Sylphium
molecular ecology

Alytes obstetricans qPCR detection kit

with eDNA qPCR hot start mix

#SYL153

Validation report

Document date: November 10, 2025

For general laboratory and research only.

Sylphium
molecular ecology

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1. *In silico* validation

Forward primer	Forward primer	Reverse primer	Probe
Length (bp)	23	21	29
GC %	43	57	48
Stability	2.1	1.9	-
T_M (°C)	61	63	71
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)	98
Combined dimer formation	No
***In silico* PCR on Genbank**	*Alytes obstetricans, Alytes maurus*
Date of in silico PCR	March 2022

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 BIO-RAD CFX96 and CFX384 Touch Real-Time PCR Detection Systems.

2.1. Optimal annealing temperature primer/probe set

The annealing temperature significantly influences the performance, robustness, and specificity of the primer/probe set. To determine the optimal annealing temperature, a temperature gradient PCR was conducted, spanning a range 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 temperatures 69.2°C to 55.0°C. The highest signal was observed from 64.6°C to 56.0°C. At these temperatures the CT values also were the lowest. The optimal annealing temperature was set to 64.6°C (**Figure 1, Table 3**).

Annealing temp.	RFU	CT
72.0°C	-	-
71.7°C	-	-
71.1°C	-	-
70.4°C	-	-
69.2°C	1400	33.0
68.0°C	2900	26.2
66.3°C	3200	25.1
64.6°C	3700	24.7
62.5°C	3500	24.8
60.8°C	3600	24.6
59.3°C	3300	24.6
58.0°C	3400	24.7
56.8°C	3200	24.8
56.0°C	3600	24.5
55.3°C	3000	24.8
55.0°C	3500	24.5

Table 3. Temperature gradient PCR on DNA of *Alytes obstetricans.*

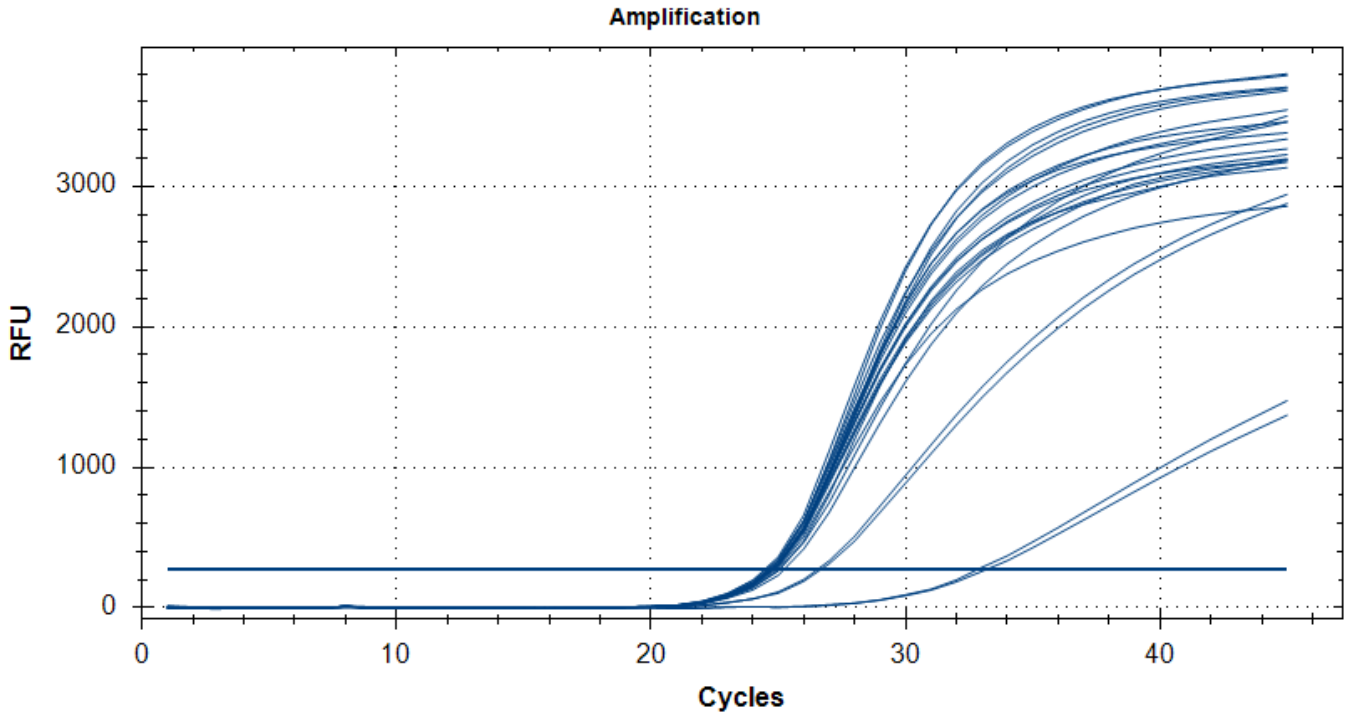

Figure 1. Temperature gradient PCR with ± 100 copies template per reaction

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 was 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 and quantitative detection were determined between 1-10 and >10^5 target copies per reaction. The fluorescence output signal was at least 100 times stronger than the background signal (3800 for positive sample, 0 for a negative sample; **Figure 3**). 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**).

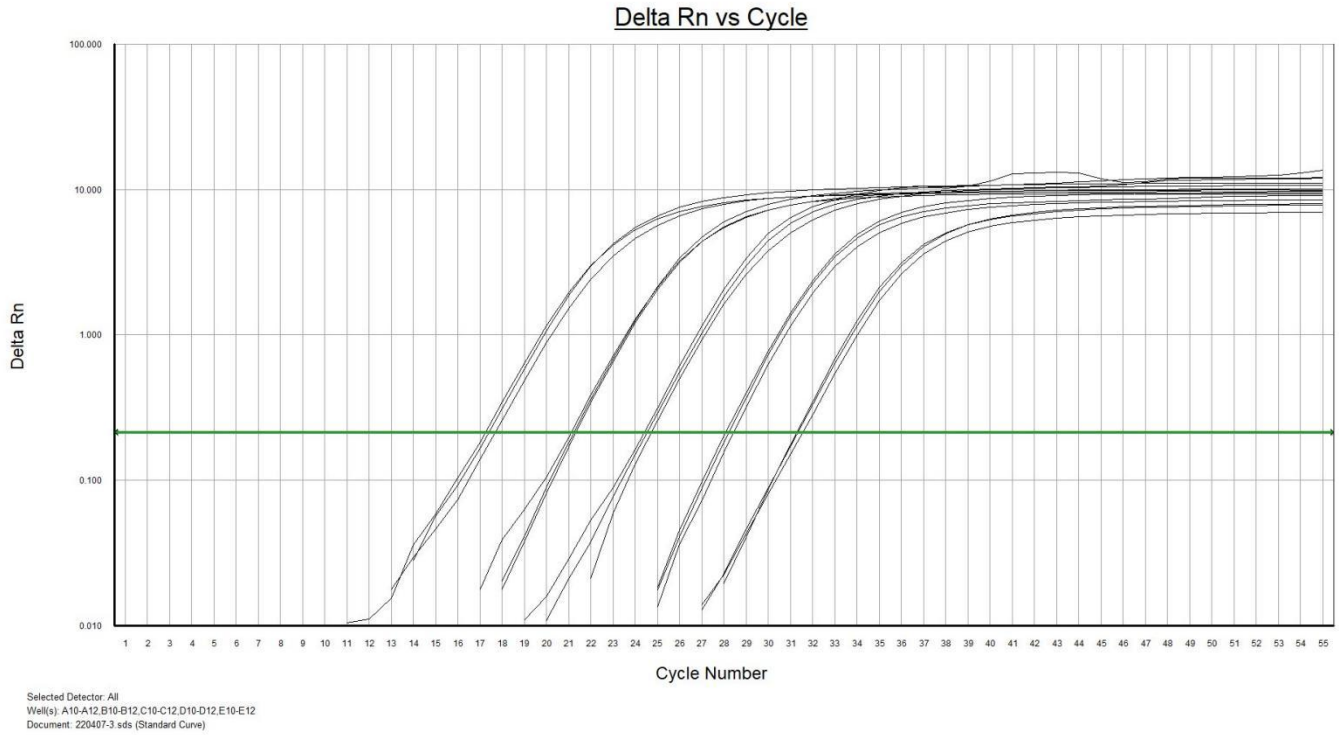

Figure 2. Obtained fluorescent signal at optimal primer/probe concentration at a annealing temperature of 60°C

Target DNA copy	Target detected	CT value
0	No	-
1	Yes	-
10	Yes	-
10^2	Yes	31.2
10^3	Yes	28.2
10^4	Yes	24.5
10^5	Yes	21.2

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

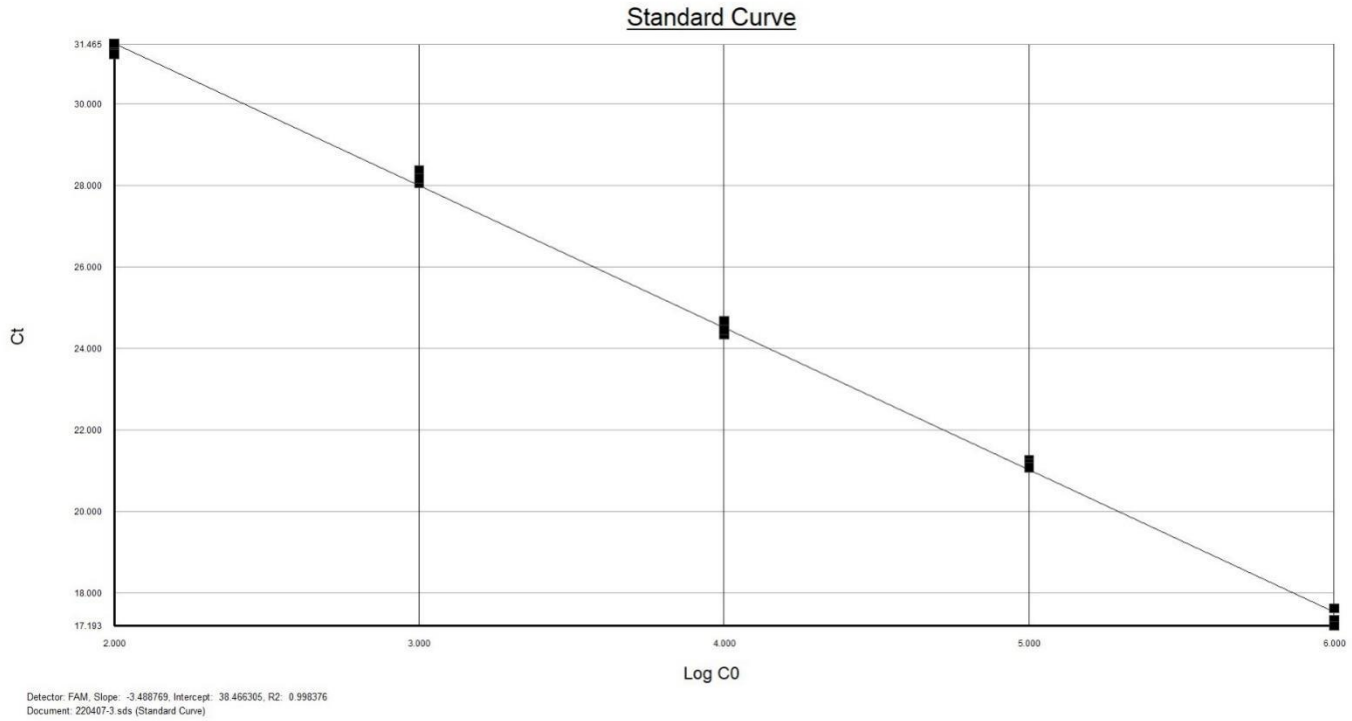

Figure 3. Standard curve of SYL153 based on 10^2, 10^3, 10^4 and 10^6 DNA target copies

Slope	-3.49
Efficiency	93.8
R^2	0.998

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 ability to perform in the presence of potential inhibitors in three types of environmental water samples (with sandy, clayey, and peaty substrates). Each sample type was tested in triplicate and spiked with 1000 copies of the target DNA. The ΔCT between spiked samples and spike-only was determined and was required to be less than 2. Standard deviations and standard errors of the mean were calculated to assess robustness, repeatability, and measurement uncertainty.

Results:

No fluorescent output signal was detected in the environmental samples without the addition of target organism DNA (spike). All spiked samples and spike-only analyses consistently produced positive signals across all replicates (**Table 6**). In all cases, the ΔCT was less than 2.

Sample	CT value	Standard deviation	ΔCT
Sandy	-	-	-
Clayey	-	-	-
Peaty	-	-	-
Sandy + spike	24.4	0.0	0.1
Clayey + spike	24.5	0.1	0.0
Peaty + spike	24.6	0.2	0.1
Spike-only	24.5	0.2	-

Table 6. CT values obtained with target organisms free environmental samples spiked with 1000 target copies

2.4. 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 amphibian species that may occur in the same habitats as *Alytes obstetricans*; *Rana arvalis*, *Rana temporaria* and *Salamandra salamandra*. 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, qPCR products obtained from confirmed *A. obstetricans*-positive environmental samples were sequenced to verify that amplification originated from the target species.

In addition, environmental DNA (eDNA) samples from locations where *A. obstetricans* 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. To determine the minimal filtration volume under field conditions, environmental samples were collected during the optimal seasons (spring, summer, and autumn) using the SYL009 – Environmental Sampling Kit, after which eDNA was isolated with the SYL002 – Environmental DNA Isolation Kit.

Results:

No amplification was detected in any of the tested non-target mammalian or amphibian species. Likewise, no amplification occurred in environmental samples from locations without *A. obstetricans* presence, and all negative controls (NTCs) remained free of signal throughout the analyses. Sequenced amplicons from positive environmental samples were confirmed to originate from A. obstetricans. The SYL153 – *Alytes obstetricans* detection kit successfully detected target DNA in environmental samples from several locations in the Netherlands, with positive samples yielding between 1 and 10 000 molecules of A. obstetricans DNA per 750 mL of filtered water.

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

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"

- Primers specific at temperature range of 55.0°C to 69.2°C (**Section 2.1**, **Table 3**).
- Influence of chemical and biological factors present in environmental samples on the performance of the test were not found (**Section 2.3**, **Table 6**).
- Fluorescent output signals of positive samples are 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 100 copies per reaction (**Section 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 93.8 %, this means that the primer/probe mixture can be regarded as optimal (**Section 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 2.3, Table 6**).

3.5 Correctness

- *"The ability of the method to do what it 'says' to do"*
- The test was able to detect target-DNA in samples from an environment in which *Alytes obstetricans* was present (**Section 2.4**)
- The method did not give any other combined BLAST hit than the target organism
- The method was able to detect all spiked target DNA in target organism free environmental samples (**Section 2.3, Table 6**)
- The method did not give any signal in target organism free environmental samples (**Section 2.3, Table 6**)
- The assay showed no cross-reactivity with DNA from common non-target species or from phylogenetically related taxa, confirming high analytical specificity of the primer/probe set

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