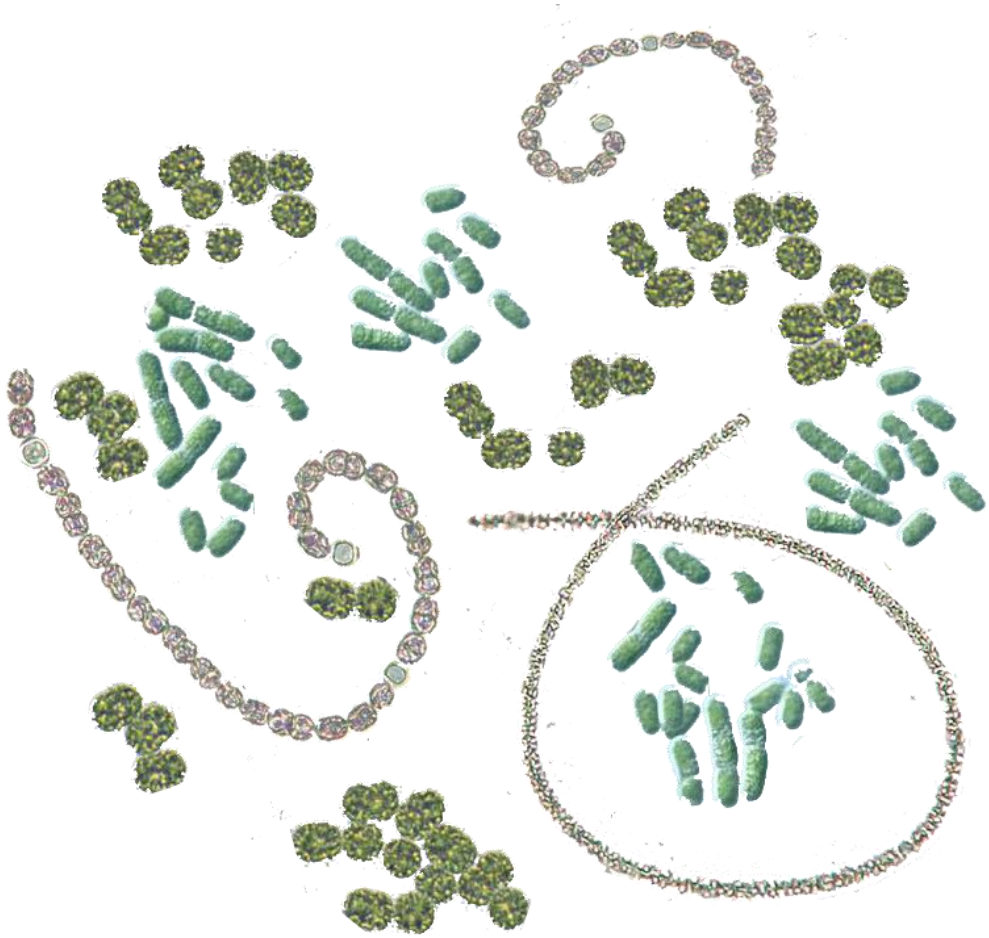
Validation report:

Anatoxin gene cluster qPCR detection kit

with eDNA qPCR hot start mix



#SYL122

Document date: 29 March 2023

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1. Validation report Anatoxin detection kit

1.1 In silico validation

Table 1: Forward primer 1 *in silico* validation.

Length	18
GC %	61
Stability	2,1
T_M (°C)	67
Target region	AnaF (DNA)
Dimer	No
Run	No
Database hit	Cyanobacteria

Table 2: Forward primer 2 *in silico* validation.

Length (bp)	23
GC %	43
Stability	1,0
T_M (°C)	64
Target region	AnaF (DNA)
Dimer	No
Run	No
Database hit	Cyanobacteria

Table 5: Forward primer 3 *in silico* validation.

Length	24
GC %	37
Stability	1,5
T_M (°C)	63
Target region	AnaF (DNA)
Dimer	No
Run	No
Database hit	Cyanobacteria

Table 6: Reverse primer 1 *in silico* validation

Length	23
GC %	47
Stability	2,2
T_M (°C)	62
Target region	AnaF (DNA)
Dimer	No
Run	No
Database hit	Cyanobacteria

Table 7: Reverse primer 2 *in silico* validation.

Length (bp)	27
GC %	Average of 33 (2 variants)
T_M (°C)	64
Target region	AnaF (DNA)
Dimer	No
Run	No
Database hit	Cyanobacteria

Table 8: Probe 1 *in silico* validation

Length	32
GC %	52
T_M (°C)	70
Target region	AnaF (DNA)
Dimer	No
Run	No
Fluorescence label	Fam

Table 3: Probe *in silico* validation

Probe name:	prSYL346
Length	31
GC %	Average of 43 (2 variants)
T_M (°C)	Unknown
Target region	AnaF (DNA)
Dimer	No
Run	No
Fluorescence label	FAM

Table 10: Combined primers and probe *in silico* validation

PCR product size (bp)	105
Combined dimer formation	No
Combined 1000 BLAST® analyses of both primers	Cyanobacteria
Date of BLAST® analyses	November 2018

1.2 Experimental validation

All validation experiments were performed with the Sylphium qPCR mix and performed on Applied biosystems 7300 qPCR machines and Biometra gradient PCR machines.

1.2.1 Gradient PCR

Influence of the annealing temperature on the performance and specificity of the primer set was tested with a temperature gradient between 50.8°C to 67.3°C in eight steps. Optimal temperature and the temperature range in which the test can perform was determined.

Results:

The expected product was formed at all tested temperatures (50°C to 70°C). The optimum range for both primer sets is determined between 52.3°C and 62.0°C. The optimal annealing temperature was is at 57°C for the primer set (See table 11).

Table. 11: Temperature gradient PCR.

Annealing temp.	**50.8°C**	**52.3°C**	**56.0°C**	**58.0°C**	**60.0°C**	**62.0°C**	**65.0°C**	**67.3°C**
Expected fragment	Weak	Strong	Strong	Strong	Strong	Strong	Weak	Weak
Primer dimer	Weak	Weak	Weak	Weak	Weak	Weak	Weak	Weak
Additional fragments	No	No	No	No	No	No	No	No

1.2.2 detection limit and fluorescence output signal

Standard solutions with 10^6, 10^5, 10^4, 10^3, 10^2, 10 and 0 target copies per 5 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.

Results:

The detection limits (low and high) for qualitative detection was determined between 10 and >10^6 target copies per reaction. The detection limits (low and high) for quantitative detection was determined between 10 and >10^6 target copies per reaction. The fluorescence output signal was at least 100 (7 for positive sample, 0,01 for a negative sample) times stronger than the background signal. (fig. 1, table 12)

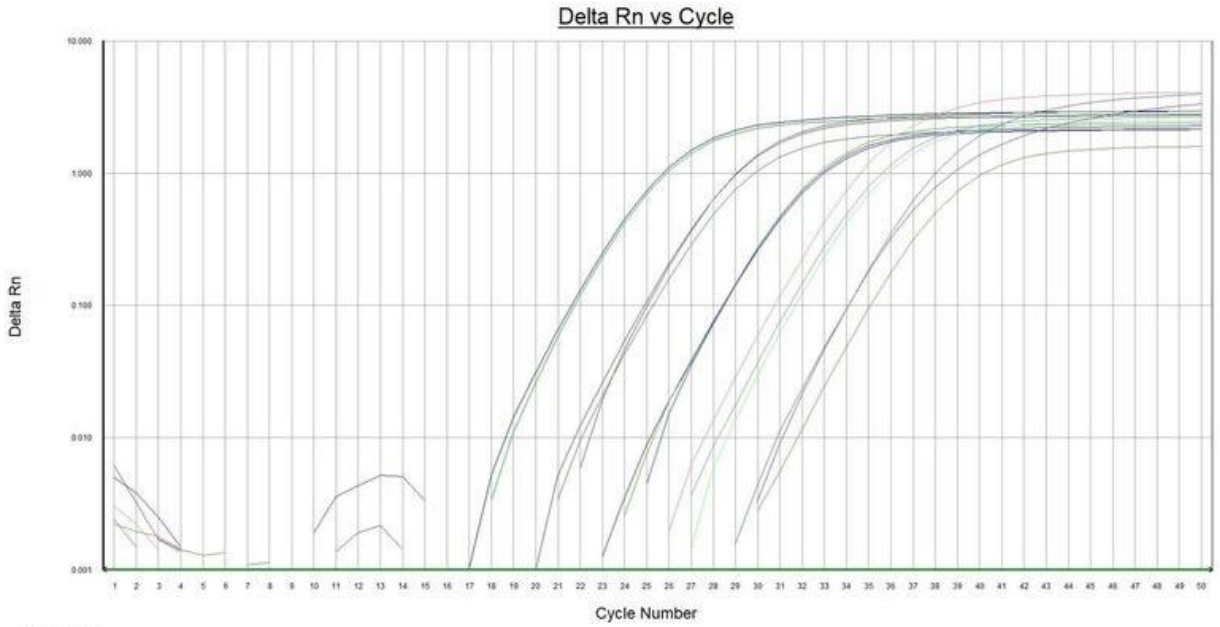

Figure. 1: Obtained fluorescent signal at optimal primer/probe concentration at a annealing temperature of 57°C.

Table 4: CT values of anatoxin 1 obtained at optimal primer/probe concentration

target DNA[1] copy	Target detected	CT value	Standard deviation
0	No	-	-
10	Yes	Nd[2]	Nd[2]
10^2	Yes	34.3	0.6
10^3	Yes	31.2	0.5
10^4	Yes	28.3	0.3
10^5	Yes	25.0	0.2
10^6	Yes	21.5	0.1

[1] Estimated by gel electrophoresis

[2] Not determined as very low amounts of DNA cannot quantitatively be detected by the qPCR technique

1.2.3 Efficiency

Based on the slope of the standard curve the efficiency of the primer/probe set was determined.

Results:

The efficiency of the test was above 90%. Which means that the efficiency of the primer/probe mixture can be regarded optimal (Fig. 2, table 13).

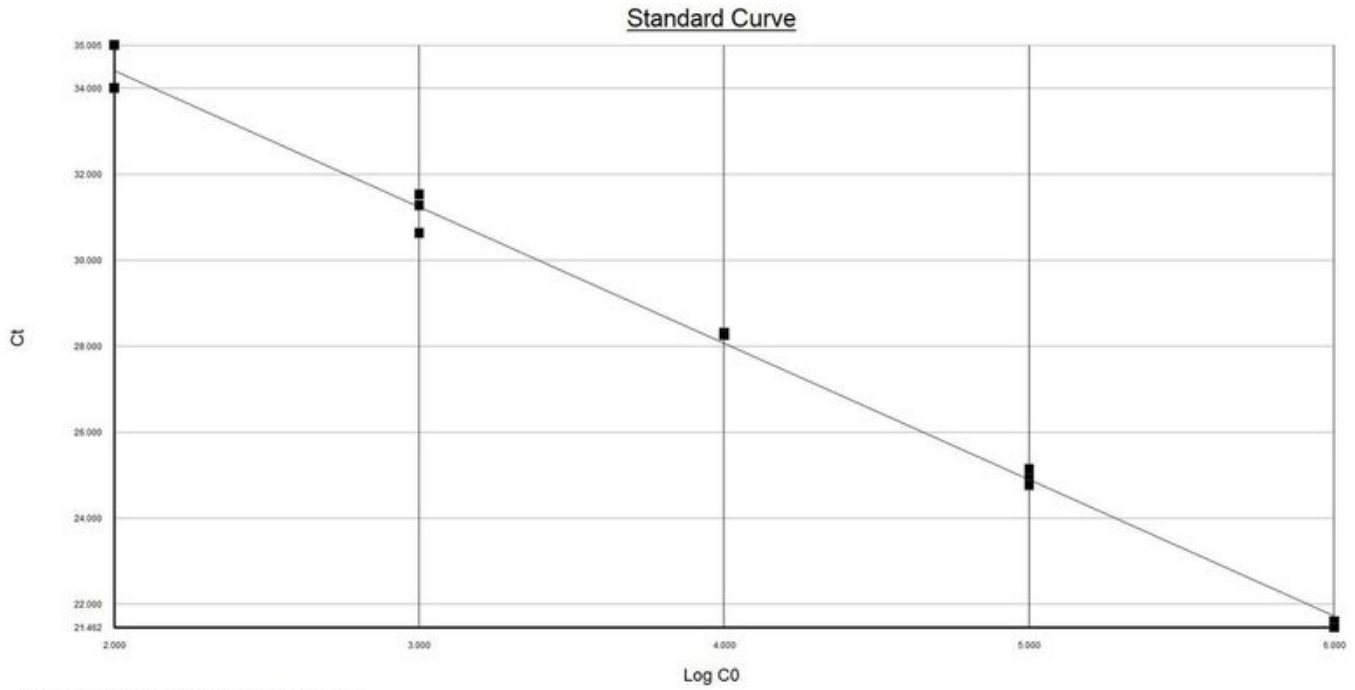

Fig. 2: Standard curve of SYL106 based on 10^2, 10^3, 10^4, 10^5 and 10^6 DNA target copies

Table 13: Values obtained from the standard curve

Slope	-3.16
Efficiency	107%
R^2	0.994

1.2.4 Influence of inhibiting factors present in environmental samples and repeatability

Performance of the test was tested on the influence of inhibiting factors present in three different kinds of environmental water samples (sandy, clayey or peaty bottoms) in three fold spiked with 10000 target DNA copies. Standard deviations, one way ANOVA and the standard error of mean were calculated to determine robustness, repeatability and measurement uncertainty.

Results:

No fluorescent output signal was detected in the environmental samples without added target organism DNA (spike). Spiked samples and the spike only analyses gave in all replicates a positive signal. (see table 10). There were no

statistically significant differences between group means as determined by one-way ANOVA (F(3,8) = 2.500, p = .29) Influence of chemical and biological factors present in environmental samples was not detected. The standard error of mean was 0.1 within these samples with an average of 28.4

Table 14: CT values obtained with target organisms free environmental samples spiked with 10000 target copies.

Sample	CT value	Standard deviation
Sandy	-	-
Clayey	-	-
Peaty	-	-
Sandy + spike	28.4	0.0
Clayey + spike	28.3	0.0
Peaty + spike	28.5	0.1
Spike only	28.3	0.0

1.2.5 Sequence conformation of specificity.

PCR products obtained from the gradient PCR experiment were sequenced for conformation of identity of the formed product. If any product was formed with target organism free environmental samples, than these products were also be sequenced.

Results:

Target organism free environmental samples did not give any PCR product. PCR products obtained from the gradient PCR experiment were sequenced in triplo via the Sanger sequencing method. The obtained sequences were identical to each other and Blast ® hits confirmed identity (<97%) of the target organism. (table 11)

Table 15: First 3 Blast ® hits obtained from the NCBI database.

Species	Sequence ID	Similarity
Cuspidothrix issatschenkoi	LT984882.1	100%
Cuspidothrix issatschenkoi	KM245025.1	100%
Cuspidothrix issatschenkoi	KM245024.1	100%

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: 52.3°C and 62.0°C (section 1.2.1, section 1.2.5, table 11, table 15)
- Statistical differences (p>0.05) due to influence of chemical and biological factors present in environmental samples on the performance of the test were not found. (section 1.2.4, table 14)
- 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"

The detection limits (lower and upper limit) for qualitative analysis was determined between 10^6 and 100 target DNA copies per reaction. The detection limits for quantitative analyses were determined between 10^6 and 1000 target copies per reaction, below this concentration the results become unreliable quantitative analyses, but still useful for qualitative analysis. (section 1.2.2, table 12)

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 107%, this means that the primer/probe mixture can be regarded as optimal. (section 4.2.3, table 13)

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 10000 target DNA copies. (section 1.2.4, table 14)

1.3.6 Correctness

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

- The method did not give any other combined BLAST hit than the target Anatoxin
- The method was able to detect all spiked target DNA in target organism free environmental samples (section 1.2.4, table 14)
- The method did not give any signal in target organism free environmental samples (section 1.2.4, table 14)
- The standard error of the mean based on the measured CT values from the spiked experiments (1000 target copies) was 0.1 on an average of 28.4.