VALIDATION

Ondatra zibethicus qPCR detection kit

SYL119

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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)	24	22	35
GC %	45	32	32
Stability	2.4	1.8	-
T_m (°C)	61	62	73
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)	143
Combined dimer formation	No
***In silico* PCR on Genbank**	*Ondatra zibethicus, Sitta yunnanensis, Cyornis ruficauda, Atlapetes albinucha, Niltava macgrigoriae*
Date of in silico PCR	June 2022

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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 10^5, 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 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 **92.4%**, 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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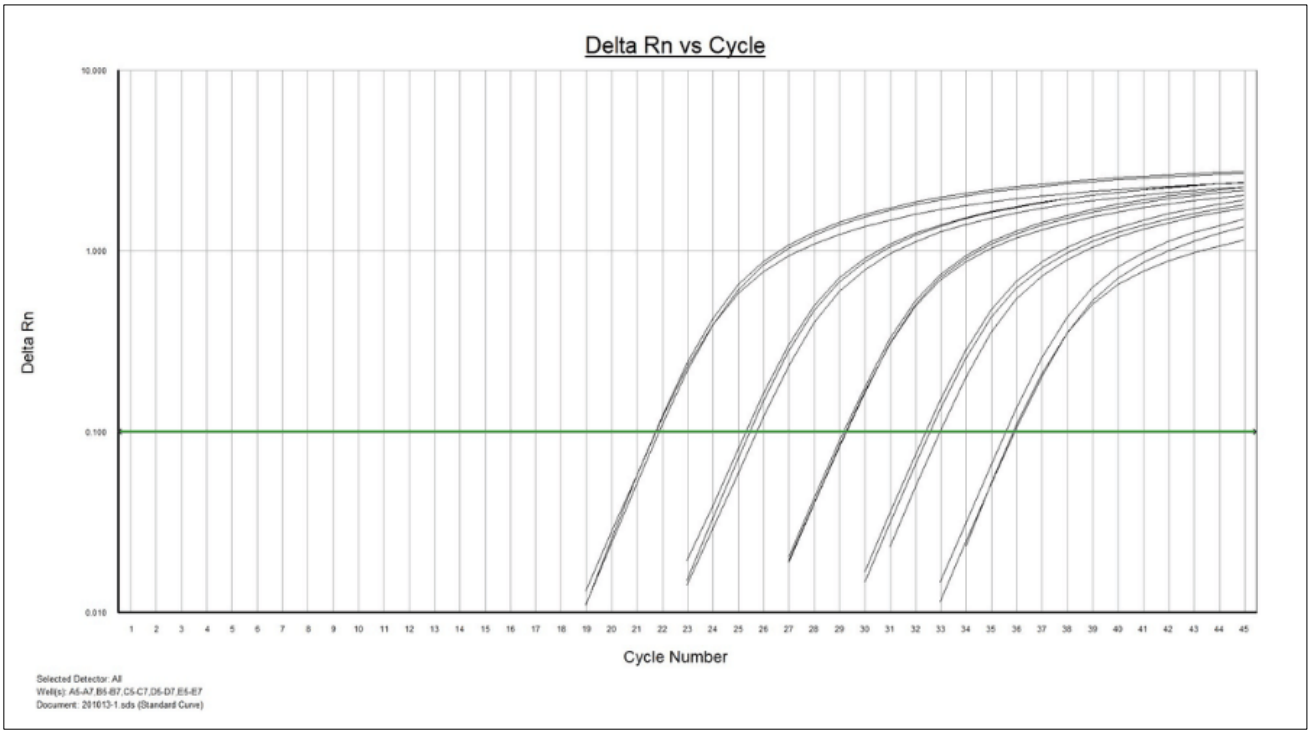


Figure 1. Amplification curves obtained at the optimal primer and probe concentrations and an annealing temperature of 58°C.

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	35.8	0.2
10^2	Yes	32.6	0.3
10^3	Yes	29.2	0.0
10^4	Yes	25.4	0.2
10^5	Yes	21.8	0.1

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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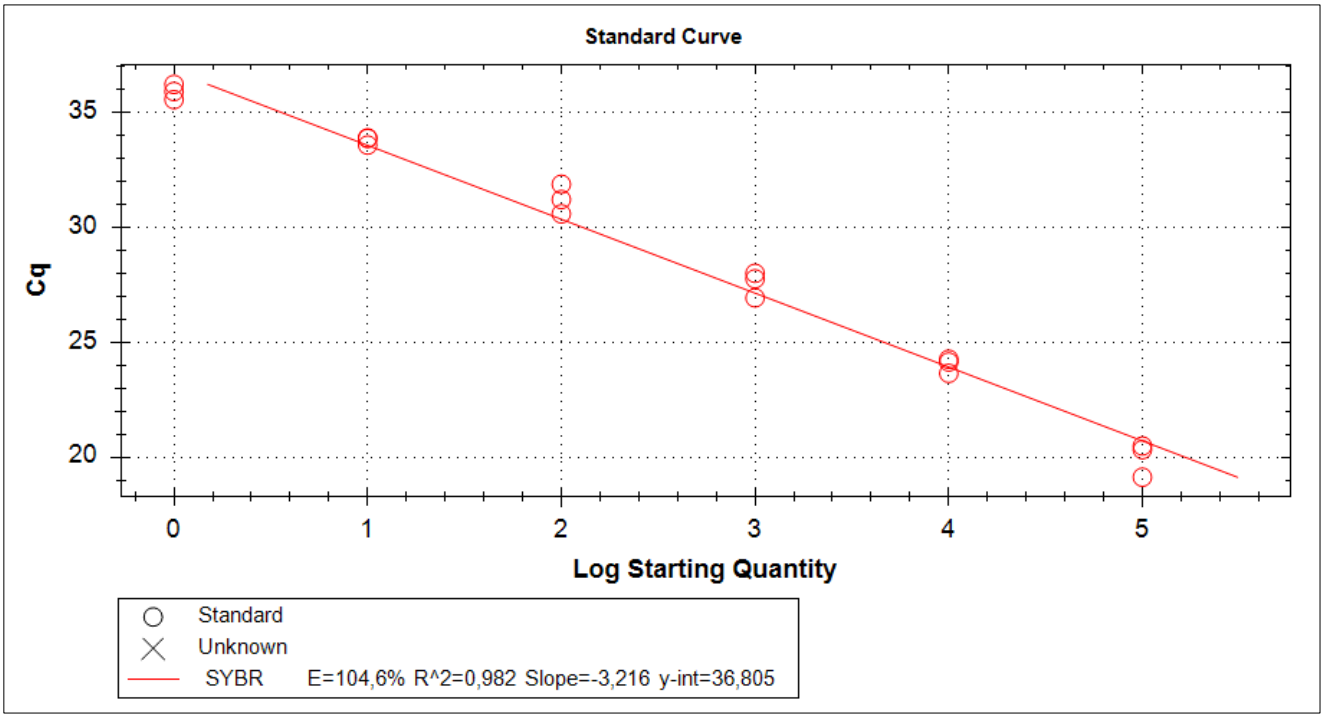


Figure 2. Standard curve generated from nominal target concentrations of 1, 10, 10^2, 10^3, 10^4, and 10^5 copies per reaction.

Table 4. Standard curve parameters obtained with the *Ondatra zibethicus* qPCR detection kit

Parameter	Result
Slope	-3.5
Amplification efficiency (%)	92.4%
R^2	0.997

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