Validation report:

Aeshna viridis qPCR detection kit

with eTaq qPCR master mix

#SYL130

Document date: 12 May 2022

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1. Validation report *Aeshna viridis* detection kit

1.1 In silico validation

Table 1: Forward primer *in silico* validation

Length	30
GC %	36
Stability	1.1
T_M (°C)	63
Target region	COI (mtDNA)
Dimer	No
Run	No
Database hit	*Aeshna viridis*

Table 2: Reverse primer *in silico* validation

Length (bp)	24
GC %	33
Stability	2.0
T_M (°C)	55
Target region	COI (mtDNA)
Dimer	No
Run	No
Database hit	*Aeshna viridis*

Table 3: Probe *in silico* validation

Length	43
GC %	25
T_M (°C)	70
Target region	COI (mtDNA)
Dimer	No
Run	No
Fluorescence label	FAM

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

PCR product size (bp)	138
Combined dimer formation	No
In silico PCR on Genbank	*Aeshna viridis, Phimodera lapponica*
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 Gradient PCR

Influence of the annealing temperature on the performance and specificity of the primer set was tested with a temperature gradient between 50.9°C to 72.0°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 62.3°C. No nonspecific additional fragments were formed. No primer dimer was formed at any tested temperature. The optimal annealing temperature was between 50.0°C and 59.7°C (table 8).

Table. 8: Temperature gradient PCR on genomic DNA of *Aeshna viridis*.

Annealing temp.	**50.0°C**	**52.1°C**	**54.4°C**	**57.0°C**	**59.7°C**	**62.3°C**	**65°C**	**67.6°C**	**69.9°C**	**72°C**
Expected fragment	Strong	Strong	Strong	Strong	Strong	weak	No	No	No	No
Primer dimer	No	No	No	No	No	No	No	No	No	No
Additional fragments	No	No	No	No	No	No	No	No	No	No

1.2.2 detection limits, fluorescence output signal and efficiency

Standard solutions with 10^5, 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 10 copy per reaction. (Fig. 5, table 9).

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. 4, table 8)

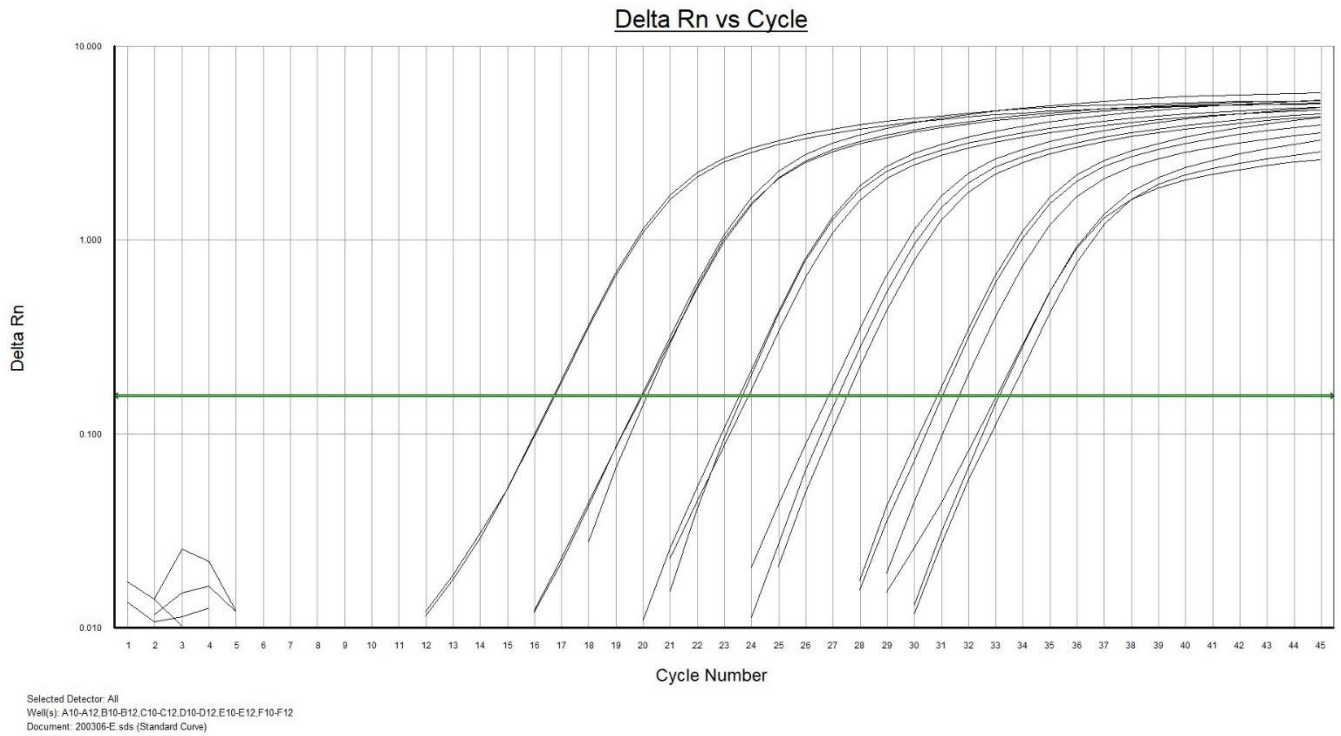

Figure. 4: Obtained fluorescent signal at optimal primer/probe concentration at an annealing temperature of 58°C.

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

target DNA[1] copy	Target detected	CT value	Standard deviation
0	No	-	-
1	Yes	33.2	0.2
10	Yes	31.1	0.4
10^2	Yes	27.1	0.3
10^3	Yes	23.6	0.2
10^4	Yes	20.0	0.1
10^5	Yes	16.7	0.0

[1] Estimated by gel electrophoresis

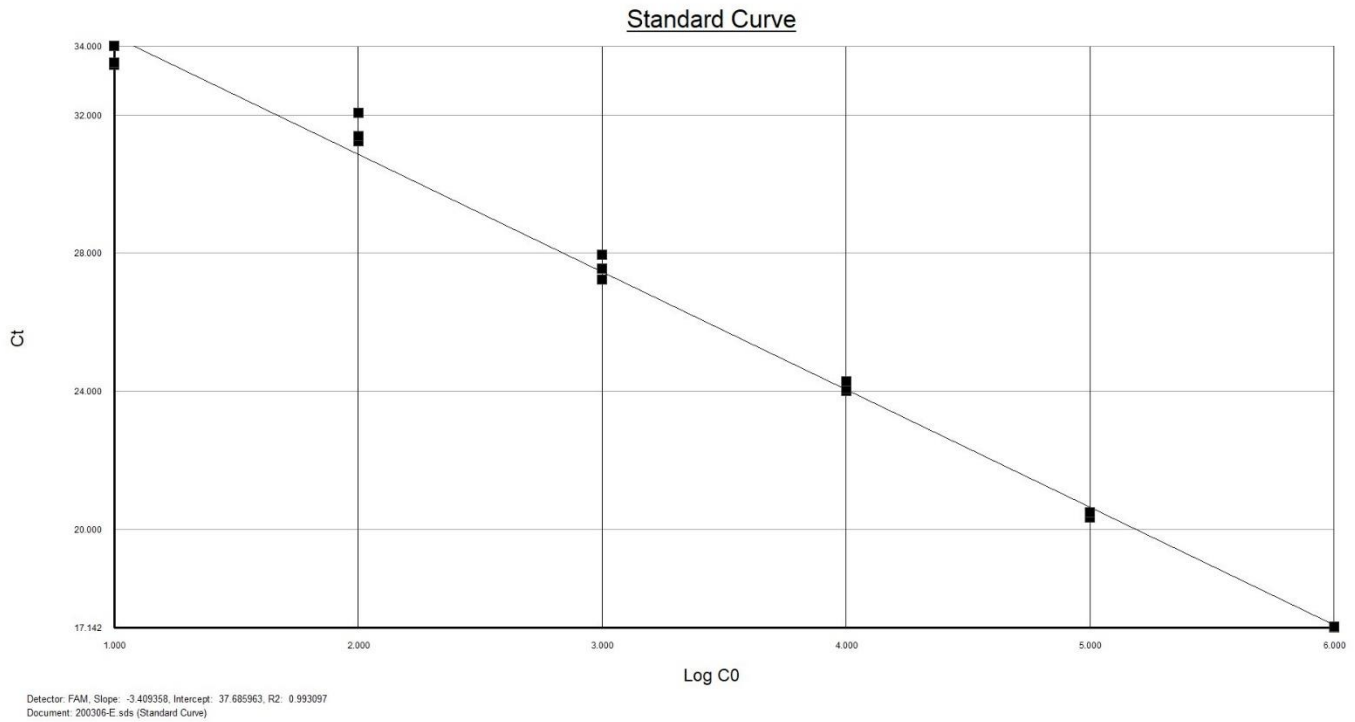

Fig. 5: Standard curve of SYL130 based on 1, 10, 10^2, 10^3, 10^4 and 10^5 DNA target copies

Table 9: Values obtained from the standard curve

Slope	-3.41
Efficiency	96.5%
R^2	0.993

1.2.3 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 100 target DNA copies. Standard deviations, difference to spike 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 11). The difference between environmental samples and the spike only sample was less than 3%. Influence of chemical and biological factors present in environmental samples was not detected. The standard error of mean was 0.05 within these samples with an average of 28.9. There were no statistical differences (p = 0.5, ANOVA) between environmental samples.

Sample	CT value	Standard deviation	Difference to spike
Sandy	-	-	-
Clayey	-	-	-
Peaty	-	-	-
Sandy + spike	28.9	0.1	0.6 %
Clayey + spike	28.9	0.1	0.6%
Peaty + spike	28.8	0.1	0.07 %
Spike only	28.8	0.3	-

Table 11: CT values obtained with target organisms free environmental samples spiked with 100 target copies.

1.2.4 Detection conformation in environmental samples

Conformation of detection with the test was performed on samples form locations where the presence of *Aeshna viridis* was suspected. Samples were taken using SYL001 - Environmental sampling kit and eDNA was isolated using SYL002 - Environmental DNA isolation kit.

Results:
See appendix A (in Dutch).

1.2.5 Sequence conformation of specificity.

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

Results:
Target organism free environmental samples did not give any PCR product. PCR products obtained from environmental samples were sequenced in triple via the Sanger sequencing method. The obtained sequences were identical to each other and Blast ® hits confirmed identity of the target organism.

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: 50.0°C - 59.7°C (section 1.2.1, section 1.2.2, table 8, table 9)
- 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 1.2.2, table 11)
- Fluorescent output signals of positive samples are at least 100-fold stronger than the background

1.3.2 Detection limits

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

Limit of detection (LDqPCR) for this kit was determined on 1 copy per reaction. Limit of quantification (LQqPCR) was also determined on 1 copy per reaction. (section 1.2.2, table 8)

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 96.5%, this means that the primer/probe mixture can be regarded as optimal. (section 1.2.2, table 9)

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 were no statistical differences (p>0.05) between different kinds of environmental water samples (sandy, clayey or peaty bottoms) spiked with 100 target DNA copies. (section 1.2.3, table 11)

1.3.6 Correctness

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

- The test was able to detect *Aeshna viridis* DNA in environmental samples from locations were the presence of *Aeshna viridis* was suspected. (section 4.2.6)
- The method did not give any other combined BLAST hit than the target organism *Aeshna viridis*
- The method was able to detect all spiked target DNA in target organism free environmental samples (section 4.2.3, table 10)
- The method did not give any signal in target organism free environmental samples (section 4.2.3, table 11)
- The standard error of the mean based on the measured CT values from the spiked experiments (100 target copies) was 0.05 on an average of 28.9.

Appendix A

Analyserapport

Opdrachtgever	Sylphium, validatiemethode
Contact persoon	N.v.t.
Aantal Monsters	2 sterivex filters
Aan te tonen organisme(s)	Groene glazenmaker
Datum rapport	13-05-2020
Uitgevoerd door	Jan Warmink

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Monstercode	Locatie	Volume
E2444	Dalweg, Wildervank, nl	350 ml gefiltreerd
E2445	Dalweg, Wildervank, nl	350 ml gefiltreerd

Tabel 1: Aangeleverde monsters.

Uitvoering en kwaliteitswaarborging:
De analyses van de monsters zijn in achtvoud uitgevoerd. Een monster wordt positief bevonden als minimaal één van deze analyses een positief signaal geeft. Als controles werden gebruikt:

- Rendement en inhibitiecontrole (RIC): Aan de monsters is xenobiotisch-DNA toegevoegd als controle. Deze controle sluit vals negatieve PCR-resultaten uit, die veroorzaakt worden door storende factoren in het DNA-isolaat. Tevens bepaald deze controle de isolatie-efficiëntie van de uitgevoerde procedure. Bij het aantreffen van storende factoren wordt het experiment herhaald bij een monsterverdunning van 2x, 4x en 8x. Op basis van deze resultaten wordt besloten op welke verdunning de doelsoortanalyse wordt uitgevoerd.
- Procedure blanco: Alleen conserveringsmiddel dat alle isolatie en analyse stappen doorloopt. Deze controle toont eventuele contaminatie met DNA tijdens de handelingen aan.
- PCR positieve controle: Doelsoort DNA toegevoegd aan PCR mix. Deze controle sluit vals negatieve PCR-resultaten uit, door fouten in het PCR-proces.
- PCR negatieve controle: Geen monster of DNA toegevoegd. Dit is een extra controle op vals positieve resultaten door contaminatie.

Resultaten:

De aangeleverde monsters zijn positief bevonden voor de aanwezigheid van groene glazenmaker DNA (tabel 2). De positieve controles blijken in alle gevallen een positief resultaat te geven. De negatieve controles geven in alle gevallen een negatief resultaat.

Monstercode	Resultaat doelsoort	Procedure blanco	Inhibitie controle	PCR negatieve controle	PCR positieve controle
E2444	2/8	Ok	Ok	Ok	Ok
E2445	2/8	Ok	Ok	Ok	Ok

Tabel 2: PCR-resultaten analyse monsters.

Conclusie:

De aangeleverde monsters zijn positief bevonden voor de aanwezigheid van groene glazenmaker DNA. Alle positieve controles gaven een positief resultaat en alle negatieve controles gaven een negatief resultaat. Deze controles geven aan dat er geen storende factoren of DNA-contaminaties van de doelsoort aanwezig waren. Vals positieve resultaten kunnen hiermee uitgesloten worden.