Lepomis gibbosus detection kit

#SYL101
200 reactions
Version: 201218

For general laboratory and research use only.
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Index

1. Introduction

The *Lepomis gibbosus* 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 on the mitochondrial DNA of the species *Lepomis gibbosus.* This kit of Sylphium molecular ecology is designed to have a sensitivity of only one molecule per reaction in environmental samples.

Primers and probe and the qPCR buffer were designed to have:

- **Highest possible sensitivity (1 DNA copy per reaction)**. Environmental water samples contain normally very low amounts of target DNA.
- **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 these kind of samples.
- **100% specificity**. Isolated DNA from environmental samples contains billions of DNA fragments from bacteria, protozoa, plants, animals, etc. Not only animals from the same order (fish, amphibians, reptiles, mammals, etc.) must be taken in account during primer/probe design, but all known DNA sequences must be checked 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 eDNA isolates purified using the eDNA isolation kit (#SYL002 or SYL008) from Sylphium molecular ecology.
Other eDNA 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 *Lepomis gibbosus* detection kit.

The *Lepomis gibbosus* qPCR detection kit is validated for use in all waters worldwide.

1.1 Kit contents

- Positive control (*Lepomis gibbosus* DNA)
- 2x Sylphium qPCR mix (100 reactions) without primers and probes
- 2x Primer/probe mix (100 reactions) for detection of *Lepomis gibbosus* (FAM dye)
- 1x Taq DNA polymerase (200 reactions)
- 2 ml dilution solution (S6)
- Batch validation document

1.2 Equipment Required

- qPCR machine multiplex capable for detection of the FAM dye
- Microcentrifuge (11,000 x g)
- Pipettors
- -20 °Freezer

1.3 Kit Storage

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

1.4 Notices and disclaimers

This product is developed, designed and sold for research purposes only. Sylphium Molecular Ecology (Trade name of Eelco Wallaart b.v.) does not take any responsibility and is not liable for any damage caused through use of this product, be it indirect, special, incidental or consequential damages (including but not limited to damages for loss of business, loss of profits, interruption or the like).

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 mitochondrial DNA of *Lepomis gibbosus*. 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 DNA of *Lepomis gibbosus* will be detected via the FAM dye channel.

2.2 PCR positive control (PPC)

The PCR positive control provided in this kit contains cloned DNA of *Lepomis gibbosus*. This is a control for checking the reactions during analyses. This control should give a positive signal. Each time the kit is used, at least two positive control reactions must be included in the run. 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 any positive findings a negative control reaction 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. A negative signal (only background) should be obtained with this control. 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 repeated.

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 eDNA 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 FAM channel. A positive signal should be obtained from this control in all cases (reactions). A negative signal indicates inhibiting substances in the eDNA isolate or a failure during isolation. In that case 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 in the environmental DNA isolation kit (SYL002 and SYL008) and should be run before doing the actual analysis for *Lepomis gibbosus* to prevent wasting of qPCR mix, prime/probe mix and DNA Taq polymerase.

2.5 Procedure blanco

During DNA isolation an additional negative control will be used. This control contains only preservation solution will be analyzed as an normal sample. If a positive signal is detected with this control, a DNA contamination was obtained during isolation. Results are not reliable anymore. The sampling and isolation procedure should be repeated after cleaning the lab and equipment.

2.6 Limit of detection (LODqPCR)

Lowest analysis limit of the qPCR which corresponds to the minimum number of DNA copies in the qPCR which gives a positive result. This will determent with dilution series of the positive control.

2.7 Limit of quantification (LOQqPCR)

Lowest limit of the qPCR where with a reliability of 95% the number of DNA copies can be quantified. This will determent with dilution series of the positive control.

3. Protocol

This kit of Sylphium molecular ecology is designed to have a sensitivity of only one molecule per reaction in environmental samples. To have maximum sensitivity, the protocol belonging to this kit differs from standard qPCR protocols (from other suppliers) in which the lowest sensitivity is not required. Read carefully and understand all steps before proceeding with the analysis.

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.
- **Prevent breakdown of Taq DNA polymerase and unwanted products**. Cool down the PCR reaction mixture after preparing to 0 – 4 °C to prevent unwanted non-specific hybridization of primers and probe and breakdown of the Taq DNA polymerase. The cooling step will increase the sensitivity of the method.
- **Use only quality checked eDNA isolates.** The eDNA isolation kits of Sylphium contains a quality control qPCR analyses to determine the isolation efficiency and the possible presence of inhibiting substances. Perform this sample quality control before performing the analysis on the target organism. This will prevent false negative results. See the protocol of the eDNA isolation for more details.

3.2 Procedure

- Thaw the Sylphium qPCR mix (100 reactions), the primer/probe mix (100 reactions), samples and positive controls completely to dissolve all precipitations. Preferably at 55 °C for 10 minutes.
- Mix all tubes by shaking and 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.
- Prepare a dilution series of the positive control, according to the scheme below. The concentration of the positive control is printed on the tube. This dilution series will be used as a positive control.

5 µl positive control + 45 µl S6 → **10 x diluted**

↓

5 µl 10 x diluted + 45 µl S6 → **100 x diluted**

↓

5 µl 100 x diluted + 45 µl S6 → **1000 x dilute**

↓

5 µl 1000 x diluted + 45 µl S6 → **10.000 x diluted**

(only necessary if 10.000 copies/2 µl is provided as positive control)

Figure 1: Pipetting scheme for preparing dilution series.

- Prepare as much mixture as needed for the analyses. See our calculator (https://drive.google.com/file/d/1-xET6ny0YgZKxfN4xBc6fc9v2rUj6cOw/view?usp=sharing) or section 3.3 for the exact number of reactions. Prepare 3 additional reactions to compensate for pipetting errors. The non-mixed qPCR buffer, primers/probe mixture and Taq DNA polymerase can be refrozen.

Component	Volume per well
qPCR buffer	20 µl
Taq polymerase	1 µl
Primer/probe mix	2.5 µl

Table 1: Components per well

- Mix thoroughly but slowly to minimize the number of air bubbles in the mixture. Cool down the mixture to 0 - 4°C on ice or cooling block.
- Put a PCR plate in a cooling block of 4 °C.
- Pipet 23 µl prepared PCR mix in each well.
- Place a cover on top of the 96 well PCR plate to prevent contamination from air.
- Add 2 - 5 µl template DNA (sample or positive control) to the PCR plate according to the preferred qPCR plate setup. Nothing will be added to the PCR negative control. It's not necessary to adapt volumes of the components if sample size will be changed.
- 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 qPCR experimental plate setup

The concentration of eDNA molecules in aquatic samples are under normal circumstances very low. It's highly recommended to perform the analyses in multiple fold per sample to increase detection of these low amounts of DNA. Two example experimental plate setups are shown figure 2 and figure 3 in which samples can be analyzed in 8 or 12 fold per 96 wells plate, respectively. Other plate setups are also possible with this kit. For statistical reasons, the controls used in this kit, except the PCR positive control should have the same replication size as an analyzed sample. The PCR positive controls must be analyzed in at least 2 fold. It's recommended to analyze the positive control in a dilutions series from 10^3 to 1 DNA copy to determine the detection limit.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10 / INC	Neg	10^3
B												
C												10^2
D												
E												10
F												
G												1
H												

Figure 2: Plate setup for analyses of 10 samples in 8 fold. Row 12 contains dilutions series of the positive control

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1											
B	Sample 2											
C	Sample 3											
D	Sample 4											
E	Sample 5											
F	Sample 6											
G	Sample 7 / INC											
H	pos 10^3		pos 10^2		pos 10		pos 1		neg		neg	

Figure 3: Plate setup for analyses of 6 samples in 12 fold. Columns G and H are reserved for the necessary controls.

3. 4 Thermal cycling conditions

Thermal cycling will be performed using a three-step program

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95	5 min	1
Denaturation	95	15 s	50
Annealing	55	15 s	
Extension	72	30 s	

Table 2: Thermal cycling conditions of the *Lepomis gibbosus* detection kit. Fluorogenic data should be collected during the extension step. *Lepomis gibbosus* DNA detection via FAM channel.

3.5 Interpretation of results

If at least one out of an analytical series is positive the whole series should be regarded as positive. This statement is also valid for all controls in this kit. The Limit of detection (LDqPCR) of the analysis can be determined with the lowest dilution series from positive control which can be detected.

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

Table 3: Interpretation of results of the *Lepomis gibbosus* detection kit. Target: detection of *Lepomis gibbosus* – 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.

4. Validation report *Lepomis gibbosus* detection kit

4.2 In silico validation

Table 3: Forward primer *in silico* validation

Length	21
GC %	57
Stability	1.6
T_M (°C)	65
Target region	COI (mtDNA)
Dimer	No
Run	No
Database hit	*Lepomis gibbosus*

Table 4: Reverse primer *in silico* validation

Length (bp)	20
GC %	60
Stability	2.8
T_M (°C)	64
Target region	COI (mtDNA)
Dimer	No
Run	No
Database hit	*Lepomis gibbosus*

Table 5: Probe *in silico* validation

Length	35
GC %	42
T_M (°C)	74
Target region	COI (mtDNA)
Dimer	No
Run	Yes (CCCC)
Fluorescence label	FAM

Table 6: Combined primers and probe *in silico* validation

PCR product size (bp)	117
Combined dimer formation	No
Combined 1000 BLAST® analyses of both primers	*Lepomis gibbosus*
Date of BLAST® analyses	*February 2018*

4.2 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.

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 50.0°C to 70.0°C in eight steps. Optimal temperature and the temperature range in which the test can perform was determined.

Results:

The expected product was formed between 50.0°C and 67.6°C. Nonspecific additional fragments were formed at 47.5°C and 49.7°C. No primer dimer were formed at any tested temperature. The optimal annealing temperature was between 55.5°C and 64.4°C (table 8).

Annealing temp.	**50.0°C**	**51.9°C**	**56.4°C**	**58.0°C**	**61.2°C**	**63.6°C**	**68.1°C**	**70.0°C**
Expected fragment	Weak	Weak	Strong	Strong	Strong	Strong	Strong	Weak
Primer dimer	No	No	No	No	No	No	No	No
Additional fragments	No	No	No	No	No	No	No	No

Table. 8: Temperature gradient PCR on genomic DNA of *Lepomis gibbosus*.

4.2.2 detection limits, fluorescence output signal and efficiency

Standard solutions with 10^4, 10^3, 10^2, 10, 1 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:

Limit of detection (LDqPCR) for this kit was determined at 1 copy per reaction. Limit of quantification (LQqPCR) was determined at 1 copy per reaction. (Fig. 2, table 9).

The fluorescence output signal was at least 100 (10 for positive sample, 0,01 for a negative sample) times stronger than the background signal. (fig. 1, table 8)

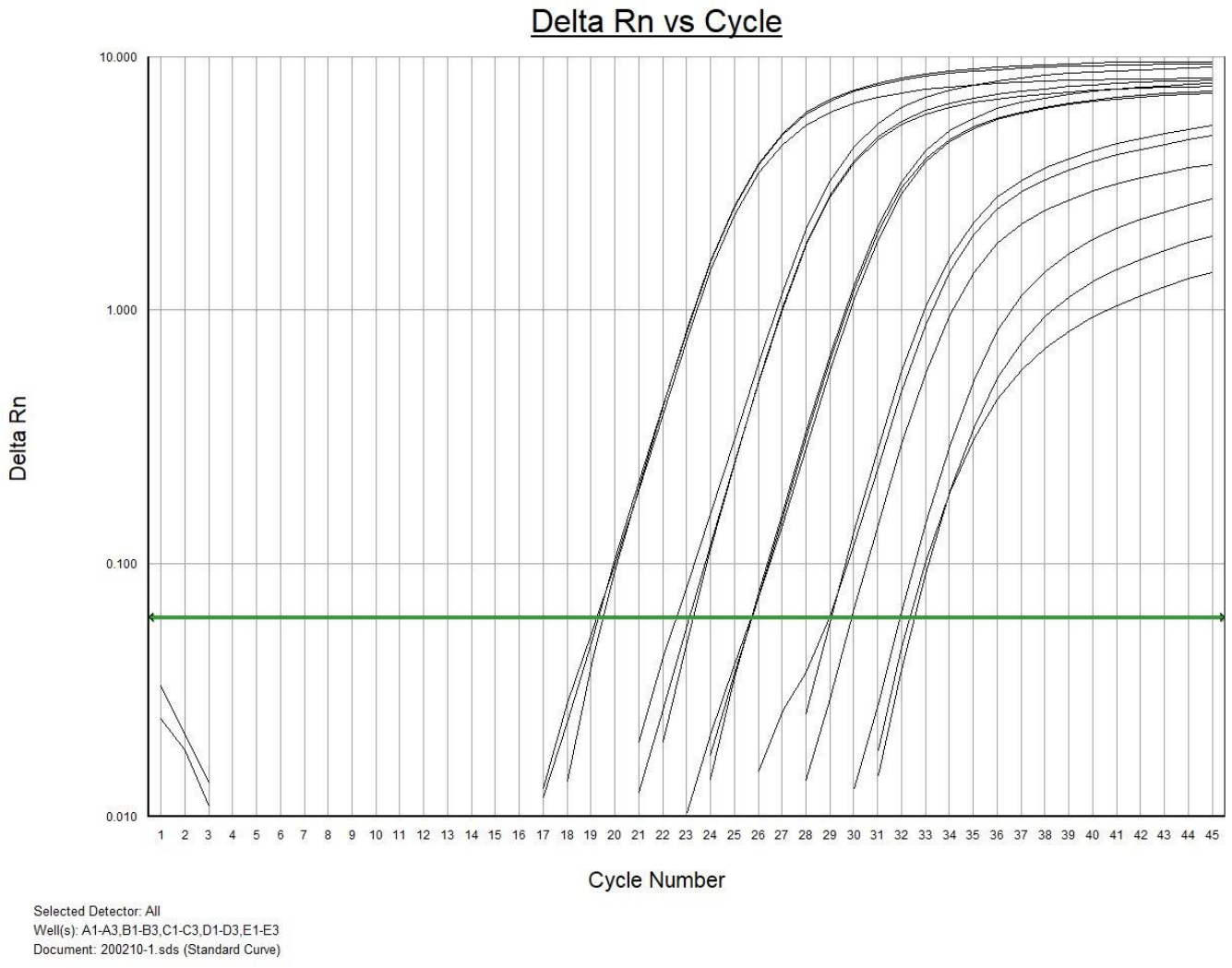

Figure. 1: Obtained fluorescent signal at optimal primer/probe concentration at a annealing temperature of 55°C.

Table 8: CT values obtained at optimal primer/probe concentration.

target DNA[1] copy	Target detected	CT value	Standard deviation
0	No	-	-
1	Yes	32.2	0.3
10	Yes	29.3	0.5
10^2	Yes	25.6	0.0
10^3	Yes	22.9	0.4
10^4	Yes	19.4	0.1

[1] Estimated by gel electrophoresis

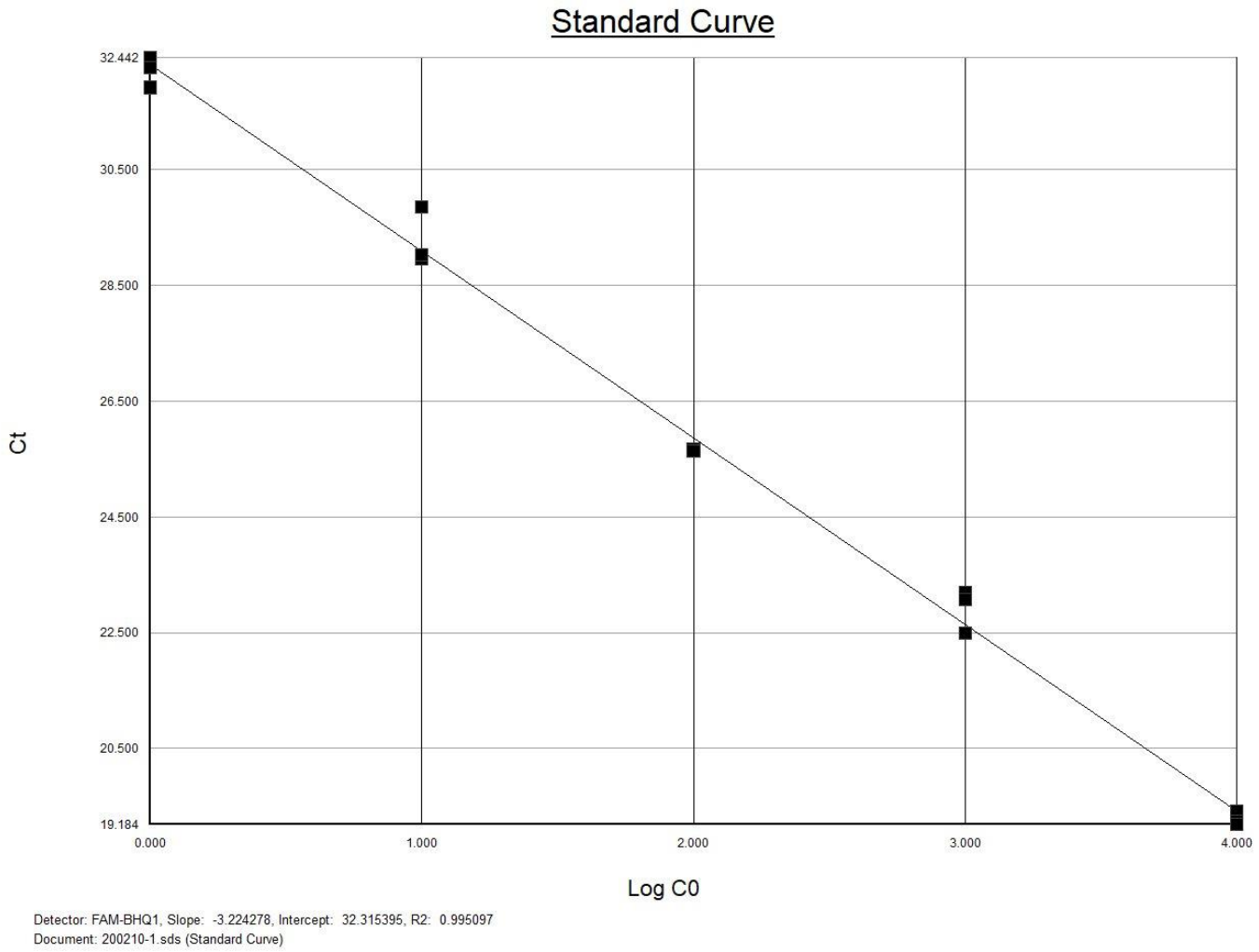

Fig. 2: Standard curve of SYL106 based on 1, 10, 10^2, 10^3 and 10^4 DNA target copies

Table 9: Values obtained from the standard curve

Slope	-3.22
Efficiency	104.4%
R^2	0.995

4.2.3 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 10^4 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 9). There were no statistically significant differences between group means as determined by one-way ANOVA ($F(3,8) = 1.464$, $p = .29$) Influence of chemical and biological factors present in environmental samples was not detected. The standard error of mean was 0.14 with an average of 26.0 for these samples.

Table 9: CT values obtained with target organisms free environmental samples spiked with 1000 target copies.

Environmental sample	CT value	Standard deviation
Sample 1	-	-
Sample 2	-	-
Sample 3	-	-
Sample 1 + spike	26,3	0,2
Sample 2 + spike	26,0	0,4
Sample 3 + spike	26,2	0,6
Spike only	25,6	0,1

4.2.4 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 triplo 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 10)

Table 10: First 3 Blast ® hits obtained from the NCBI database.

Species	Sequence ID	Similarity
Lepomis gibbosus	KY231843.1	100%
Lepomis gibbosus	MF621726.1	100%
Lepomis gibbosus	MF621725.1	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°C – 70.9°C (section 4.2.1, section 4.2.5, fig. 3, table7)
- 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 4.4.2, table 9)
- Fluorescent output signals of positive samples is at least 100 fold stronger than the background

4.3.2 Detection limit

"Lowest limits in which the analysis can be reliably applied"

Limit of detection (LDqPCR) for this kit was determined on 10 copies per reaction. Limit of quantification (LQqPCR) was also determined on 10 copies per reaction. (section 4.2.2, table 8)

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 104%, this means that the primer/probe mixture can be regarded as optimal. (section 4.2.2, table 9)

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 was no statistical differences (p>0.05) between different kinds of environmental water samples spiked with 1000 target DNA copies. (section 4.2.4, table 9)

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 organism *Lepomis gibbosus*
- The method was able to detect all spiked target DNA in target organism free environmental samples (section 4.4.2, table 9)
- The method did not give any signal in target organism free environmental samples (section 4.4.2, table 9)
- The standard error of the mean based on the measured CT values from the spiked experiments (10000 target copies) was 0,14 with an average of 26.0 . (section 4.2.4).