**Validation report:**

# *Cabomba* qPCR detection kit

**with eDNA qPCR hot start mix**

**#SYL164**

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For general laboratory and research use only

# Index

# 1. Validation report *Cabomba* detection kit

## 1.1 In silico validation

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

| | |
|---|---|
| **Length** | 20 |
| **GC %** | 40 |
| **Stability** | 1.6 |
| **$T_M$ (°C)** | 56 |
| **Target region** | Mat K (chloroplast DNA) |
| **Dimer** | No |
| **Run** | No |
| **Database hit** | *Cabomba* |
| | |

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

| | |
|---|---|
| **Length (bp)** | 20 |
| **GC %** | 35 |
| **Stability** | 2.0 |
| **$T_M$ (°C)** | 59 |
| **Target region** | Mat K (chloroplast DNA) |
| **Dimer** | No |
| **Run** | No |
| **Database hit** | *Cabomba* |
| | |

**Table 3: Probe *in silico* validation**

| | |
|---|---|
| **Length** | 38 |
| **GC %** | 31 |
| **$T_M$ (°C)** | 70 |
| **Target region** | Mat K (chloroplast DNA) |
| **Dimer** | No |
| **Run** | No |
| **Fluorescence label** | FAM |

**Table 4: Combined primers and probe *in silico* validation**

| | |
|---|---|
| **PCR product size (bp)** | 154 |
| **Combined dimer formation** | No |
| ***In silico* PCR on Genbank** | *Cabomba aquatic, Cabomba furcate, Cabomba caroliniana,* |
| **Date of *In silico* PCR** | May 2022 |

## 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 Optimal annealing temperature primers

The annealing temperature has a strong influence on the performance, robustness and specificity of the primer set. To determine the optimal annealing temperature, a temperature gradient PCR between 50°C and 72°C was performed in eight steps.

Results:

The expected product was formed between 50.0°C and 65.0°C. No non-specific additional fragments were formed at the temperatures tested. No primer dimers were formed at any temperature tested. The optimum annealing temperature was determined to be 60.0°C (see Table 5).

**Table. 5: Temperature gradient PCR on DNA of *Cabomba.***

| **Annealing temp.** | **50.0°C** | **52.1°C** | **54,4°C** | **57.0°C** | **59.7.°C** | **62.3°C** | **65.0°C** | **69.9°C** | **72.0** |
|---|---|---|---|---|---|---|---|---|---|
| **Expected fragment** | **Strong** | **Strong** | **Strong** | **Strong** | **Strong** | **Strong** | **Strong** | **No** | **No** |
| **Primer dimer** | **No** | **No** | **No** | **No** | **No** | **No** | **No** | **No** | **No** |
| **Additional fragments** | **No** | **No** | **No** | **No** | **No** | **No** | **No** | **No** | **No** |

### 1.2.2 detection limit, fluorescence output signal and efficiency

Standard solutions with $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, 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 100 and >$10^6$ 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. (fig. 1). 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 7).

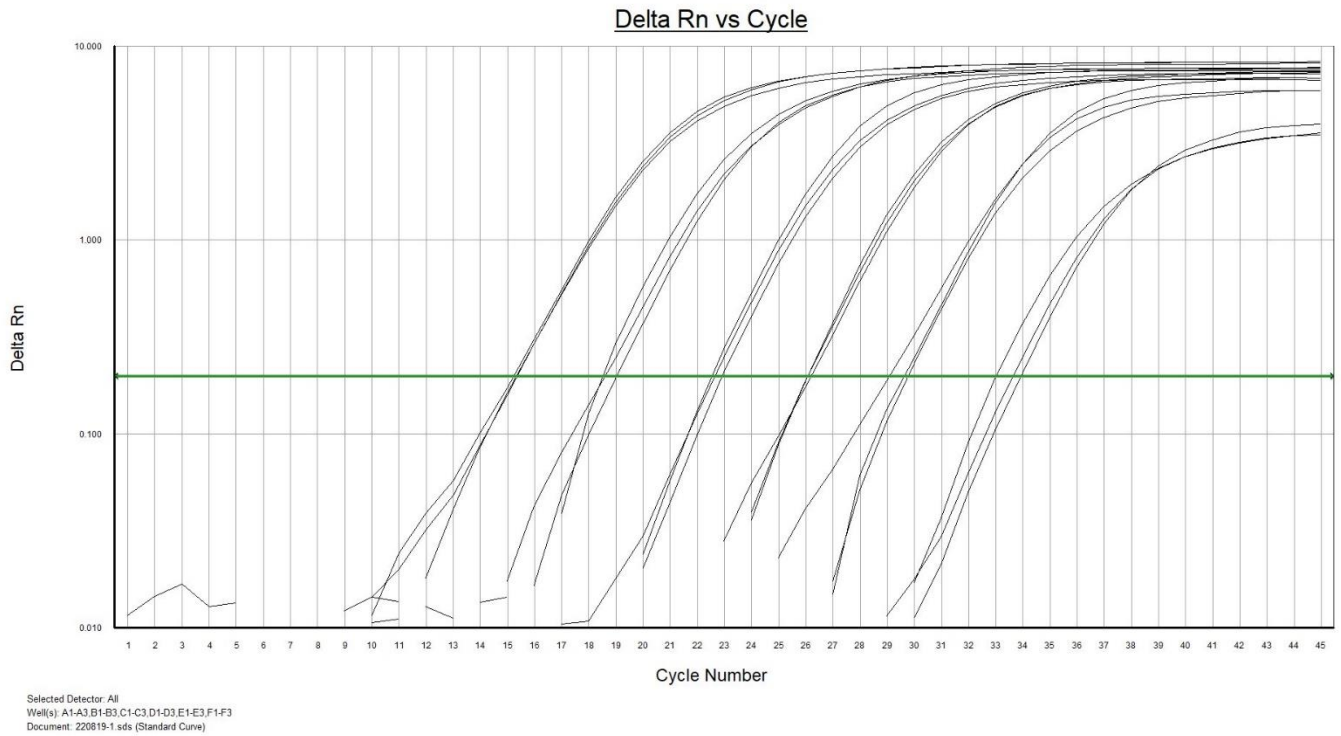


**Figure. 1: Obtained fluorescent signal at optimal primer/probe concentration at a annealing temperature of 60°C.**

**Table 6: CT values obtained at optimal primer/probe concentration.**

| target DNA copy | Target detected | CT value | Standard deviation |
|---|---|---|---|
| 0 | No | n.a. | n.a. |
| 1-10 | Yes | 36.7 | 1.1 |
| $10^2$ | Yes | 30.1 | 0.3 |
| $10^3$ | Yes | 26.7 | 0.1 |
| $10^4$ | Yes | 23.2 | 0.2 |
| $10^5$ | Yes | 19.3 | 0.3 |
| $10^6$ | Yes | 16.0 | 0.1 |

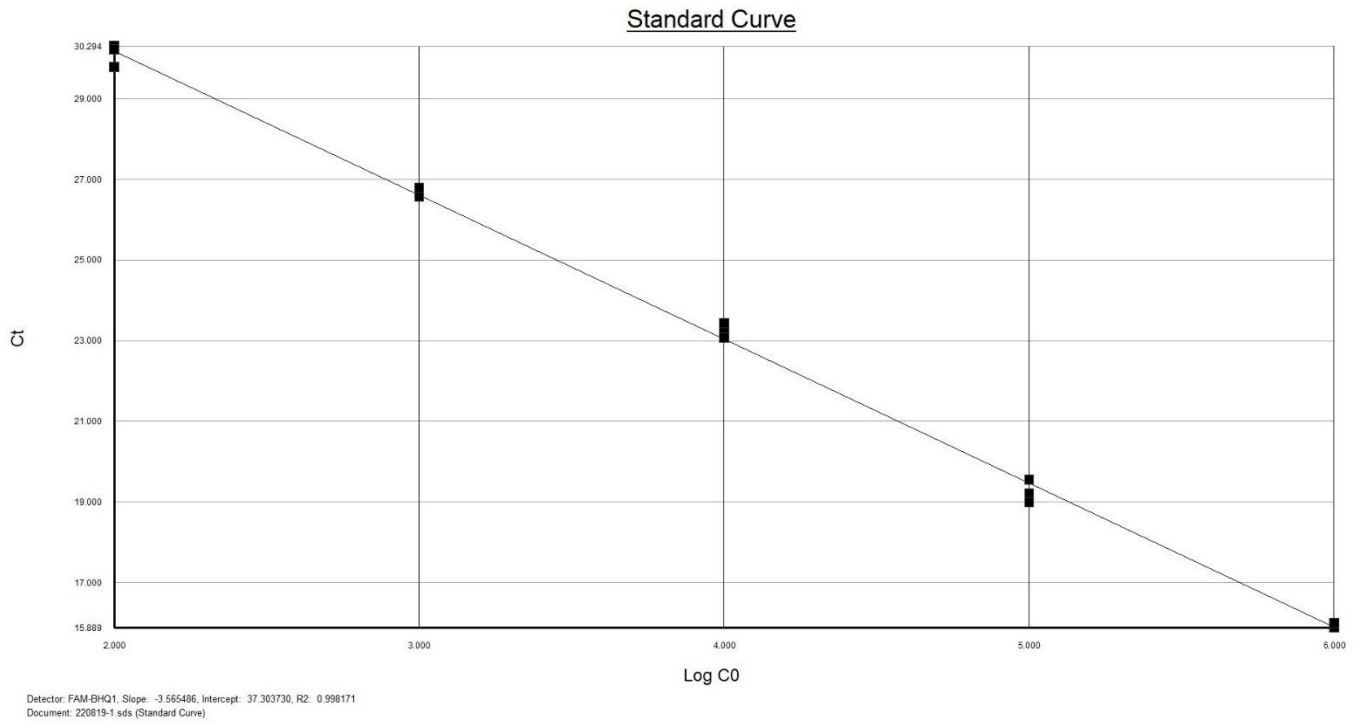


**Fig. 2: Standard curve of SYL164 based on 10, $10^2$, $10^3$, $10^4$, $10^5$ and $10^6$ DNA target copies**

**Table 7: Values obtained from the standard curve**

| | |
|---|---|
| Slope | -3.56 |
| Efficiency | 90.9% |
| $R^2$ | 0.998 |

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

Performance of the assay 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 1000 target DNA copies. The ΔCT between spiked samples and spike only was determined and should be less than 2. Standard deviations 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 8). In all cases the ΔCT was less than 2. The standard error of the mean was 0.07 within these samples with an average of 23.6.

**Table 8: CT values obtained with target organisms free environmental samples spiked with 1000 target copies.**

| Sample | CT value | Standard deviation | ΔCT |
|---|---|---|---|
| Sandy | - | - | - |
| Clayey | - | - | - |
| Peaty | - | - | - |
| Sandy + spike | 23.8 | 0.2 | 0.5 |
| Clayey + spike | 23.8 | 0.1 | 0.5 |
| Peaty + spike | 23.7 | 0.1 | 0.4 |
| Spike only | 23.3 | 0.1 | - |

### 1.2.4 Detection conformation of *Cabomba* in environmental samples

No field validation tests have been conducted yet. These tests are planned for the coming season. We do not foresee any problems with the detection of *Cabomba*. Based on the tank experiments with *Cabomba*, it can be stated that water plants have good detectability.

### 1.2.5 Sequence conformation of specificity.

No field validation tests have been conducted yet. 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.

## 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 – 65°C (section 1.2.1, table 5)
- Influence of chemical and biological factors present in environmental samples on the performance of the test were not found. (section 1.2.3, table 8)
- 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 this kit was determined on 1 copy per reaction. Limit of quantification (LOQ) was also determined on 10 copies per reaction. (section 1.2.2, table 6)

### 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 90.9 %, this means that the primer/probe mixture can be regarded as optimal. (section 1.2.2, table 7)

### 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 difference (ΔCT <2) between different kinds of environmental water samples (sandy, clayey or peaty bottoms) spiked with 1000 target DNA copies. (section 1.2.3, table 8)

### 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 organism *Cabomba*
- The method was able to detect all spiked target DNA in target organism free environmental samples (section 1.2.3, table 8)
- The method did not give any signal in target organism free environmental samples (section 1.2.3, table 8)