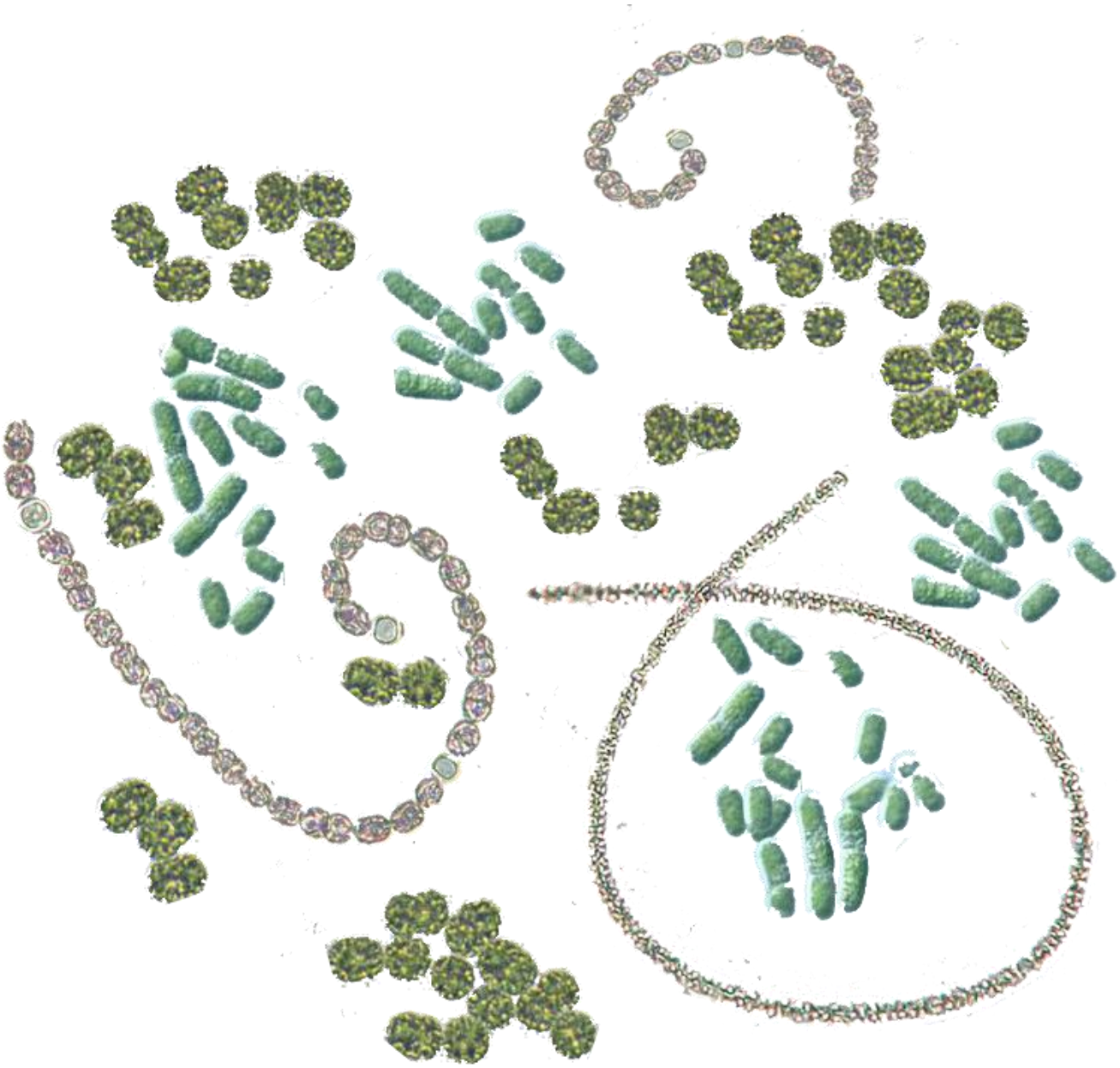
Cylindrospermopsin Cyanotox qPCR detection kit



#SYL102
48 samples (200 tests)
Version: 190911

For general laboratory and research use only

Inhoudsopgave

1. Introduction

Cylindrospermopsin Cyanotox qPCR detection kit is based on the fast, sensitive, and proven primers/probe qPCR technique. The kit contains primers and a probe for detecting a highly specific sequence present in the *cyrA* gene, which is part of the cylindrospermopsin biochemical pathway of cyanobacteria. The primers and probe are designed according to designing parameters as stated in the Sylphium primer design and validation protocol for DNA.

Primers and probe designed according these rules have:

- **Lowest possible sensitivity (<100 DNA copies per reaction)**.
- **Strong fluorescence signal with low background noise**. Isolated environmental samples contain residues of naturally occurring auto fluoresce substances that will interfere with the measurements. A strong fluorescence signal from the analyses is required for this kind of samples.
- **100% specificity**. Isolated DNA from environmental samples contains billions of DNA fragments from bacteria, protozoa, plants, animals, etc. Not only species from the same order must be taken in account during primer/probe design, but all known DNA sequences must be check for nonspecific binding of the primers and probe. This is validated by experimental and bioinformatical studies.

The kit is developed and optimized to be used on DNA isolates purified using the DNA isolation kit (#SYL002) from Sylphium molecular ecology.
Other DNA isolation methods/kits can be used as well. Please send an e-mail to info@sylphium.com to get more information how to use the obtained isolates from other methods/kits to get reliable results with the cylindrospermopsin gene qPCR detection kit.

The cylindrospermopsin Cyanotox qPCR detection kit is validated for use in all waters worldwide.

1.1 Kit contents

- Positive control (10^8 *cyrAJ* gene copies per 5 µl)
- 2x Sylphium qPCR mix (100 reactions) without primers and probes
- 2x Primer/probe mix (100 reactions) for detection of *cyrA* (FAM dye)
- 1x Taq DNA polymerase (200 reactions)

1.2 Equipment Required

- qPCR machine multiplex capable for detection of the FAM dye
- Microcentrifuge (13,000 x g)
- Pipettors
- -20°C Freezer
- PCR cooling rack

1.3 Kit Storage

Store all reagents and kit components in the dark in a freezer (-20°C).

1.4 Notices and disclaimers

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2. Principals of the kit

2.1 qPCR

During PCR amplification, one set of primers and a probe hybridize to a gene located on the DNA of cyanobacteria. The fluorogenic probe is labeled with a discriminating 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of qPCR machines. The target gene of cyanobacteria will be detected via the FAM dye channel.

2.2 PCR positive control (PPC)

The PCR positive control provided in this kit contains a cloned *cyrA* gene at a concentration of 10^8 copies per 5 µl. This is a control for checking the reactions during analyses and can be used to make a calibration curve to determine the gene copy number in the sample. A positive result indicates that the primers and probes for detecting the target worked properly in that particular experimental scenario. If a negative result is obtained, the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false-positive results. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

2.3 PCR negative control (PNC)

To validate the results, a negative control should be included every time the kit is used. DNA contaminations will be checked with this control. Nothing will be added to the PCR mix present in the wells. This control should give a negative signal. A positive signal indicates a DNA contamination somewhere during the procedure. In that case the qPCR results are not reliable and the analysis should be redone.

2.4 Internal positive control (IPC)

The Internal positive control is an efficiency control of the DNA isolation procedure and a quality and purity control of the isolated DNA. The internal positive control is a small piece of synthetic DNA present in the conservation buffer of the DNA isolation kit. The chosen DNA sequence of the positive control is unknown to the aquatic environment and will not interfere in any detection of target organisms. The IPC will be detected via the HEX channel. A positive signal should be obtained from this control in all cases (reactions). A negative signal indicates inhibiting substances in the DNA isolate or a failure during isolation. The sampling and isolation procedure should be repeated. If inhibiting substances are present dilution of the sample is a possible solution, an additional purification step can also be done. Note: This test is not included in this kit. It is part of the environmental DNA isolation kit (SYL102) and should be ran before doing the actual analysis for cylindrospermopsin to prevent wasting of PCR mix and prime/probe mix.

2.5 Internal negative control (INC)

An additional negative control will be used during the DNA isolation. This sample will be analyzed as a normal sample and should be negative. If this control gives a positive signal, a DNA contamination has occurred during the DNA isolation. If that is the case, the results are not reliable anymore. The sampling and isolation procedure should be repeated after cleaning the lab and equipment.

2.6 qPCR experimental plate setup

It is highly recommended to perform the analyses in multiple fold per sample to make a reliable calculation of the amount of DNA. An example experimental plate setup is shown figure 2 in which samples can be analyzed in 3-fold. Other plate setups are also possible with this kit. For statistical reasons, the controls used in this kit should have the same analyses size as an analyzed sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1			Sample 2			Sample 3			10^7 PPC		
B	Sample 4			Sample 5			Sample 6			10^6 PPC		
C	Sample 7			Sample 8			Sample 9			10^5 PPC		
D	Sample 10			Sample 11			Sample 12			10^4 PPC		
E	Sample 13			Sample 14			Sample 15			10^3 PPC		
F	Sample 16			Sample 17			Sample 18			INC		
G	Sample 19			Sample 20			Sample 21			PNC		
H	Sample 22			Sample 23			Sample 24			PNC		

Figure 2: Plate setup for analyses of 24 samples in 3-fold.

3. Protocol

3.1 Precautions

- **Prevent contaminations.** Before starting your experiments, clean the table surface and all equipment with thin bleach (10 times diluted) or another DNA removing agent to remove DNA from previous experiments. Wear gloves to prevent DNA contaminations between samples and clean your gloves with a soaked paper towel with diluted thin bleach between every handling.
- **Reduce long exposure to light.** It's preferable to limit the amount of light during preparations of the PCR plate and mixtures. The PCR mix contains light sensitive probes and under influence of light the signal strength will be reduced.
- **Keep cool as long as possible**. Keep samples and mixtures in a PCR cooling rack after addition of the Taq DNA polymerase to prevent unwanted non-specific hybridization of primers and probe and extension by the used DNA polymerase at room temperature. The life span of Taq DNA polymerase in its own storage buffer will be at room temperature at least a month at room temperature. In the PCR mixture this is reduced to a few hours at room temperature.

3.2 Procedure

- Thaw one tube Sylphium qPCR mix (100 reactions) and one tube with primer/probe mix (100 reactions) on ice.
- Pulse-spin each tube in a centrifuge before opening. This will ensure mixtures are in the base of the tube and is not spilt upon opening the tubes
- Add the complete primer/probe mix (+/- 250 µl) to the Sylphium qPCR mix
- Add 100 µl Taq DNA polymerase to the mix.
- Mix thoroughly but slowly to minimize the number of air bubbles in the mixture.
- Cool down the 96 well PCR plate in a PCR cooling rack and place a cover on top to prevent contamination from air.
- Pipet 23 µl prepared PCR mix in each well. Any remaining mixture may be stored in freezer until next analyses. After rethawing the mixture, check for precipitation of magnesium. If any precipitation is formed try to dissolve it by gently shaking the mixture.
- Make a calibration line of 10^7, 10^6, 10^5, 10^4 and 10^3 from the 10^8 from the positive control stock. By transferring 5 µl to a new tube with 45 µl Tris buffer. Repeat this step until all dilutions were made. Every day this should be freshly prepared.
- Add 5 µl template DNA and add 5 µl of each control to the PCR plate according to the preferred qPCR plate setup. Nothing will be added to the PCR negative control.
- Centrifuge briefly if needed. Bubbles will interfere with fluorescence detection.
- Program the thermal cycler according to the scheme below, place the samples in the cycler and start the program.

3. 3 Thermal cycling conditions

Thermal cycling will be performed using a three-step program

Table 2: Thermal cycling conditions of Cylindrospermopsin Cyanotox qPCR detection kit. Fluorogenic data is collected during the extension step. Cylindrospermopsin gene detection via FAM channel.

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	94	5 min.	1
Denaturation	95	15 sec.	35
Annealing	57	15 sec.	
Extension	72	30 sec.	

3.4 Interpretation of results

Obtained results are only valid as if all criteria are met as seen in table 3.

Table 3: Interpretation of results of Cylindrospermopsin Cyanotox qPCR detection kit. Target: detection of cylindrospermopsin gene (*cyrA*) – IPC: Internal positive control – INC: Internal negative control – PPC: PCR positive control (part of pSYL002: Environmental DNA isolation kit) – PNC: PCR negative control. See "Principals of kit" section of this document for possible solutions to solve some of these problems.

Target	IPC	INC	PPC	PNC	Result
+	+	-	+	-	Positive detection
-	+	-	+	-	Negative detection
-	-	-	-	-	Failing PCR procedure
-	-	-	+	-	PCR inhibited or isolation failed
+	+	+	+	-	DNA contamination during isolation
+	+	+	+	+	DNA contamination during PCR preparation

4. Validation report cylindrospermopsin detection kit SYL102

This kit consists of one primer/probe set for the detection of the *cyrA* gene, which is an important gene in the production process of cylindrospermopsin in cyanobacteria.

4.2 *In silico* validation

Table 4: Forward primer *in silico* validation

Length	23
GC %	47
T_M (°C)	64
Stability	2.8
Target region	CyrA (DNA)
Database hit	Cyanobacteria

Table 5: Reverse primer *in silico* validation

Length (bp)	16
GC %	68
T_M (°C)	64
Stability	2.5
Target region	CyrA (DNA)
Database hit	Cyanobacteria

Table 6: Probe *in silico* validation

Length	29
GC %	44
T_M (°C)	72
Target region	CyrA (DNA)
Dimer	No
Fluorescence label	HEX (VIC)

Table 7: Combined primers and probe *in silico* validation

PCR product size (bp)	71
Combined dimer formation	No
Combined 1000 BLAST® analyses of both primers	*cyrA* gene of Cyanobacteria
Date of BLAST® analyses	November 2018

4.2 Experimental validation

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

4.2.1 Gradient PCR

Influence of the annealing temperature on the performance and specificity of the primer set was tested with a temperature gradient between 50°C to 70°C in eight steps. The optimal temperature and the temperature range in which the test can perform is determined.

Results:

The expected product (71 bp) was formed between 50.8°C and 62.0°C. Nonspecific additional fragments were not formed at 51.9°C and 56.4°C. Weak primer dimers were formed at all tested temperature. The optimal annealing temperature was between 52.3°C and 58.0°C. (See Table 8).

Table. 8: Temperature gradient PCR on the *cyrA* gene

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	Strong	Strong	Strong	Strong	Strong	Weak	No	No
Primer dimer	No	No	No	No	No	No	No	No
Additional fragments	No	No	No	No	Yes	Yes	Yes	Yes

4.2.2 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 with optimal primer/probe concentrations. Fluorescent output signals were compared with the background to have at least a 100-fold increase in fluorescence signal. Reaction conditions were adjusted if the fluorescence signal was too low in contrast to the background noise.

Results:

The detection limits (lower and upper limit) for qualitative detection was determined between 100 and >10^6 target copies per reaction. The detection limits (lower and upper limit) for quantitative detection was determined between 1000 and >10^6 target copies per reaction. The fluorescence output signal was at least 100 (5 for positive sample, 0,05 for a negative sample) times stronger than the background signal (fig. 3). The CT values and the standard deviation between triplicates are given in table 9.

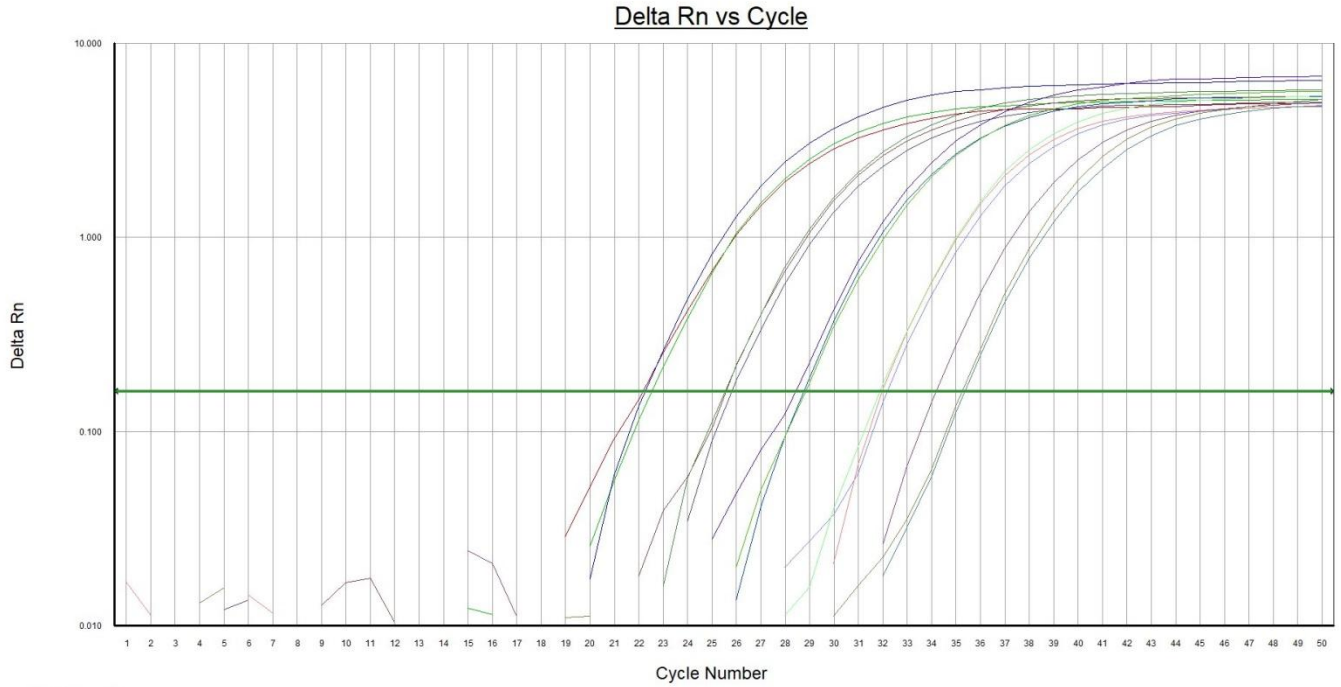

Figure 3: Obtained fluorescent signal at optimal primer/probe concentration at an annealing temperature of 57°C.

Table 9: CT values 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	34.9	0.6
10^3	Yes	32.0	0.2
10^4	Yes	28.6	0.2
10^5	Yes	25.6	0.2
10^6	Yes	22.3	0.2

[1] Estimated by gel electrophoresis

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

4.2.3 Efficiency

The efficiency of the primer/probe set was determined with use of the slope of the standard curve. The efficiency is optimal between 90% and 110%.

Results:

The efficiency of the standard curve is 107%, which means that the efficiency of the primer/probe mixture can be regarded as optimal (see fig. 4 for the standard curve and table 10 for the values).

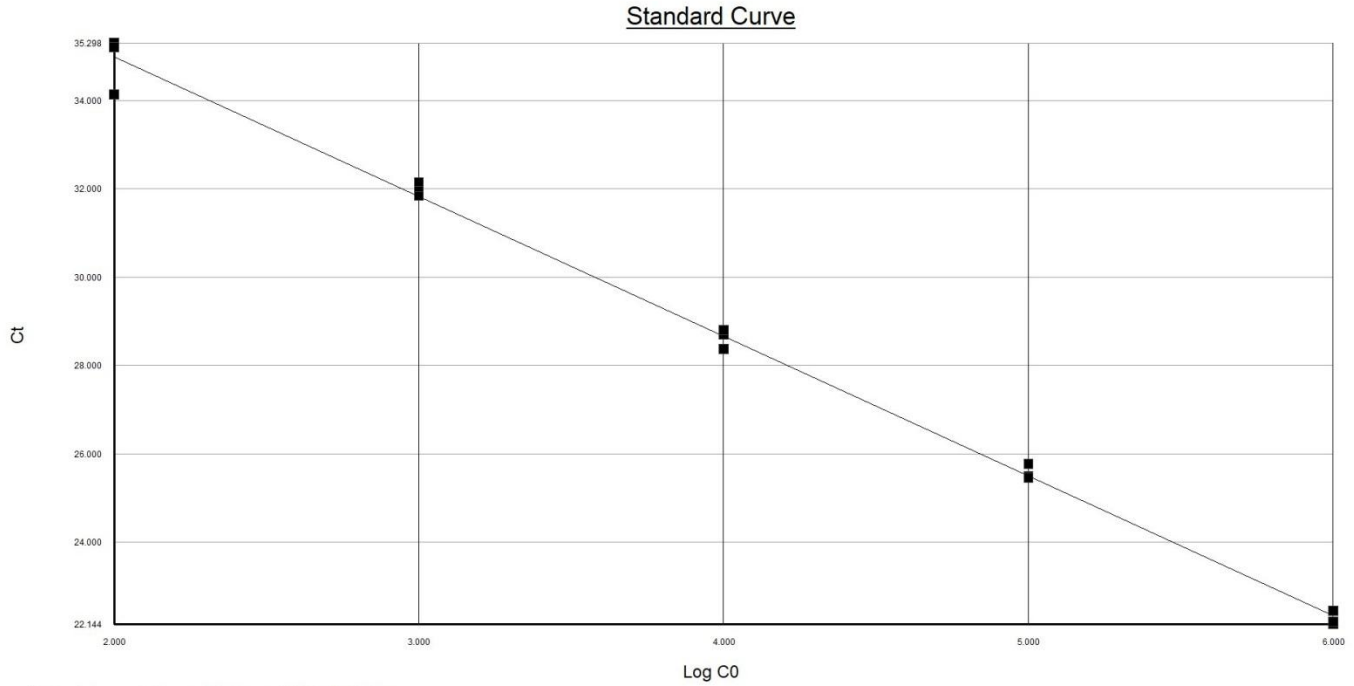

Figure 4: Standard curve of cylindrospermopsin based on 10^2, 10^3, 10^4, 10^5 and 10^6 DNA target copies.

Table 10: Values obtained from the standard curve

Slope	-3.16
Efficiency	107%
R^2	0.996

4.2.4 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. Cyanobacteria are always present in water, it is possible that samples without a spike give a positive result. If the concentrations are low (below 100 molecules), the positive samples can be ignored.

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 11). There were no statistically significant differences between group means as determined by one-way ANOVA ($p = 0.41$) Influence of chemical and biological factors present in environmental samples was not detected. The standard error of mean was 0.4 within these samples.

Table 11: CT values obtained with target organisms free environmental samples spiked with 1000 target copies.

Sample	CT value	Standard deviation
Sandy	-	-
Clayey	-	-
Peaty		-
Sandy + spike	28.8	0.1
Clayey + spike	28.6	0.2
Peaty + spike	28.5	0.2
Spike only	28.3	0.0

4.2.5 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, these 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 triplicates via the Sanger sequencing method. The obtained sequences were identical to each other and Blast® hits confirmed identity (<97%) of the target organism. (See table 12)

Table 12: First 3 Blast ® hits obtained from the NCBI database.

Species	Sequence ID	Similarity
Aphanizomenon ovalisporum	AF395828.1	100%
Uncultured cyanobacterium	KJ139758.1	100%
Uncultured cyanobacterium	KJ139757.1	100%

4.3 Summary of validation

4.3.1 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: 50.8°C – 60.0°C (section 4.2.1, section 4.2.2, table 8, figure 3)
- 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 4.2.4, table 11)
- Fluorescent output signals of positive samples are at least 100-fold stronger than the background

4.3.2 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 10 target DNA copies per reaction. The detection limits for quantitative analyses were determined between $>10^6$ and 100 target copies per reaction, below this concentration the results become unreliable quantitative analyses, but still useful for qualitative analysis. (section 4.2.2, table 9)

4.3.4 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 10).

4.3.5 Repeatability

"The degree of similarity between the results of measurements of the same measured quantity that were performed under different measurement conditions"

There were no statistical differences ($p>0.05$) between different kinds of environmental water samples (sandy, clayey or peaty bottoms) spiked with 1000 target DNA copies (section 4.2.4, table 11).

4.3.6 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 gene
- The method was able to detect all spiked target DNA in target organism free environmental samples (section 4.2.4, table 11).
- The method did not give any significant signal in target organism free environmental samples (section 4.2.4, table 11).
- 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 28.8. Below 100 target copies per reaction the measurement uncertainty is increasing and this level only qualitative measurements are valid (section 4.2.2 and 4.2.4).