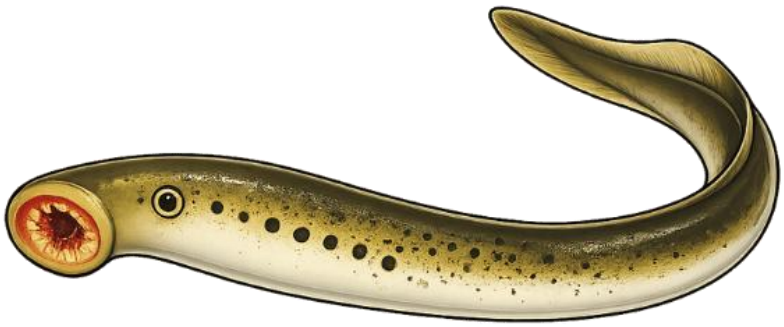
VALIDATION

Lampetra fluviatilis & *Lampetra planeri* qPCR detection kit

SYL144

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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)	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 formation	No	No	No
Nucleotide runs	No	No	No

*Probe labelled with FAM.

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

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

Conclusion:

The *in silico* analysis identified the target organisms 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 **1-10 target copies per reaction**, demonstrating single-copy detection capability 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 **95.0%**, 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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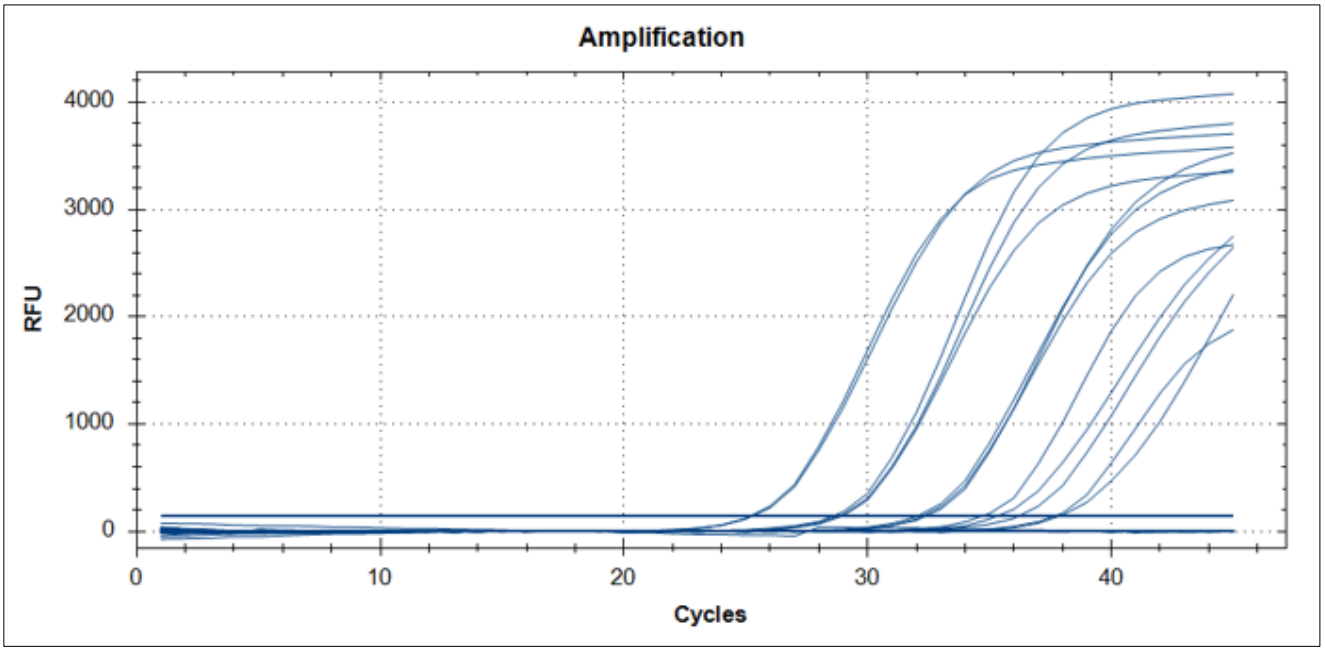


Figure 1. Amplification curves obtained at the optimal primer and probe concentrations and an annealing temperature of 62.5°C.

Table 3. Detection results and Cq values obtained at the optimal primer and probe concentrations and an annealing temperature of 62.5°C

Nominal target copies per reaction	Target detected	Mean Cq
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

n.d.: not determined, very low amounts of DNA could not be quantitively detected in a reliable manner with the used qPCR instrument.

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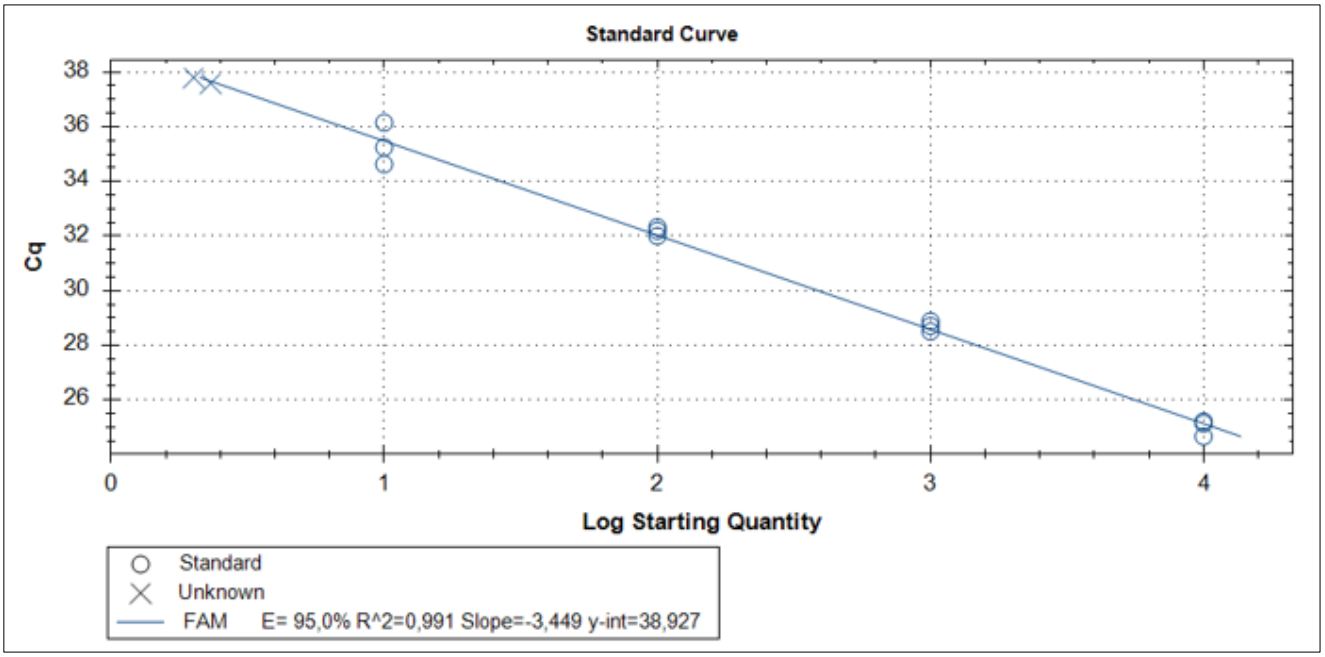


Figure 2. Standard curve generated from nominal target concentrations of 1, 10, 10^2, 10^3, 10^4, 10^5, and 10^6 copies per reaction.

Table 4. Standard curve parameters obtained with the *Lampetra fluviatilis & Lampetra planeri* qPCR detection kit

Parameter	Result
Slope	-3.449
Amplification efficiency (%)	95.0
R^2	0.991

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2.2. Specificity testing

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.

Table 5. Amplification results

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

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