VALIDATION

# *Mesotriton alpestris* qPCR detection kit

## SYL166

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Sylphium
molecular ecology

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## 1. *In silico* validation

**Table 1**. *In silico* validation of primers and probe

| Parameter | Forward primer | Reverse primer | Probe* |
|---|---|---|---|
| **Length (bp)** | 23 | 20 | 29 |
| **GC %** | 52 | 60 | 44 |
| **Stability** | 1.6 | 2.5 | - |
| **$T_m$ (°C)** | 64 | 59 | 70 |
| **Target region** | CytB (mtDNA) | CytB (mtDNA) | CytB (mtDNA) |
| **Dimer formation** | No | No | No |
| **Nucleotide runs** | No | No | No |

*Probe labelled with FAM.

**Table 2**. *In silico* validation primers and probe combination

| | |
|---|---|
| **PCR product size (bp)** | 116 |
| **Combined dimer formation** | No |
| ***In silico* PCR on Genbank** | *Mesotriton alpestris* |
| **Date of in silico PCR** | June 2022 |

*Conclusion:*

The *in silico* analysis identified the target organism 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 $10^8$, $10^7$, $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, 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 **1-10 target copies per reaction**, demonstrating single-copy detection capability under the tested conditions (**Figure 1; Table 3**). 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 **92%**, indicating that the primer and probe set performed within the predefined acceptable efficiency range of 90-110% (**Figure 2; Table 4**).


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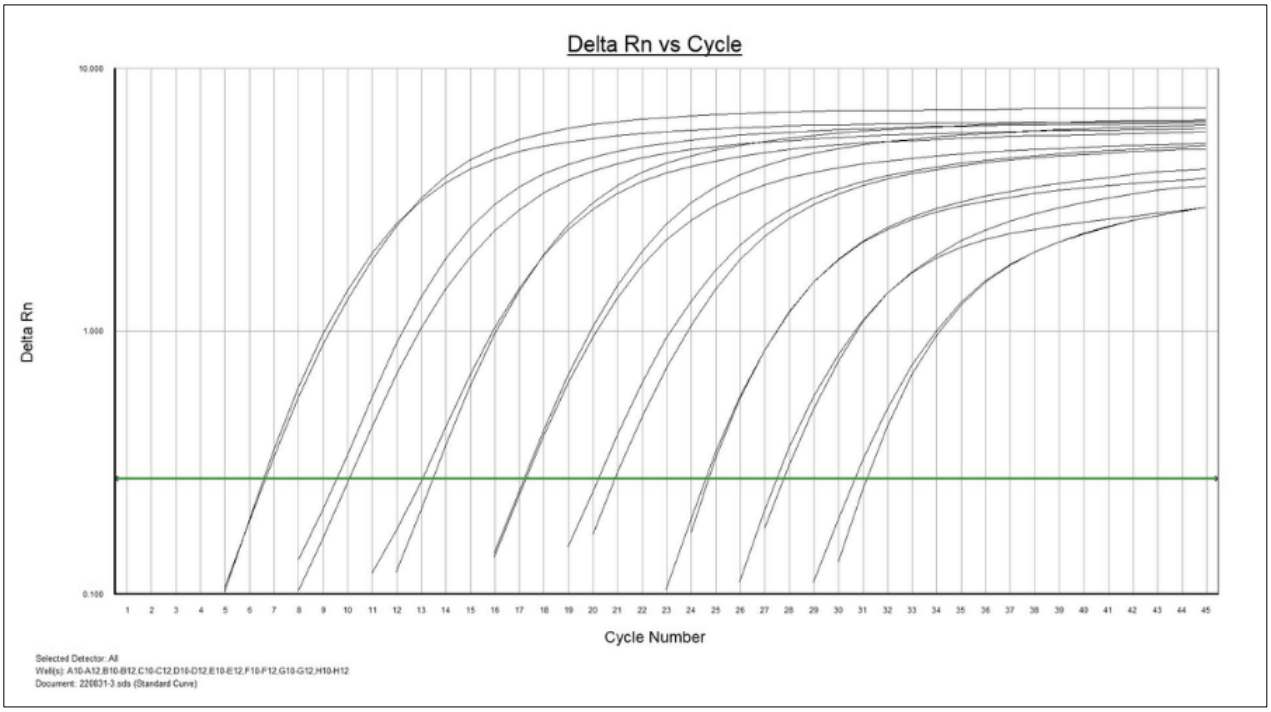



**Figure 1**. Amplification curves obtained at the optimal primer and probe concentrations and an annealing temperature of 60°C.

**Table 3**. Detection results and Cq values obtained at the optimal primer and probe concentrations and an annealing temperature of 60°C

| Nominal target copies per reaction | Target detected | Mean Cq | SD |
|---|---|---|---|
| 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 |


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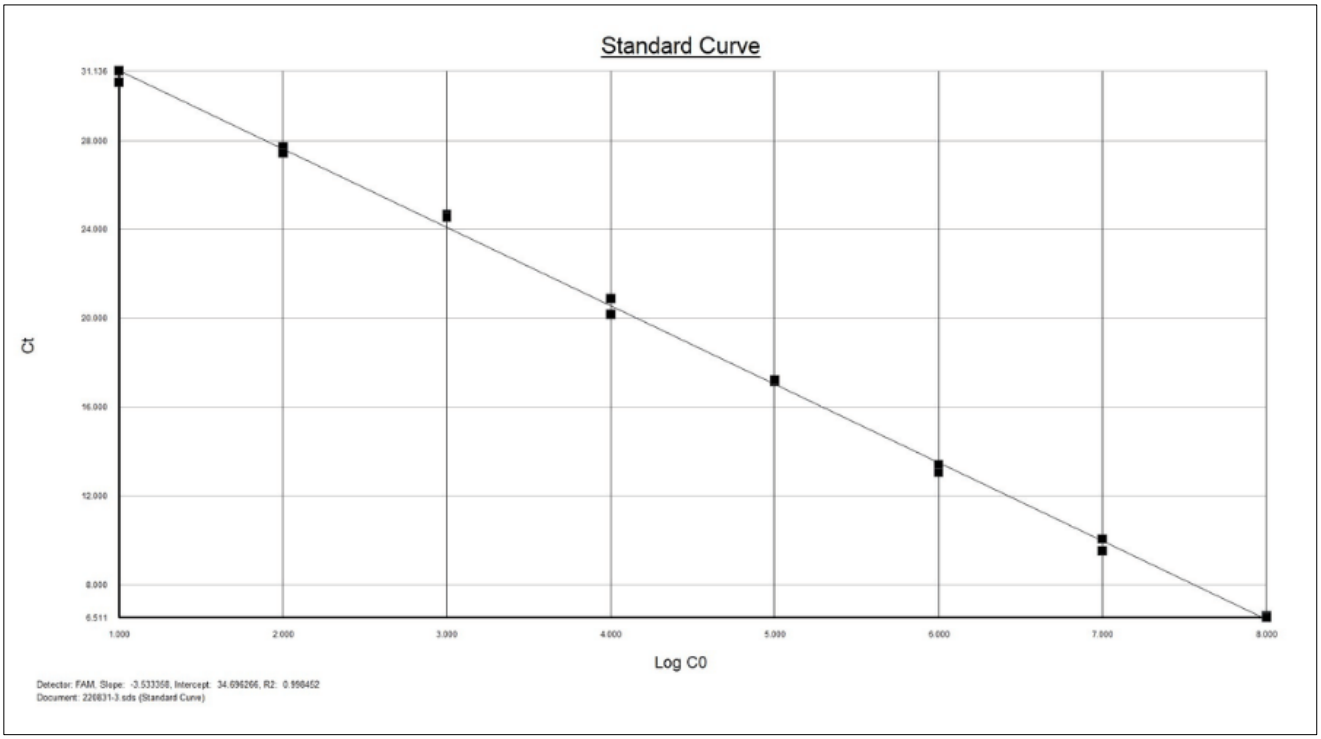



**Figure 2**. Standard curve generated from nominal target concentrations of 10, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$ and $10^8$ DNA copies per reaction.

**Table 4**. Standard curve parameters obtained with the *Mesotriton alpestris* qPCR detection kit

| Parameter | Result |
|---|---|
| **Slope** | -3.53 |
| **Amplification efficiency (%)** | 92 |
| **$R^2$** | 0.998 |


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## 2.2. Detection confirmation of *Mesotriton alpestris* in environmental samples

The amount of eDNA released by a target organism into the environment depends on the target species, the environmental conditions, and season (CEN / TC230-water analysis, proposal documented in N 1229). Samples were collected with the **SYL009 Complete eDNA sampling set** and eDNA was isolated using the **SYL002 Environmental DNA isolation kit**.

*Results:*

The kit SYL166 - *Mesotriton alpestris* detection kit successfully detected *Mesotriton alpestris* DNA in environmental samples from different locations in the Netherlands when 750 mL water was filtered.

PCR products obtained from positive environmental samples were analyzed by Sanger sequencing. The resulting sequences were identical, and BLAST analysis confirmed that the amplified products originated from *Mesotriton alpestris*. No amplification was observed in the tested target-organism-free environmental samples.


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