VALIDATION

Cottus perifretum/rhenanus

qPCR detection kit

SYL143

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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	21	37
GC %	55	47	32
Stability	2.0	1.3	-
T_m (°C)	60	60	66
Target region	CO1 (mtDNA)	CO1 (mtDNA)	CO1 (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)	99
Combined dimer formation	No
***In silico* PCR on Genbank**	*Cottus perifretum/rhenanus, Cottus Gobio, Cottus microstomus, Cottus rhenanus,*
Date of in silico PCR	*November 2020*

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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, 1 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 target copy 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 **99.5%**, 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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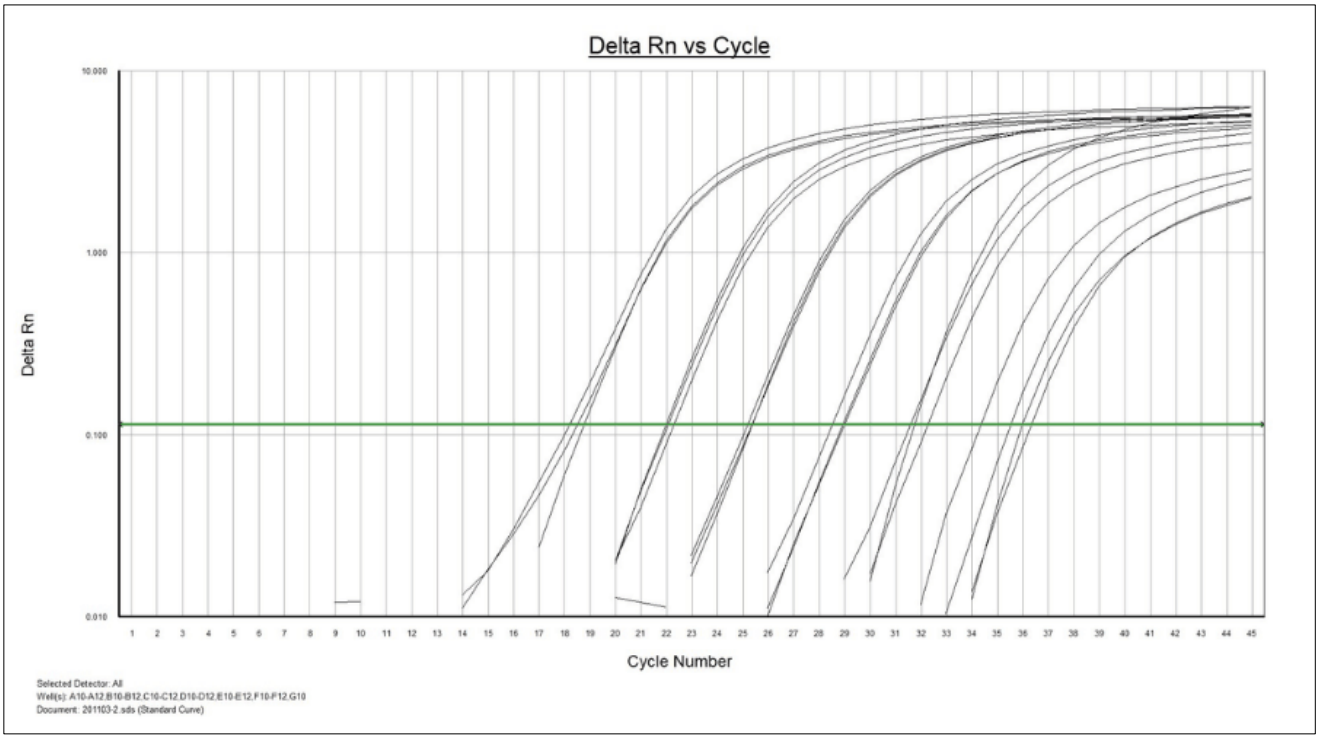


Figure 1. Amplification curves obtained at the optimal primer and probe concentrations and an annealing temperature of 58°C.

Table 3. Detection results and Cq values obtained at the optimal primer and probe concentrations and an annealing temperature of 58°C

Nominal target copies per reaction	Target detected	Mean Cq	SD
0	No	-	-
1	Yes	n.d.	n.d.
10	Yes	35.5	1.1
10^2	Yes	31.8	0.4
10^3	Yes	28.8	0.3
10^4	Yes	25.3	0.1
10^5	Yes	22.1	0.1
10^6	Yes	18.5	0.3

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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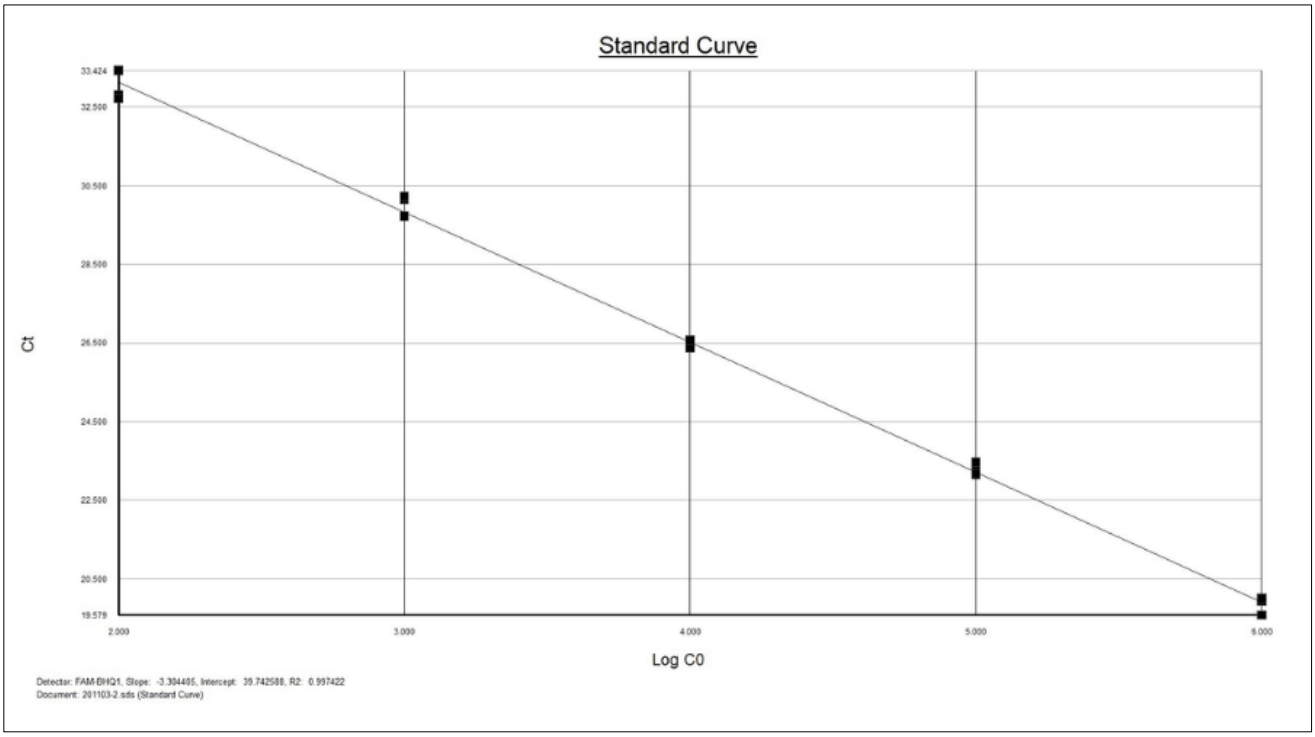


Figure 2. Standard curve generated from nominal target concentrations of10^2, 10^3, 10^4, 10^5, and 10^6 copies per reaction.

Table 4. Standard curve parameters obtained with the *Cottus perifretum/rhenanus* qPCR detection kit

Parameter	Result
Slope	-3.30
Amplification efficiency (%)	99.5
R^2	0.997

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2.2. Detection confirmation of *Cottus perifretum/rhenanus* 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 SYL143 - *Cottus perifretum/rhenanus* detection kit successfully detected *Cottus perifretum/rhenanus* DNA in environmental samples from different locations in the Netherlands 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 *Cottus perifretum/rhenanus*. No amplification was observed in the tested target-organism-free environmental samples.

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