Validation report:

Mesotriton alpestris qPCR detection kit

with eDNA qPCR hot start mix

#SYL166

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For general laboratory and research use only

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1. Validation report *Mesotriton alpestris* detection kit

1.1 In silico validation

Table 1: Forward primer *in silico* validation

Length	23
GC %	52
Stability	1.6
T_M (°C)	64
Target region	CytB (mtDNA)
Dimer	No
Run	No
Database hit	*Mesotriton alpestris*

Table 2: Reverse primer *in silico* validation

Length (bp)	20
GC %	60
Stability	2.5
T_M (°C)	59
Target region	CytB (mtDNA)
Dimer	No
Run	No
Database hit	*Mesotriton alpestris*

Table 3: Probe *in silico* validation

Length	29
GC %	44
T_M (°C)	70
Target region	CytB (mtDNA)
Dimer	No
Run	No
Fluorescence label	FAM

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

PCR product size (bp)	116
Combined dimer formation	No
***In silico* PCR on Genbank**	*Mesotriton alpestris*
Date of *In silico* PCR	June 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 72.0°C. Weak non -specific additional fragments were formed at all 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 *Mesotriton alpestris.*

Annealing temp.	**50.0°C**	**50.7**	**52.1**	**54.4**	**57.0**	**59.7**	**62.3**	**65.0**	**67.6**	**69.9**	**71.3**	**72**
Expected fragment	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**Weak**	**Weak**	**Weak**
Primer dimer	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**
Additional fragments	**Weak**	**Weak**	**Weak**	**Weak**	**Weak**	**Weak**	**Weak**	**Weak**	**Weak**	**Weak**	**Weak**	**Weak**

1.2.2 detection limit, fluorescence output signal and efficiency

Standard solutions with 10^8, 10^7, 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 >10^8 target copies per reaction. The detection limits (low and high) for quantitative detection was determined between 10 and >10^8 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).

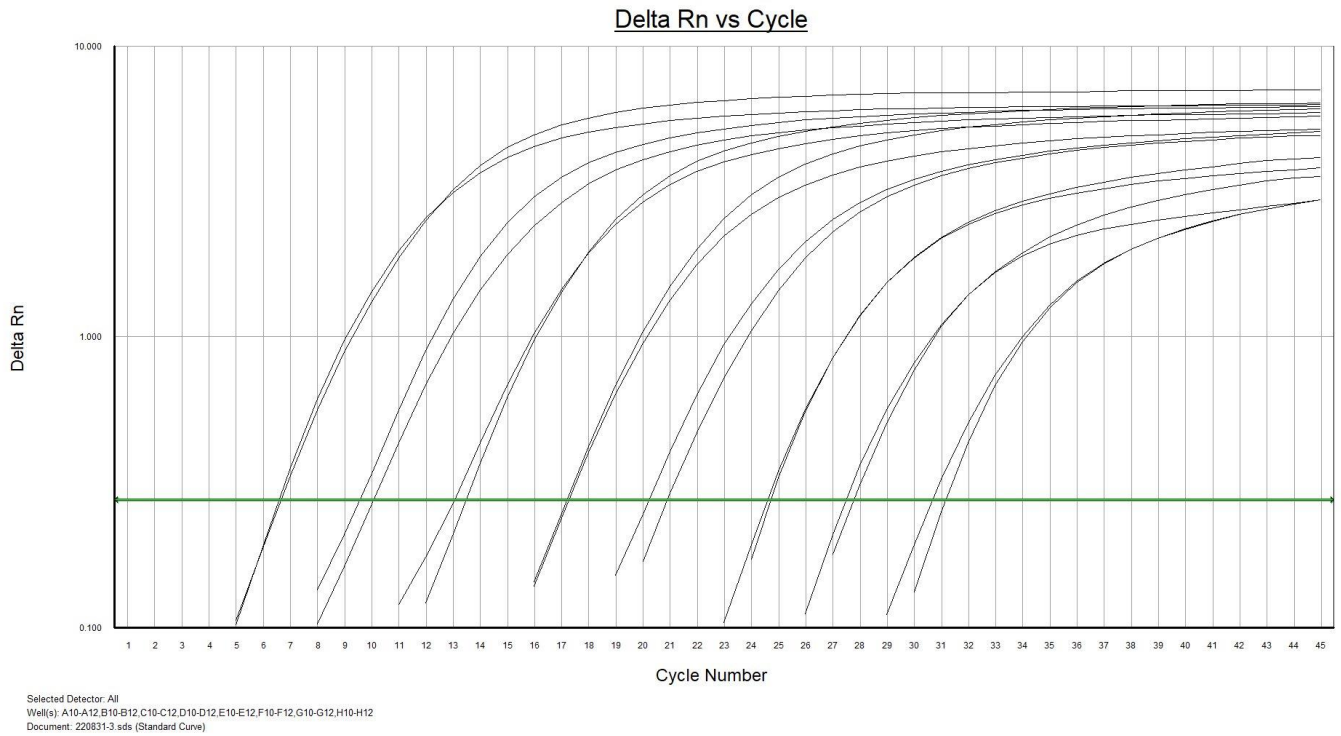

Figure. 1: Obtained fluorescent signal at optimal primer/probe concentration at a annealing temperature of 60°C.

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

target DNA copy	Target detected	CT value	Standard deviation
0	No	-	-
10	Yes	30.9	0.4
10^2	Yes	27.6	0.2
10^3	Yes	24.6	0.1
10^4	Yes	20.5	0.5
10^5	Yes	17.2	0.0
10^6	Yes	13.2	0.3
10^7	Yes	9.8	0.4
10^8	Yes	6.6	0.1

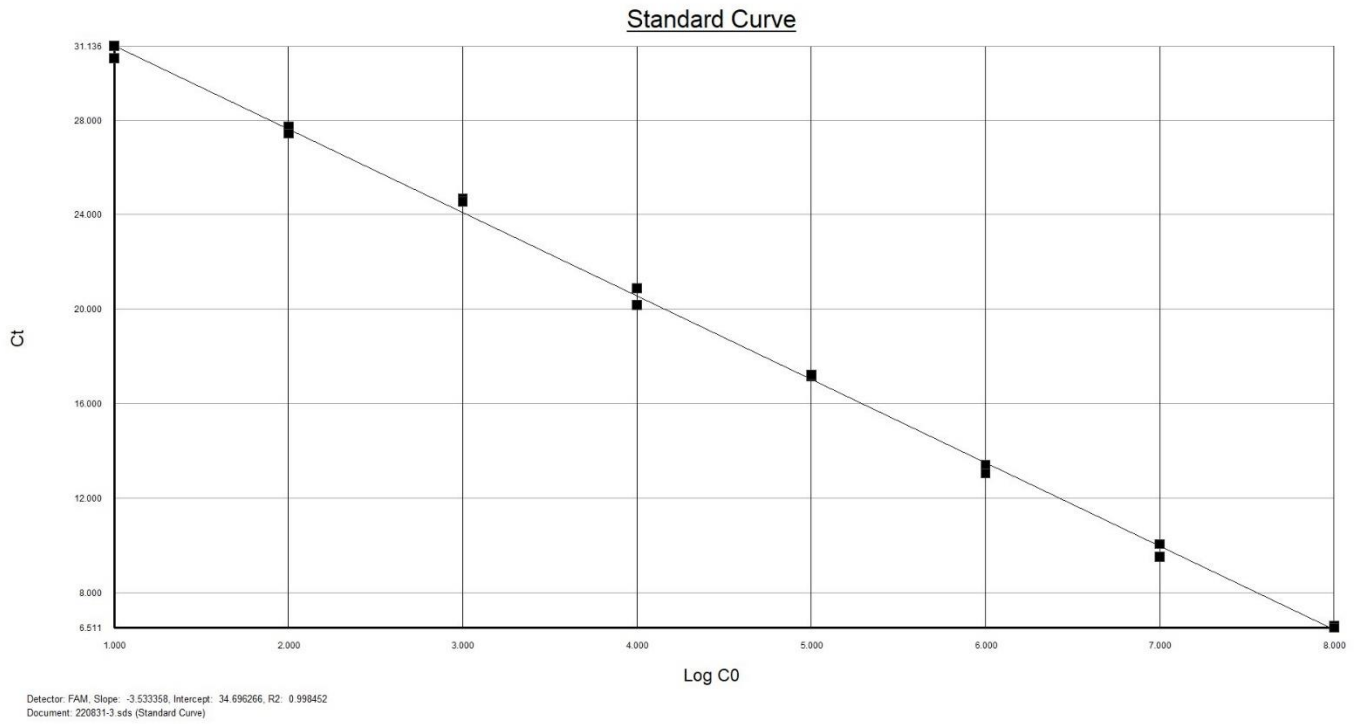

Fig. 2: Standard curve of SYL166 based on 10, 10^2, 10^3, 10^4, 10^5, 10^6,10^7 and 10^8 DNA target copies

Table 7: Values obtained from the standard curve

Slope	-3.53
Efficiency	92.0%
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 based on the measured CT values from the spiked experiments (1000 target copies) was 0.02 on an average of 21.7.

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	21.8	0.2	0.1
Clayey + spike	21.5	0.1	0.4
Peaty + spike	21.8	0.2	0.1
Spike only	21.9	0.1	-

1.2.4 Detection conformation of *Mesotriton alpestris* in environmental samples

The amount of eDNA that a target organism leaves in the environment depends on the type of target organism, environmental factors and season (CEN / TC230-water analysis, proposal documented in N 1229). To determine the minimal filtration volume, samples were taken at locations where *Mesotriton alpestris* may occur, during the optimal seasons (spring, summer and autumn). Samples were taken with SYL009 - Environmental Sampling Kit and eDNA was isolated with SYL002 - Environmental DNA Isolation Kit.

Results:
The kit SYL166 - *Mesotriton alpestris* detection kit was able to detect *Mesotriton alpestris* in environmental samples from different location in the Netherlands. Positive samples gave 1 to 500 molecules *Mesotriton alpestris* DNA when 750 ml water was filtrated.

1.2.5 Sequence conformation of specificity.

PCR products obtained from the environmental samples where the presence of *Mesotriton alpestris* was suspected were sequenced for conformation of identity of the formed product. 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. PCR products obtained from environmental samples were sequenced in triplo 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°C – 67°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 92.0 %, 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 10000 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 test was able to detect *Mesotriton alpestris* DNA in environmental samples from locations were the presence of *Mesotriton alpestris* was suspected. (section 1.2.5)
- The method did not give any other combined BLAST hit than the target organism *Mesotriton alpestris*
- 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)
- The standard error of the mean based on the measured CT values from the spiked experiments (1000 target copies) was 0.02 on an average of 21.7.