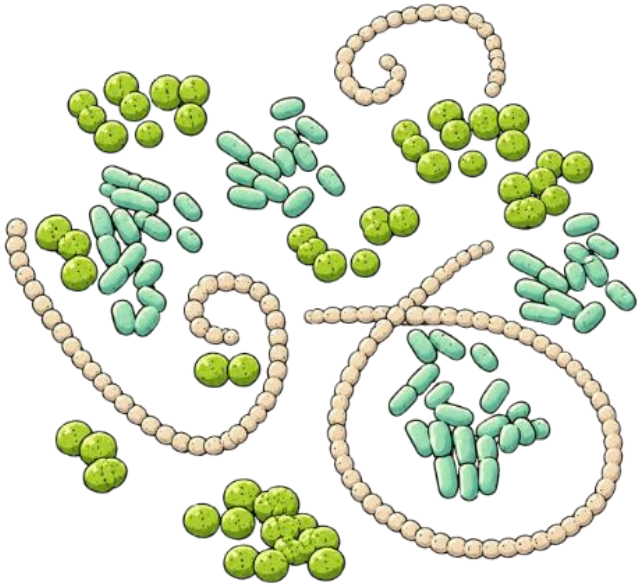
VALIDATION

Anatoxin gene cluster qPCR detection kit

SYL122

Document date: 28 August 2026



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1. *In silico* validation

Table 1. *In silico* validation of forward primers

Parameter	Forward primer 1	Forward primer 2	Forward primer 3
Length (bp)	18	23	24
GC %	61	43	37
Stability	2,1	1,0	1,5
T_m (°C)	67	64	63
Target region	AnaF (DNA)	AnaF (DNA)	AnaF (DNA)
Dimer formation	No	No	No
Nucleotide runs	No	No	No

Table 2. *In silico* validation of reverse primers

Parameter	Reverse primer 1	Reverse primer 2
Length (bp)	23	27
GC %	47	Average of 33 (2 variants)
Stability	2,2	64
T_m (°C)	62	
Target region	AnaF (DNA)	AnaF (DNA)
Dimer formation	No	No
Nucleotide runs	No	No

*Probe labelled with FAM.

Table 3. *In silico* validation of probes

Parameter	Probe 1*	Probe 2*
Length (bp)	32	31
GC %	52	Average of 43 (2 variants)
T_m (°C)	70	Unknown
Target region	AnaF (DNA)	AnaF (DNA)
Dimer formation	No	No
Nucleotide runs	No	No

*Probes labelled with FAM.

Table 4. *In silico* validation primers and probe combination

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PCR product size (bp)	105
Combined dimer formation	No
***In silico* PCR on Genbank**	*Anatoxin gene cluster*
Date of in silico PCR	November 2018

Conclusion:

The *in silico* analysis identified Cyanobacteria exclusively, with no predicted amplification of non-target taxa.

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2. Experimental validation

All validation experiments were conducted using the Sylphium qPCR mix and analyzed with the Applied biosystems 7300 qPCR instrument.

2.1. Analytical sensitivity, fluorescence output signal and efficiency

Standard solutions containing nominal target concentrations of 10^6, 10^5, 10^4, 10^3, 10^2, 10, and 0 target copies per reaction were analyzed at the optimal primer and probe concentrations. Fluorescence output was compared with the background signal, with a minimum 100-fold increase in fluorescence considered acceptable. Reaction conditions were optimized when fluorescence signals were insufficiently distinguishable from background noise.

The amplification efficiency of the primer and probe set was calculated from the slope of the standard curve and was considered acceptable when between 90% and 110%.

Results

Positive amplification was observed down to the lowest tested nominal concentration of **10 target copies per reaction**, demonstrating single-copy detection capability under the tested conditions (**Figure 1; Table 5**). The fluorescence output of positive reactions exceeded the predefined criterion of a 100-fold increase relative to the background signal.

The amplification efficiency of the assay was **107%**, indicating that the primer and probe set performed within the predefined acceptable efficiency range of 90-110% (**Figure 2; Table 6**).

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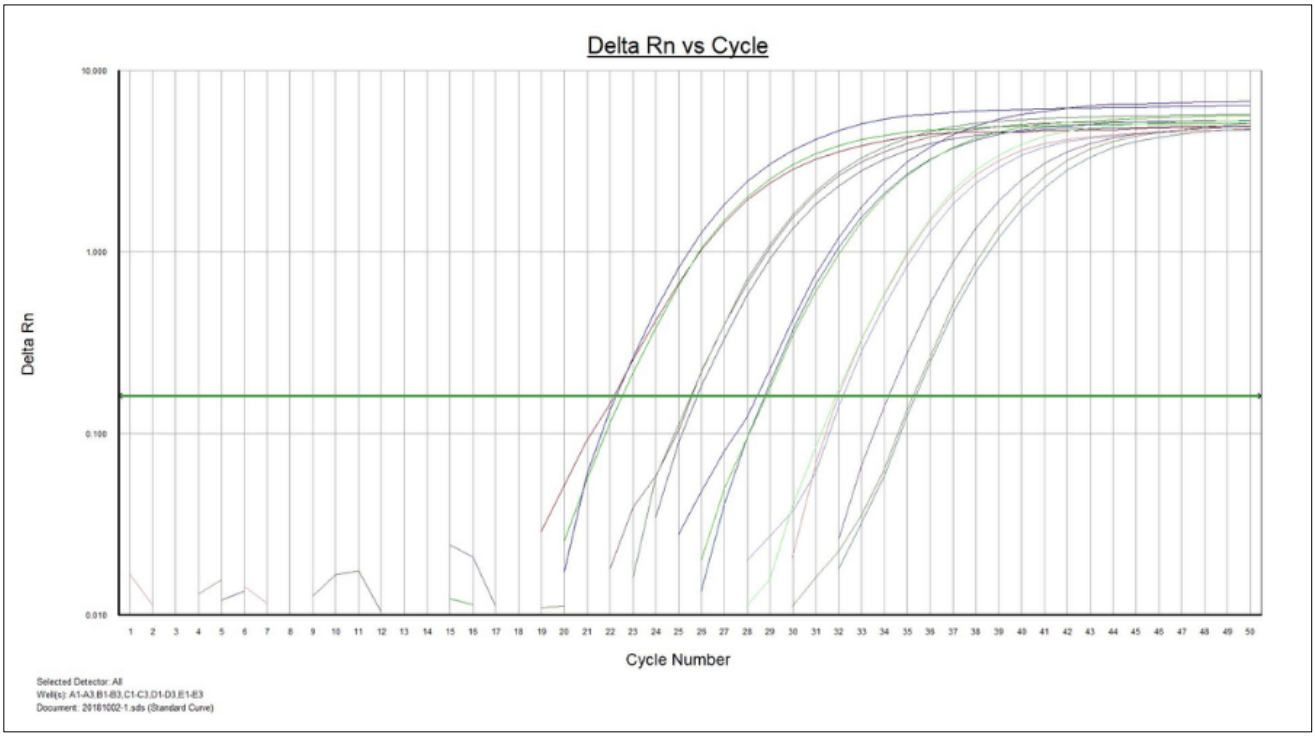


Figure 1. Amplification curves obtained at the optimal primer and probe concentrations and an annealing temperature of 57°C.

Table 5. Detection results and Cq values obtained at the optimal primer and probe concentrations and an annealing temperature of 57°C

Nominal target copies per reaction*	Target detected	Mean Cq	SD
0	No	-	-
10	Yes	n.d.	n.d.
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

n.d.: not determined, very low amounts of DNA could not be quantitively detected in a reliable manner with the used qPCR instrument.
***As estimated by gel electrophoresis.

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Applied biosystems 7300 qPCR

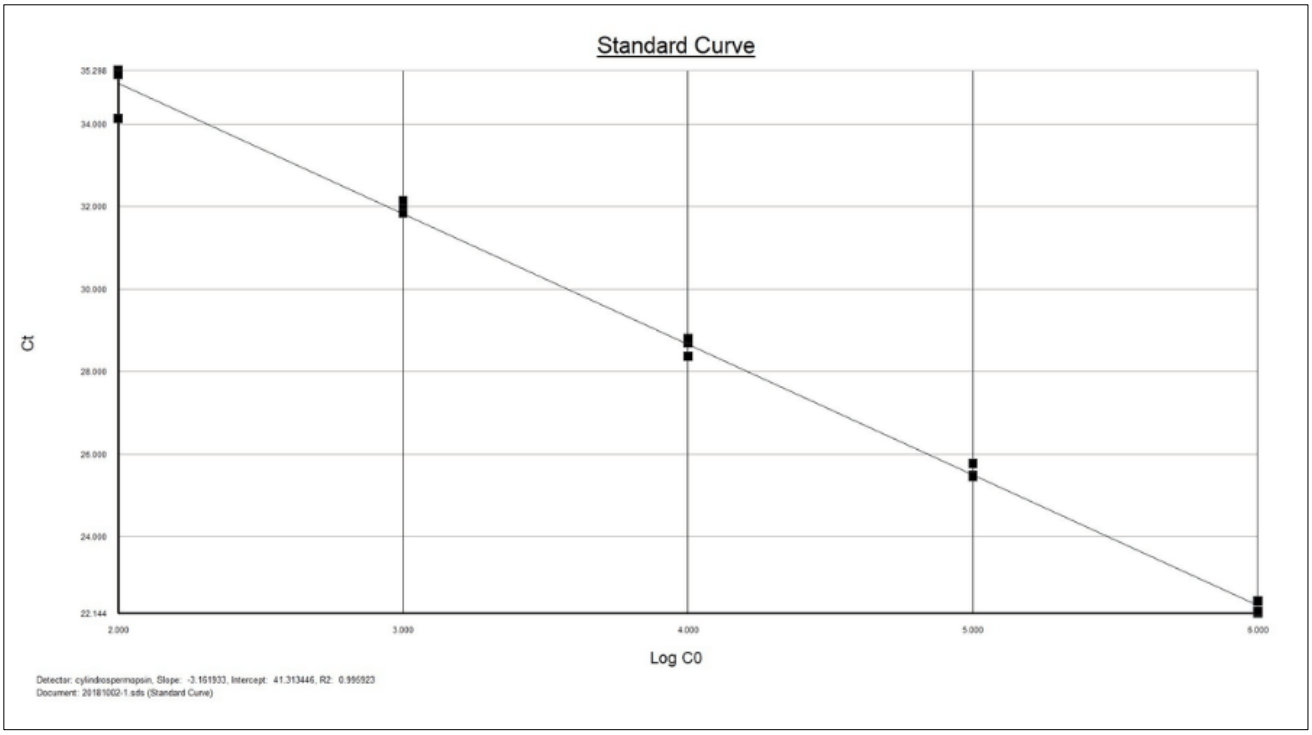


Figure 2. Standard curve generated from nominal target concentrations of 10^2, 10^3, 10^4, 10^5, and 10^6 copies per reaction.

Table 6. Standard curve parameters obtained with the *Anatoxin gene cluster* qPCR detection kit

Parameter	Result
Slope	-3.16
Amplification efficiency (%)	107
R^2	0.994

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