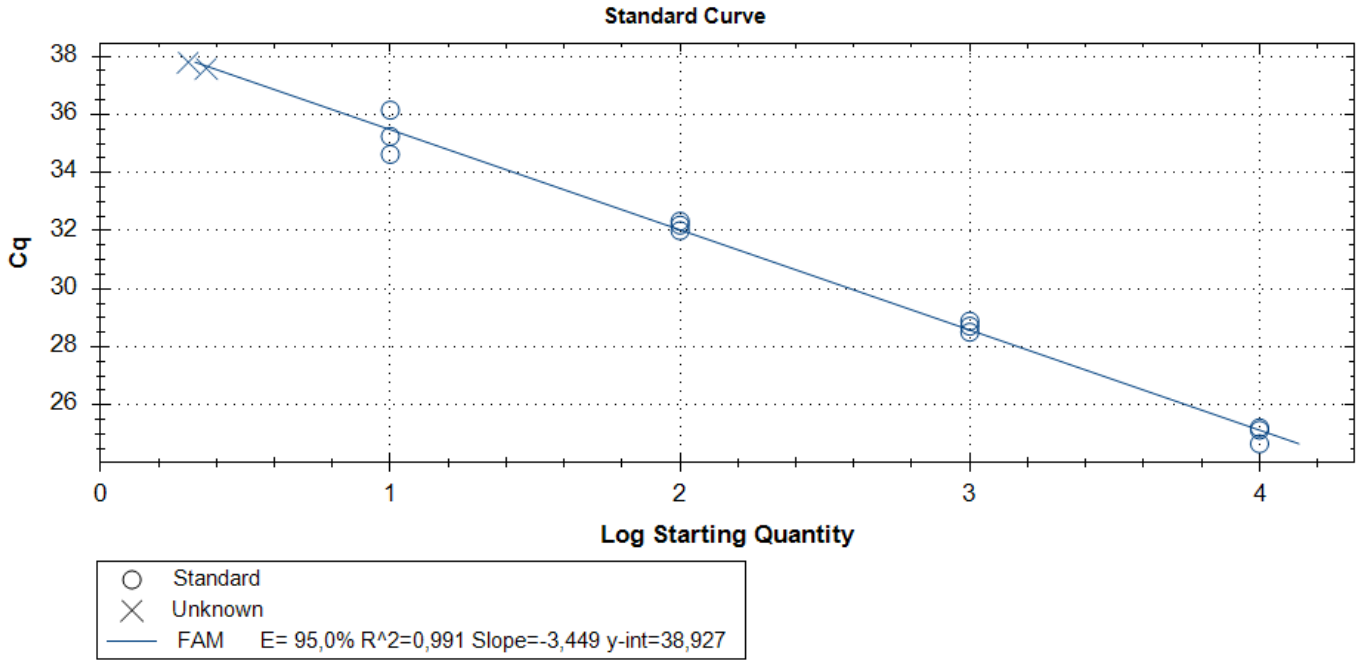

Figure 3. Standard curve of SYL144 based on 1, 10, 10^2, 10^3, 10^4 and 10^5 DNA target copies.

Slope	-3.449
Efficiency	95.0%
R^2	0.991

Table 5. Values obtained from the standard curve.

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

The assay was evaluated for its performance in the presence of potential inhibitors in three types of environmental water samples, characterized by sandy, clayey, and peaty substrates. Each sample type was tested in triplicate and spiked with approximately 1000 copies of the target DNA. The ΔCT between spiked environmental samples and the spike-only control was determined, with an acceptance criterion of less than 2. Standard deviations and standard errors of the mean were calculated to assess robustness, repeatability, and measurement uncertainty.

Results:
No fluorescence signal was detected in the unspiked environmental samples, confirming the absence of the target organism DNA. All spiked samples and spike-only controls consistently yielded positive signals across all replicates (Table 6). In all cases, the ΔCT values remained below 2, indicating that potential environmental inhibitors did not significantly affect assay performance.

Sample	CT value	Standard deviation	ΔCT
Sandy	-	-	-
Clayey	-	-	-
Peaty	-	-	-
Sandy + spike	28.7	0.2	0.3
Clayey + spike	28.9	0.1	0.5
Peaty + spike	28.8	0.1	0.4
Spike-only	28.4	0.0	-

Table 6. CT values obtained with target organisms free environmental samples spiked with 1000 target copies. (251005-1)

2.4. Specificity testing of the primer and probe set

The specificity of the primer and probe set was evaluated using two complementary approaches.

First, DNA from several non-target species was tested in triplicate under identical qPCR conditions. The selected species included two common non-target mammals (*Homo sapiens* and *Bos taurus*) and three fish species that are frequently encountered in the same habitats as *Lampetra fluviatilis*. Among these, *Petromyzon marinus* (sea lamprey) was included as the only closely related species from the same family (*Petromyzontidae*), while *Rutilus rutilus* (roach) and *Anguilla anguilla* (European eel) represented distantly related fish species. Genomic DNA was extracted from tissue material. Each qPCR run included a positive control (target DNA) and a no-template control (NTC). The absence of amplification (Cq > 40 or undetermined) in all non-target samples was considered evidence of acceptable assay specificity.

In addition, environmental DNA (eDNA) samples from locations where *L. fluviatilis* is known to be absent were analyzed to further verify assay specificity under environmental conditions. These target-free samples served to confirm that no unintended amplification occurred in natural eDNA matrices.

Results

No amplification was detected in any of the tested non-target or phylogenetically related species. Likewise, no amplification occurred in environmental samples from sites without *L. fluviatilis* presence. All negative controls (NTCs) remained free of signal throughout the analyses.

Species	Phylogenetic relation to target	Amplification	Interpretation
Homo sapiens	Unrelated (mammal)	No	Specific
Bos taurus	Unrelated (mammal)	No	Specific
Rutilus rutilus	Distantly related fish species	No	Specific
Anguilla anguilla	Distantly related fish species	No	Specific
Petromyzon marinus	Closely related species (same family)	No	Specific

Table 7 Summarizing amplification results for each species

3. Summary of validation

3.1. Robustness

"Influence of temperature variations and inhibiting factors present in environmental samples on the performance and specificity of the primer set"

- The primers showed specific amplification across the tested temperatures; however, an annealing temperature of 62.5°C was identified as the optimal setting for reliable and stringent performance (Section 1.2.1, Table 3).
- No chemical or biological inhibitory effects originating from environmental samples were observed that influenced test performance (Section 1.2.3, Table 6).
- Fluorescent output signals of positive samples were at least 100-fold stronger than the background.

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**).

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

3.4. 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**).

3.5. Correctness

- *"The ability of the method to do what it 'says' to do"*
- 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**)
- Specificity testing showed no cross-reactivity with any non-target species, including closely related *P. marinus*. No amplification occurred in non-target or target-free environmental samples, confirming high analytical specificity (Section 2.4; Table 7).

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