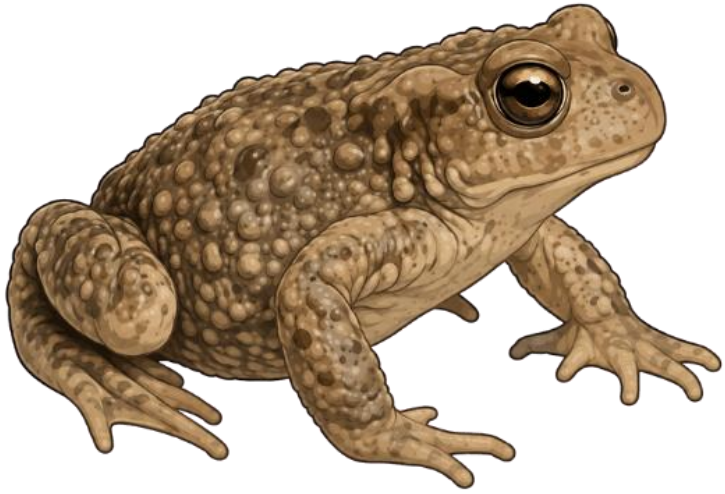
VALIDATION

# *Alytes obstetricans* qPCR detection kit

## SYL153

Document date: 13 November 2026



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 | 21 | 29 |
| **GC %** | 43 | 57 | 48 |
| **Stability** | 2.1 | 1.9 | - |
| **$T_m$ (°C)** | 61 | 63 | 71 |
| **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)** | 98 |
| **Combined dimer formation** | No |
| ***In silico* PCR on Genbank** | *Alytes obstetricans, Alytes maurus* |
| **Date of in silico PCR** | March 2022 |

*Conclusion:*

The *in silico* analysis identified the target organism and *Alytes maurus*, 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 Bio-Rad CFX96 Touch Real-Time PCR Detection System.

## 2.1. Analytical sensitivity, fluorescence output signal and efficiency

Standard solutions containing nominal target concentrations of $10^5$, $10^4$, $10^3$, $10^2$, 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 **93.8%**, 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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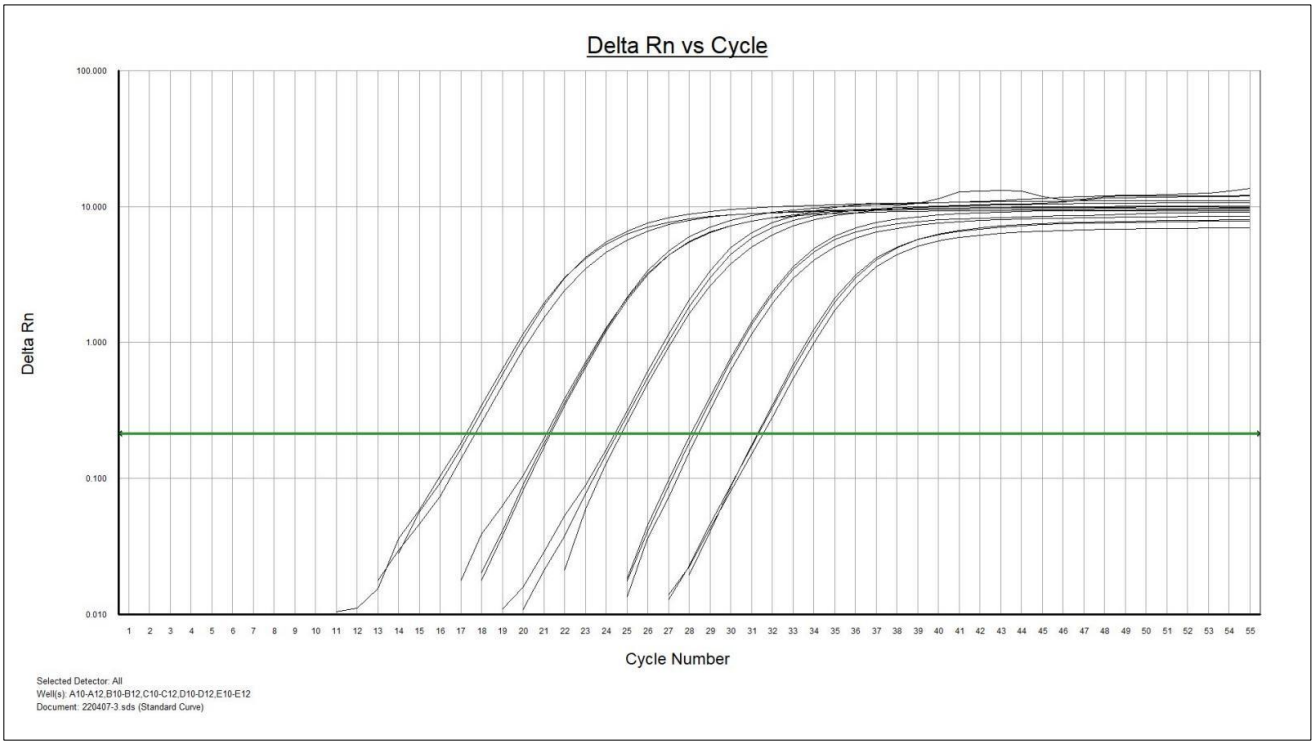



**Figure 1**. Amplification curves obtained at the optimal primer and probe concentrations and an annealing temperature of 60°C.

**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 |
|---|---|---|
| 0 | No | - |
| 1 | Yes | n.d. |
| 10 | Yes | n.d. |
| $10^2$ | Yes | 31.2 |
| $10^3$ | Yes | 28.2 |
| $10^4$ | Yes | 24.5 |
| $10^5$ | Yes | 21.2 |

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


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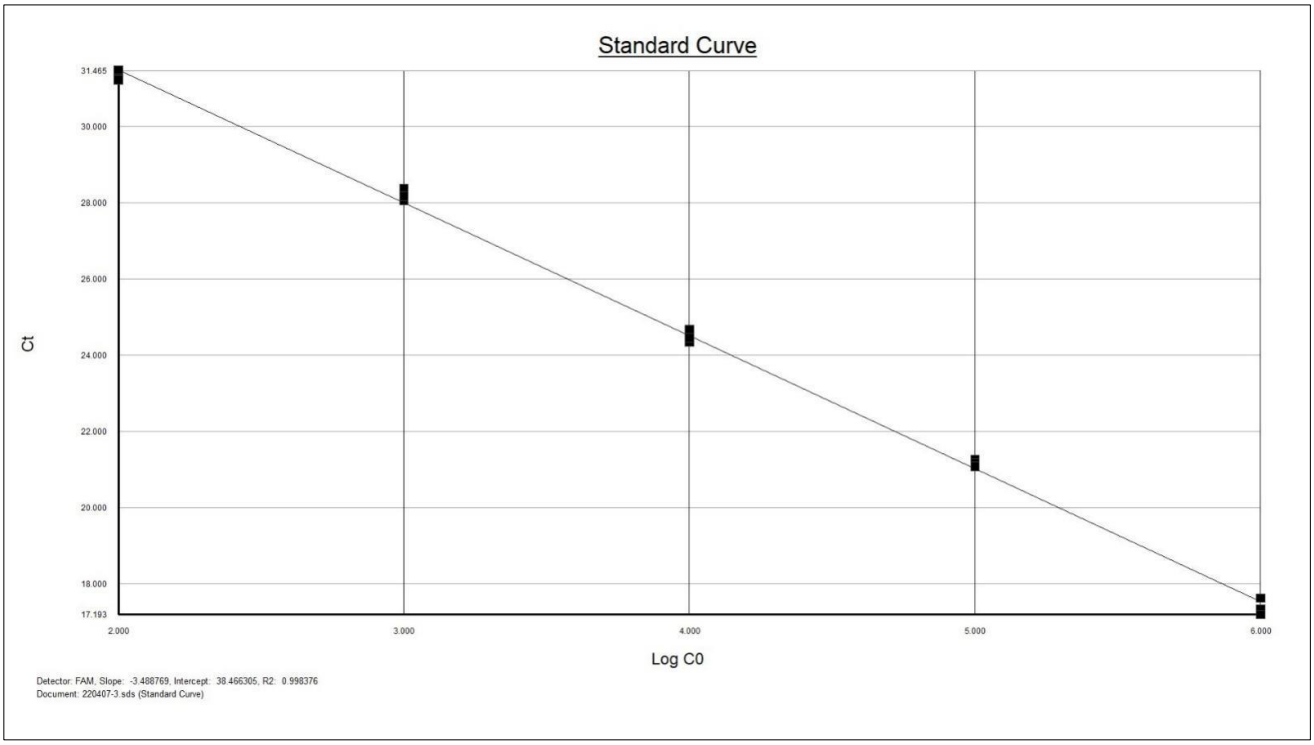



**Figure 2**. Standard curve generated from nominal target concentrations of $10^2$, $10^3$, $10^4$, and $10^6$ copies per reaction.

**Table 4**. Standard curve parameters obtained with the *Alytes obstetricans* qPCR detection kit

| Parameter | Result |
|---|---|
| **Slope** | -3.49 |
| **Amplification efficiency (%)** | 93.8 |
| **$R^2$** | 0.998 |


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## 2.2. Specificity testing

Specificity of the primers and probe set was evaluated by testing DNA from *Homo sapiens*, *Canis familiaris*, and *Bos taurus* as common non-target species, together with DNA from three species genetically related to the target organism. Genomic DNA was extracted from tissue material, and each sample was analyzed in triplicate under the same qPCR conditions used for the target species. A positive control containing target DNA and a no-template control (NTC) were included in each run. Specificity was considered acceptable when no amplification was observed in any non-target (Cq > 40 or undetermined).

*Results:*

**Table 5**. Amplification results for each species

| Species | Relation to target | Cq mean ± SD | Amplification? | Interpretation |
|---|---|---|---|---|
| ***Homo sapiens*** | Common species | - | No | Specific |
| ***Bos taurus*** | Common species | - | No | Specific |
| ***Rana arvalis*** | Relative | - | No | Specific |
| ***Rana temporaria*** | Relative | - | No | Specific |
| ***Salamandra salamandra*** | Relative | - | No | Specific |

No amplification was observed for any of the tested non-target species, including the phylogenetically related taxa, confirming the analytical specificity of the primer and probe set under the tested conditions.


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