VALIDATION

Bombina variegata qPCR detection kit

SYL152

Document date: 13 November 2026



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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	20	34
GC %	41	55	35
Stability	4.1	3.1	-
T_m (°C)	61	62	70
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)	101
Combined dimer formation	No
***In silico* PCR on Genbank**	*Bombina variegata, Bombina pachypus*
Date of in silico PCR	March 2022

Conclusion:

The *in silico* analysis identified the target organism 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 **90.3%**, 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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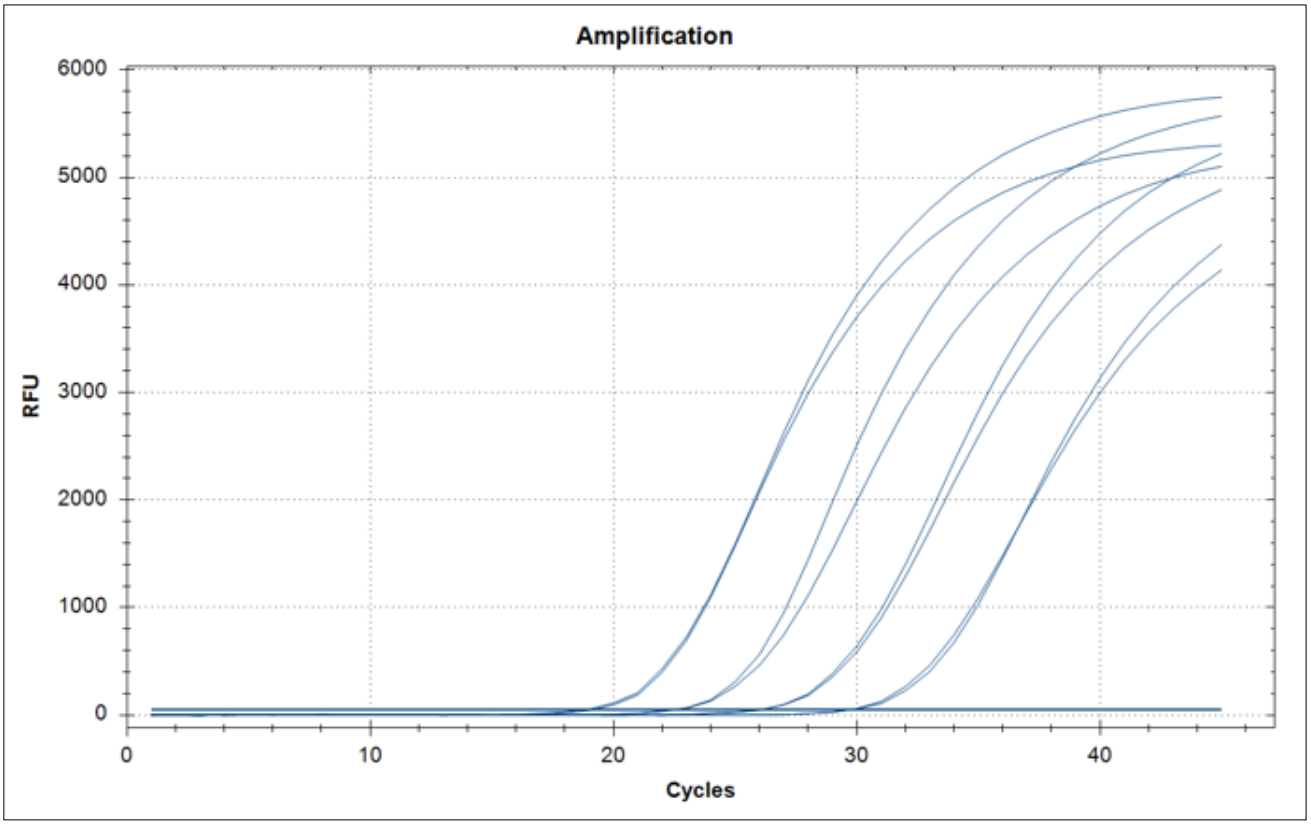


Figure 1. Amplification curves obtained at the optimal primer and probe concentrations and an annealing temperature of 62°C.

Table 3. Detection results and Cq values obtained at the optimal primer and probe concentrations and an annealing temperature of 62°C

Nominal target copies per reaction	Target detected	Mean Cq
0	No	-
1	Yes	34.3
10	Yes	29.9
10^2	Yes	26.2
10^3	Yes	22.6
10^4	Yes	19.1

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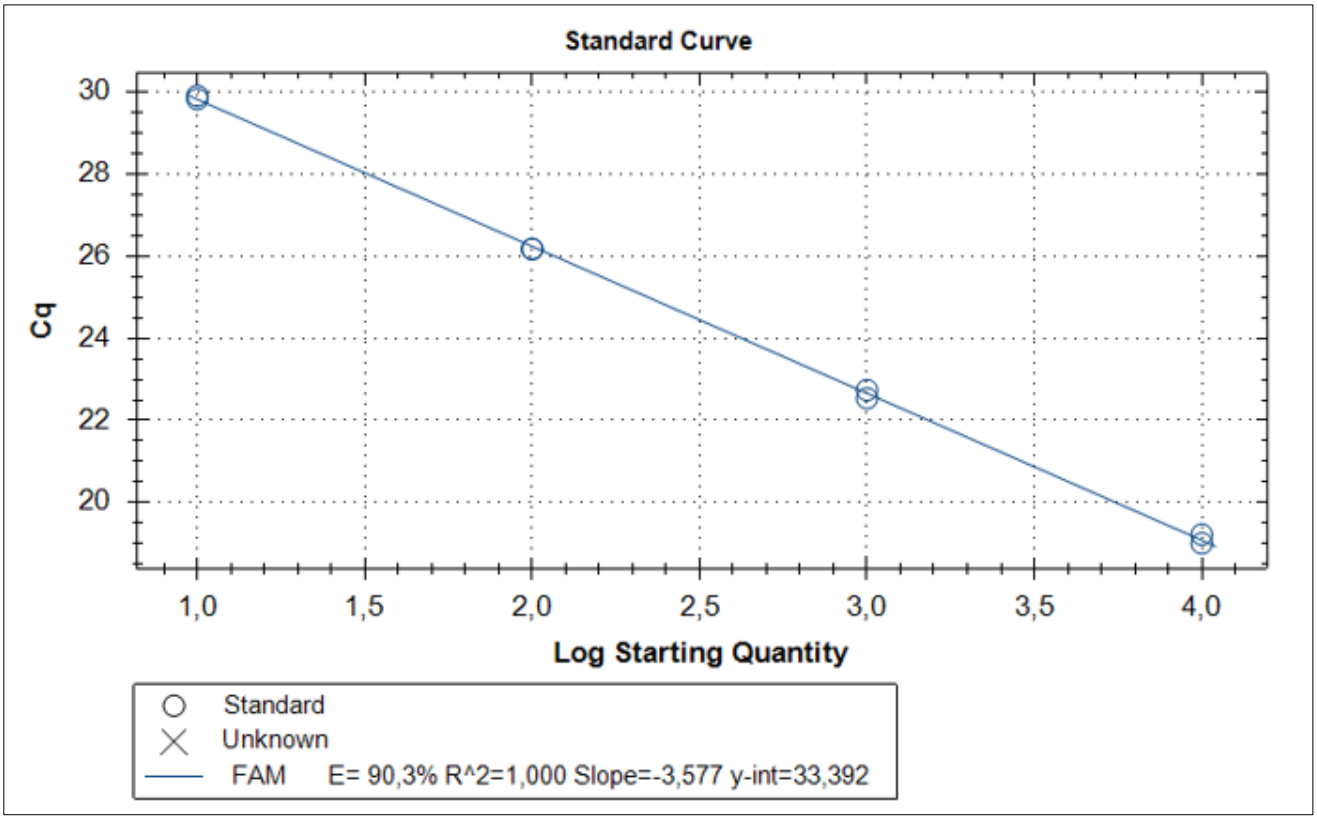


Figure 2. Standard curve generated from nominal target concentrations of 10, 10^2, 10^3, and 10^4copies per reaction.

Table 4. Standard curve parameters obtained with the *Bombina variegata* qPCR detection kit

Parameter	Result
Slope	-3.577
Amplification efficiency (%)	90.3
R^2	1.000

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2.2. Specificity testing

Specificity of the primers and probe set was evaluated by testing DNA from *Homo sapiens*, *Canis familiaris*, and *Bos taurus* as common non-target species, together with DNA from three species genetically related to the target organism. Genomic DNA was extracted from tissue material, and each sample was analyzed in triplicate under the same qPCR conditions used for the target species. A positive control containing target DNA and a no-template control (NTC) were included in each run. Specificity was considered acceptable when no amplification was observed in any non-target (Cq > 40 or undetermined).

Results:

Table 5. Amplification results for each species

Species	Relation to target	Cq mean ± SD	Amplification?	Interpretation
Homo sapiens	Common species	-	No	Specific
Bos taurus	Common species	-	No	Specific
Rana arvalis	Relative	-	No	Specific
Rana temporaria	Relative	-	No	Specific
Salamandra salamandra	Relative	-	No	Specific

No amplification was observed for any of the tested non-target species, including the phylogenetically related taxa, confirming the analytical specificity of the primer and probe set under the tested conditions.

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