Sylphium
molecular ecology

Protocol eDNA qPCR hot start mix

For environmental samples

#SYL1003
400 reactions
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For general laboratory and research use only.

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1. Components

The eDNA qPCR hot start mix for primer/probe based assays offers outstanding performance for sensitive, robust and precise probe-based qPCR detection and quantitation of target (e)DNA extracted from environmental samples. eDNA qPCR hot start mix is highly resistant to inhibiting factors, like humic acids present in environmental samples. The enzyme originally isolated from *Thermus aquaticus* has been genetically modified to obtain hot start properties.

The eDNA qPCR hot start mix has:

- **High resistance to inhibiting factors**. Environmental DNA (eDNA) extracts contain often multiple qPCR inhibiting factors. Normal qPCR hot start mixes are sensitive to these substances.
- **Strong fluorescence signal with low background noise**. Isolated environmental samples contain residues of naturally occurring auto fluoresce substances that will interfere with the measurements. A strong fluorescence signal from the analyses is required for this kind of samples.
- **Highest possible sensitivity** (1 DNA copy per reaction). Environmental water samples may contain very low amounts of target DNA.
- **Hot-start properties.** The DNA polymerase enzyme has been genetically modified to have similar or better hot-start properties when compared to antibody-mediated hot-start enzymes. Working on ice is not necessary during the preparation of the PCR mixture.

1.1. Kit contents and required equipment

Kit contents	Required equipment
• 2 x 2500 µL eTaq qPCR mix (2x) • 2 x 200 µL eTaq DNA polymerase • 1 x PCR grade water	• qPCR machine • Pipettors • -20°C freezer

1.2. Kit storage

Store all reagents and kit components in a -20°C freezer.

1.3. Ordering information

This eDNA qPCR hot start mix (SYL1003), a wide variety of compatible eDNA qPCR detection assays, eDNA sampling materials or equipment and eDNA isolation kits can be found and ordered at www.sylphium.com/webshop

1.4. Notices and disclaimers

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2. Principles of the kit

2.1. qPCR

The eDNA qPCR hot start mix is designed for primer/probe based assays for environmental DNA samples with high concentration of inhibiting substances. During PCR amplification, one set of primers and a probe hybridize to a sequence located on the target DNA. The fluorogenic probe is labeled with a discriminating 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of qPCR machines. The target DNA will be detected via the FAM dye channel.

2.2. PCR positive control (PPC)

The PCR positive control provided in this kit contains target DNA. This is a control for checking the reactions during analyses. This control should give a positive signal. Each time the kit is used, at least two positive control reactions must be included in the run. A positive result indicates that the primers and probes for detecting the target worked properly in that particular experimental scenario. If a negative result is obtained, the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false-positive results. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

2.3 PCR negative control (PNC)

To validate any positive findings, a negative control reaction should be included every time the kit is used. This control checks for DNA contamination. DNA-free water is added to the eDNA qPCR hot start mix in the wells. A negative signal (background only) should be observed with this control. A positive signal indicates DNA contamination occurred at some point during the procedure. In such cases, the qPCR results are unreliable, and the analysis should be repeated. Refer to **Appendix A** for possible solutions.

2.4. Internal positive control (IPC)

The Internal positive control is an efficiency control of the DNA isolation procedure and a quality and purity control of the isolated DNA. The internal positive control is a small piece of synthetic xenobiotic DNA added to the conservation buffer of the eDNA isolation kit (SYL002). The selected DNA sequence of the positive control is unknown to the aquatic environment and will not interfere in any detection of target organisms. The IPC can be detected with the IPC detection kit, which is optionally supplied with eDNA isolation kit (SYL002). A positive signal should be observed with this control in all reactions. A negative signal or a higher CT value (ΔCT >2) indicates the presence of inhibiting substances. **Appendix B** lists several options for solving problems with inhibiting substances.

2.5 Blank procedure

During DNA isolation, an additional negative control will be included. This control, containing only preservation solution, will be processed and analyzed like a normal sample. If a positive signal is detected in this control, it indicates that DNA contamination occurred during the isolation process. In such cases, the results are no longer reliable, and the sampling and isolation procedure must be repeated after thoroughly cleaning the laboratory and equipment. Refer to Appendix A for potential solutions.

2.6. Limit of detection (LODqPCR)

Lowest analysis limit of the qPCR which corresponds to the minimum number of DNA copies in the qPCR which gives a positive result. This will determent with dilution series of the positive control.

2.7. Limit of quantification (LOQqPCR)

Lowest limit of the qPCR where with a reliability of 95% the number of DNA copies can be quantified. This will determent with dilution series of the positive control.

3. Protocol

The eDNA qPCR hot start mix is designed to have a sensitivity of only one molecule per reaction in environmental samples. To have maximum sensitivity, please read this document carefully and make sure to understand all steps before proceeding with the analysis.

3.1. Precautions

- **Use separated rooms/PCR cabinets for DNA isolation, hot start mix preparation and PCR machines.** To prevent unwanted spread of target DNA, use separated rooms/PCR cabinets especially from high concentrated positive controls and PCR products and follow an one-way workflow from clean to contaminated rooms. See **Appendix B** for more details.
- **Prevent contaminations.** Before starting your experiments, clean the table surface and all equipment with bleach (1% Sodium hypochlorite) or another DNA removing agent to remove DNA from previous experiments. Wear gloves to prevent DNA contaminations between samples and clean your gloves with a soaked paper towel with diluted thin bleach between every handling.
- **Reduce long exposure to light.** It's preferable to limit the amount of light during preparations of the PCR plate and mixtures. The PCR mix contains light sensitive probes and under influence of light the signal strength will be reduced.
- **Prevent formation of unwanted products**. Cool down the PCR reaction mixture after preparing to 0 – 4 °C to prevent unwanted non-specific hybridization of primers and probe. The cooling step will increase the sensitivity of the method.

3.2. qPCR experimental plate setup

The concentration of eDNA molecules in aquatic samples is typically very low under normal circumstances. It is highly recommended to perform the analyses in multiple replicates per sample to improve the detection of these low DNA amounts. Two example experimental plate setups are shown **Figure 1** and **Figure 2** in which samples can be analyzed in 8- or 12-fold per 96-wells plate, respectively. Other plate setups are also possible with this kit.

For statistical reasons, the controls used in this kit, with the exception of the PCR positive control, should have the same number of replicates as the analyzed samples. The PCR positive controls must be analyzed in at least 2 replicates. It is recommended to analyze the positive control in a dilutions series from 10^3 to 1 DNA copy to determine the detection limit of the assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10/ INC	Neg	10^3
B												
C												10^2
D												
E												10
F												
G												1
H												

Figure 1. 96-wells plate setup for the analyses of 10 samples in 21 samples in octuple (8-fold). Column 12 contains the dilution series of the positive control.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1											
B	Sample 2											
C	Sample 3											
D	Sample 4											
E	Sample 5											
F	Sample 6											
G	Sample 7 / INC											
H	10^3		10^2		10		1		Neg		Neg	

Figure 2. 96-wells plate setup for the analyses of 6 samples in twelvefold. Row H contains the dilution series of the positive control and the PCR negative controls.

3.3. Preparing the positive control dilution series

Prepare a dilution series of the positive control around the expected concentration of a sample. In the case of eDNA, this will often be between 1 and 100 copies of the target DNA per µl. eDNA assay kits of Sylphium contain positive controls of known concentrations and are printed on the tube. The concentration of custom made positive controls must be determined before making a dilution series. A dilution series can be made according to the scheme below.

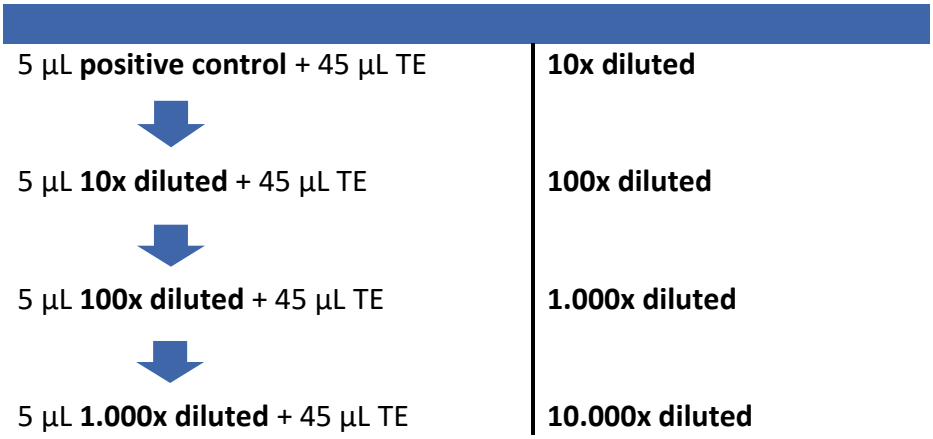

Figure 3. Pipetting scheme for preparing the dilution series.

3.4. Thermal cycling conditions

The eDNA qPCR hot start mix can be used with a two- or three-step thermal cycling program. This depends on the annealing temperatures of the primers/probe mix used and on the properties of the qPCR machine. The temperatures and times of qPCR detection assays of Sylphium can be found in the manual for these kits. Below diagrams are examples of a two-step and a three-step program.

Step	Temperature	Time*	Number of cycles
Initial denaturation	95°C	10 min	1
Denaturation	95°C	15 s	50
Annealing/Extension	60°C	30 s	

Table 1. **Two-step thermal cycling program**. Fluorogenic data should be collected during the annealing/extension step. ***Given times are indicative and depend on the used qPCR machine.

Step	Temperature	Time*	Number of cycles
Initial denaturation	95°C	10 min	1
Denaturation	95°C	15 s	50
Annealing	X[§]	15 s	
Extension	72°C	30 s	

Table 2. **Three-step thermal cycling program**. Fluorogenic data should be collected during the extension step. ***Given times are indicative and are depend on the used qPCR machine. **§** Annealing temperature of primer/probe mix.

3.5. Procedure

1. Completely thaw the eDNA PCR mix (2x), primer/probe mix, samples and positive controls to dissolve all precipitates.

2. Mix all tubes by shaking and briefly pulse-spin them in a centrifuge before opening. This ensures that the tube contents settle in the bottom and prevents spillage when opening the tubes. Prepare only as much mixture needed for the analysis, with a few additional reactions to account for pipetting errors.

Component	Reaction volume 25 µl	Reaction volume 50 µl
eTaq qPCR mix (2x)	12.5 µl	25 µl
eTaq DNA polymerase	1 µl	2 µl
10x Primers/probe mix*	2.5 µl	5 µl
Sample	2 - 8 µl	2 - 16 µl
ROX Reference Dye§	X µl	X µl
PCR grade water	Fill to 25 µl	Fill to 50 µl

Table 3. Components per well for 25 µl and 50 µl reaction volume. *If using custom primer/probe mixes, formulate to 10x initial concentration. **§** Not supplied in kit, see manual qPCR machine if needed.

3. Mix thoroughly but gently to minimize air bubbles in the mixture.

4. Pipette the prepared PCR mix into the wells of a PCR plate (total reaction volume minus the sample volume per well). To avoid pipetting errors, use a minimum sample volume of 2 µl for qualitative measurements and at least 5 µl for quantitative assays. Place a seal cover on top of the 96-well PCR plate to protect against airborne contaminations.

5. Add template DNA (sample or positive control) to the PCR plate according to the desired qPCR plate setup. **Note:** all hot-start enzymes exhibit some background activity at room temperature. To maximize sensitivity, keep the mixture at room temperature or lower for as little time as possible after adding the sample. Using a cooling block (4 °C) is recommended after adding the sample.

6. Briefly spin the plate if necessary to remove air bubbles, as these can interfere with fluorescence detection.

7. Program the thermal cycler according to the scheme in **Section 3.4**.

3.6. Interpretation of results

If at least one out of an analytical series is positive the whole series should be regarded as positive. This statement is also valid for all controls in this kit. The Limit of detection (L0DqPCR) of the analysis can be determined with the lowest dilution series from positive control which can be detected.

Target	IPC	INC	PPC	PNC	Result	Solution
+	+	-	+	-	Positive detection	-
-	+	-	+	-	Negative detection	-
-	-	-	-	-	Failing PCR procedure	Repeat PCR procedure
-	-	-	+	-	PCR inhibited or isolation failed	see **appendix B**
+	+	+	+	-	DNA contamination during isolation	see **appendix A**
+	+	+	+	+	DNA contamination during PCR preparation	see **appendix A**

Table 2. **Interpretation of results.** IPC: Internal positive control, INC: Internal negative control, PPC: PCR positive control (included in SYL002: Environmental DNA isolation kit), PNC: PCR negative control. See **Section 2** "Principals of the kit" section of this document for problem-solving.

Appendix A: Lab room layout and one-way workflow

To prevent unwanted spread of target DNA, use separated rooms/PCR cabinets especially for DNA isolation, qPCR hot start mix preparations and for PCR machines (**Figure 3**).

Provide a one-way workflow to reduce the risk of contamination. Do not carry materials (such as pens, markers), consumables, or laboratory equipment (such as pipettes) from the DNA isolation area into the hot start mix preparation area. Do not take anything from the PCR room into the DNA isolation room or into the hot start mix room. Color coding for materials, lab coats and disposables from any room can help ensure the one-way workflow and thus reduce the chance of contamination. Do not move work instructions and notebooks from room to room. When using these rooms for work other than PCR analysis, personnel should adhere to the workflow that applies to these PCR rooms (**Figure 3**).

In exceptional cases where transport of materials, consumables or laboratory equipment between the different rooms is unavoidable: clean these items well with a suitable agent. Only work from a clean environment to a 'polluted' environment.

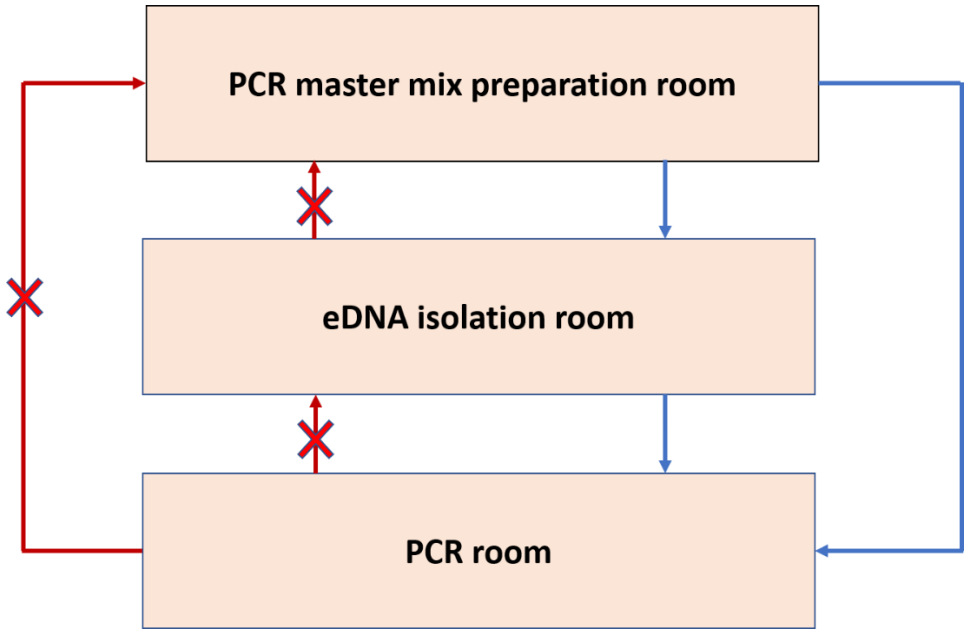

Figure 3. Lab Room Layout and One-Way Workflow. Transport allowed only via blue arrows, transport via red arrows will lead to target DNA contaminations. A PCR cabinet can be seen as a separate room, but is not recommended.

Appendix B: Steps to overcome inhibition

Environmental samples often contain inhibitory factors such as humic acids. The eDNA qPCR hot start mix has a high resistance to such compounds, but in some cases inhibition will still occur. No fluorescent signal or a higher CT value (ΔCT >2) indicates inhibitory substances.

There are three ways to overcome inhibitions.

1. **Increase the reaction volume**. Normal PCR assays are performed in a reaction volume of 25 µl. Increasing the reaction volume to 50 or 75 µl results in a lower concentration of inhibitory factors in the qPCR reaction mix, but does not reduce the chance of detecting target DNA.
2. **Dilution of the sample**. Dilution of the sample leads to a reduction of the inhibitory compounds until the PCR assay gives reliable results. This reduces the chance of detection of target DNA.
3. **Use of a PCR cleanup kit**. Commercial kits are available that can lower inhibitory compounds in the sample. The sample volume will be increased while using this kit and some target DNA will be lost. This reduces the chance of detection of target DNA.

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