Sylphium
molecular ecology

Internal Positive Control qPCR quantification kit

With eDNA qPCR hot start mix

#SYL113

Validation report

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For general laboratory and research use only.

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1. Validation report Internal Positive Control detection kit

1.1. In silico validation

Forward primer	Forward primer	Reverse primer	Probe
Length (bp)	22	20	33
GC %	45	50	45
Stability	2.5	2.2	74
T_M (°C)	60	61	n.a.
Target region	n.a.	n.a.	No
Dimer	No	No	No
Run	No	No	33

Table 1. *In silico* validation of probe and forward and reverse primers.

PCR product size (bp)	109
Combined dimer formation	No
***In silico* PCR on Genbank**	Artemisia annua amorpha-411-diene synthase (ADS)
Date of *in silico* PCR	May 2023
DNA sequence	GACGCCTAAATGATCTCATGACCCACAAGGCCGAGCAAGAAAGAAAACAT AGTTCATCGAGCCTTGAAAGTTATATGAAGGAATATAATGTCAATGAGGAG TATGCCCA

Table 2. Combined primers and probe *in silico* validation.

1.2. 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.

1.2.1. 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 50°C to 72°C in eight incremental steps. The template concentration was approximately 1-10 molecules per reaction.

Results:

A fluorescent signal (RFU) was detected at annealing temperatures between 50°C and 63.8°C. The highest signal was observed 58.4°C. (**Table 3**, **Figure 1**).

Annealing temp.	50°C	51.4°C	54.3°C	58.4°C	63.8°C	68.2°C	70.7°C	72.0°C
RFU	1000	1000	950	1350	1000	-	-	-
CT	34.3	35.0	34.6	34.0	35.1	-	-	-

Table 3. Temperature gradient PCR on DNA of *Internal Positive Control.*

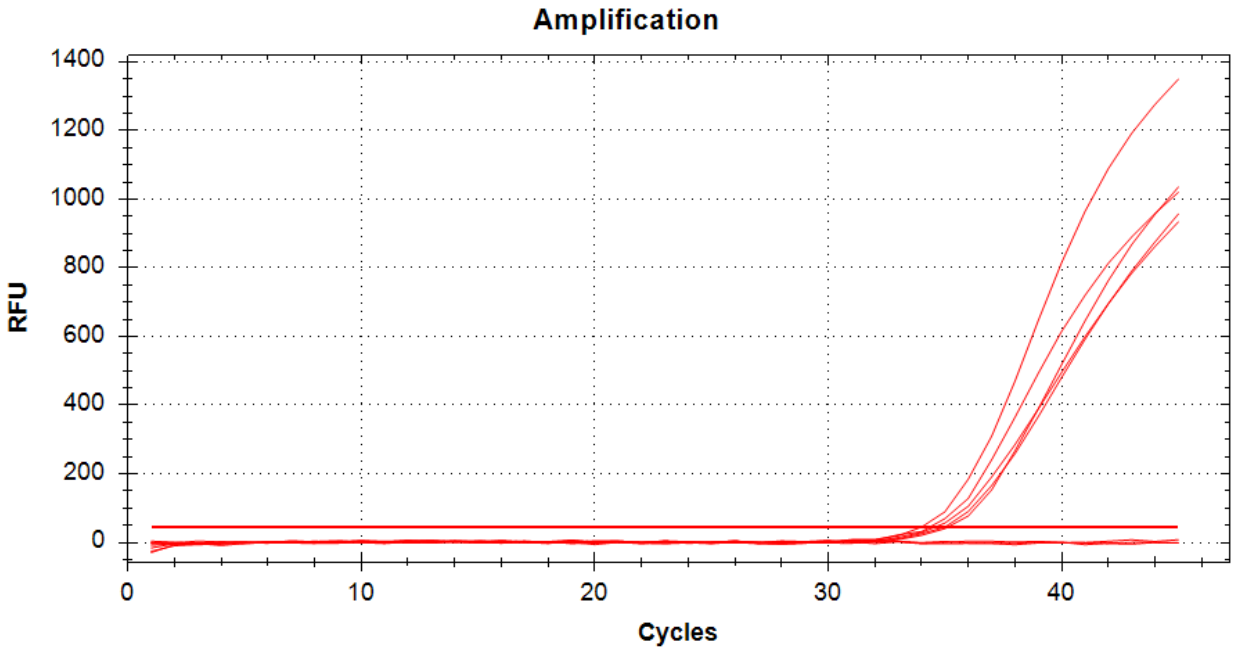

Figure 1. Temperature gradient PCR with ± 10 copies template per reaction.

1.2.2. 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 were 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 detection was determined between 1-10 and >10^6 target copies per reaction. The detection limits (low and high) for quantitative detection was determined between 1-10 and >10^5 target copies per reaction. The fluorescence output signal was at least 100 (8 for positive sample, 0.01 for a negative sample) times stronger than the background signal (**Figure 2**). The efficiency of the test was above 90%, which means that the efficiency of the primer/probe mixture can be regarded as optimal (**Figure 3**; **Table 5**).

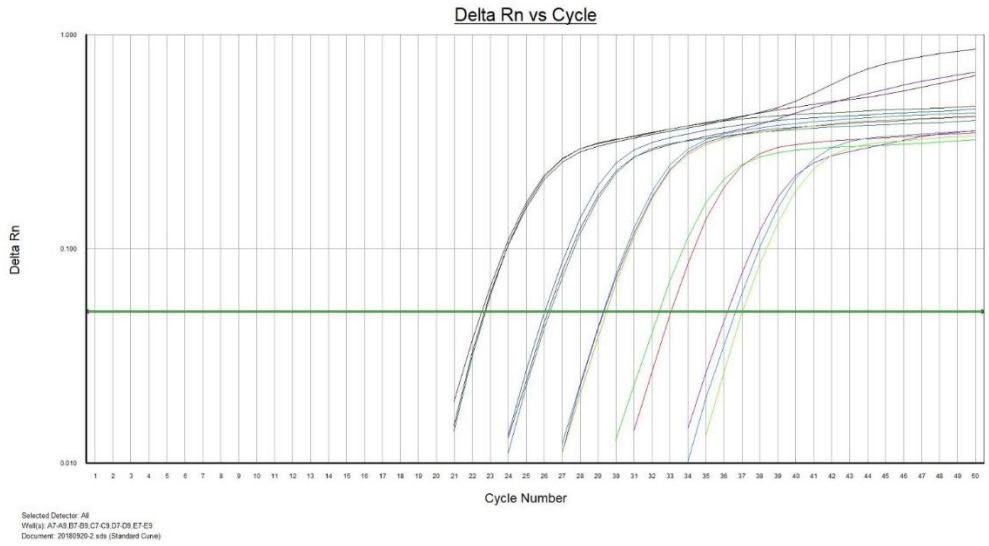

Figure 2. Obtained fluorescent signal at optimal primer/probe concentration at a annealing temperature of 55°C.

target DNA copy	Target detected	CT value	Standard deviation
0	No	-	-
1	Yes	36.6	0.4
10	Yes	32.8	0.3
10^2	Yes	29.3	0.1
10^3	Yes	26.2	0.1
10^4	Yes	22.6	0.1

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

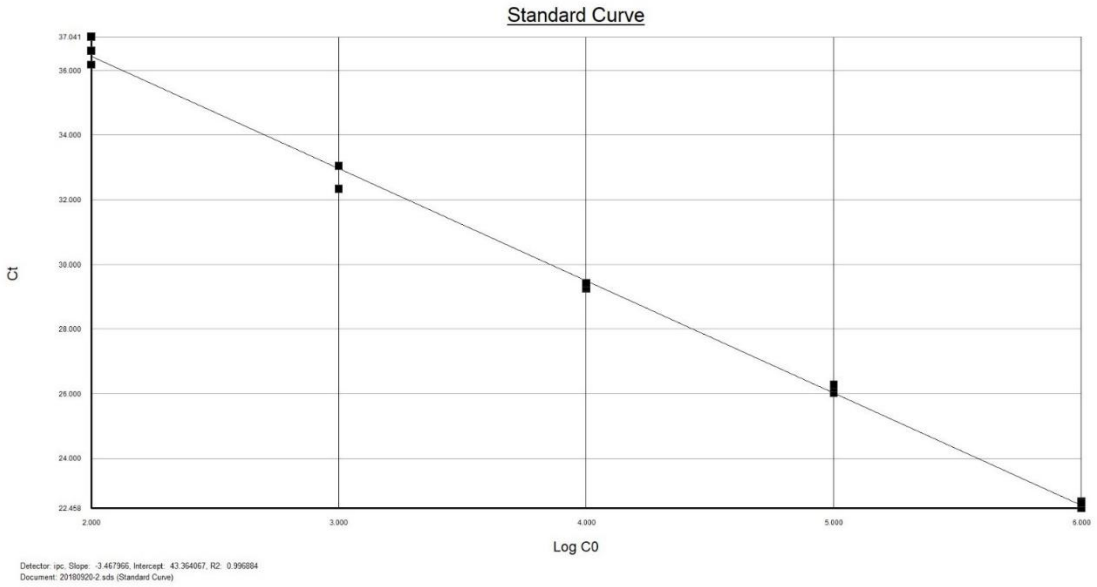

Figure 3. Standard curve of SYL113 based on 1, 10, 10^2, 10^3, 10^4 and 10^5 DNA target copies.

Slope	-3.39
Efficiency	97.2%
R^2	0.995

Table 5. Values obtained from the standard curve.

1.2.3. Influence of inhibiting factors present in environmental samples and repeatability

The essay 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 10^7 copies of the target DNA. 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**). There were no statistically significant differences between group means as determined by one-way ANOVA (**F**(3,8) = 2.12, *p* = .18) Influence of chemical and biological factors present in environmental samples was not detected. The standard error of mean was 0.17 with an average of 17.6 with these samples.

Sample	CT value	Standard deviation
Sandy	-	-
Clayey	-	-
Peaty	-	-
Sandy + spike	17.4	0.1
Clayey + spike	17.2	0.9
Peaty + spike	17.7	0.2
Spike-only	17.1	0.0

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

1.3. Summary of validation

1.3.1. 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: 50°C to 63.8°C (**Section 1.2.1**, **Table 3**).
- Influence of chemical and biological factors present in environmental samples on the performance of the test were not found (**Section 1.2.3**, **Table 6**).
- Fluorescent output signals of positive samples is at least 100-fold stronger than the background.

1.3.2. Detection limit

"Limits (lower and upper limit) within which the analysis can be reliably applied"

- Limit of detection (LOD) for qualitative was determined to be between 100 and 10^5 target DNA copies per reaction. The detection limits for quantitative analyses were determined between100 and 10^5 target copies per reaction. Below this concentration, the results become unreliable quantitative analyses, but may still be useful for qualitative analysis (**Section 1.2.2**, **Table 4**).

1.3.4. 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 97.2%, this means that the primer/probe mixture can be regarded as optimal (**Section 1.2.2**; **Table 5**).

1.3.5. 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 statistical differences (p>0.05) between different kinds of environmental water samples (sandy, clayey or peaty bottoms) spiked with 10^7 target DNA copies. (**Section 1.2.3**.; **Table 6**).

1.3.6. Correctness

"The ability of the method to do what it 'says' to do"

- The test was able to detect all spiked target DNA in organism free environmental samples (**section 1.2.2.**).
- The method did not give any signal in target organism-free environmental samples (**section 1.2.2.)**.
- The standard error of the mean based on the measured CT values from the spiked experiments (1000 target copies) was 0.4 on an average of 30.6. Below 100 target copies per reaction, the measurement uncertainty is increasing and on this level only qualitative measurements are valid (**section 1.2.3.**).

Sylphium

The eDNA company

Sylphium molecular ecology

P.O. box 11107
9700 CC Groningen
Netherlands

Tel.: +31 6 82 42 64 44
E-mail: info@sylphium.com

www.sylphium.com