Sylphium
molecular ecology

IPC qPCR quantification

With eDNA master mix

#SYL113

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For general laboratory and research use only.

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1. Introduction

All Sylphium sample sets include an Internal Positive Control (IPC) within the preservation solution, located in the small syringe of the sampling set (SYL009). The IPC serves as an efficiency check for the DNA isolation procedure, ensures the quality and purity of the isolated eDNA, and helps prevent false negative results. This internal control consists of a small piece of synthetic xenobiotic DNA. The selected positive control DNA sequence is not found in the aquatic environment and does not interfere with the detection of target organisms. Additionally, the IPC Quantification Kit can be ordered separately or together with the eDNA Isolation Kit. The IPC is detected through the FAM channel.

1.1. Kit contents, required equipment and consumables

Kit contents	Required equipment and consumables
• Positive control • eTaq qPCR mix (2x) • Primer/probe mix for detection of the internal positive control (FAM dye) • eTaq DNA polymerase • PCR water	• qPCR machine multiplex capable for detection of the FAM dye (optional) • Pipettors • -20 °Freezer • PCR cooling rack (optional) • 96 wells qPCR plates + seal cover

1.2. Kit storage

Store all reagents and kit components after arrival of the IPC qPCR quantification kit in the dark and in a freezer (-15 to -20°C).

1.3. Notices and disclaimers

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2. Principles of the kit

2.1. qPCR

The primer and probe mix provided in this kit is a probe-based PCR method. During PCR amplification, one set of primers and a probe hybridize to synthetic DNA present in the sample preservation solution(small syringe). 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. 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 DNA present in the sample preservation solution(small syringe) of the eDNA sampling kit. The chosen DNA sequence of the positive control is unknown to the aquatic environment and will not interfere in any detection of target organisms. The IPC will be detected via the FAM channel. A positive signal should be obtained from this control in all cases (reactions). A negative signal indicates inhibiting substances in the eDNA isolate or a failure during isolation. The sampling and isolation procedure should be repeated. If inhibiting substances are present dilution of the sample is a possible solution, an additional purification step can also be done.

2.3 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.

2.4. Internal Negative Control (INC)

During DNA isolation an additional internal negative control will be used. This is provided in the kit as S1. This sample will be analyzed as a normal sample and should be negative. If a positive signal is detected with this control, a DNA contamination was obtained during isolation. Results are not reliable anymore. The sampling and isolation procedure should be repeated after cleaning the lab and equipment.

2.5. qPCR experimental plate set-up

Two example experimental plate setups are shown **Figure 1** and **Figure 2** in which samples can be analyzed in 4- or 2-fold per 96 wells plate, respectively. Other plate setups are also possible with this kit.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	Sample 13	Sample 15	Sample 17	Sample 19	Sample 21	**PNC**
B												
C												
D												
E	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	**INC**	**PPC**
F												
G												
H												

Figure 1. 96-wells plate setup for the analyses of 21 samples in quadruplicate (4-fold).

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	5	9	13	17	21	25	29	33	37	41	45
B												
C	2	6	10	14	18	22	26	30	34	38	42	**INC**
D												
E	3	7	11	15	19	23	27	31	35	39	43	**PNC**
F												
G	4	8	12	16	20	24	28	32	36	40	44	**PPC**
H												

Figure 2. 6-wells plate setup for the analyses of 25 samples in duplicate (2-fold).

3. Protocol

The IPC qPCR Quantification Kit can be used to check the eDNA isolated with the Sylphium eDNA Isolation Kit or any other isolation kit. The IPC is included in the sample preservation solution of the Sylphium eDNA sampling set. However, if another sampling set or isolation kit/protocol is being used, the IPC should be added to the preservation buffer.

3.1. Precautions

- **Prevent contaminations.** Before starting your experiments, clean the table surface and all equipment with thin bleach (10 times diluted) 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.
- **Keep cool as long as possible**. Keep mixtures for the internal positive control in a PCR cooling rack after addition of the Taq DNA polymerase. This to prevent unwanted non-specific hybridization of primers and probe and extension by the used DNA polymerase at room temperature. The life span of Taq DNA polymerase in its own storage buffer will be at room temperature at least a month. In the PCR mixture this is reduced to a few hours at room temperature.

3.2. Adding internal positive control to sample preservation solution

The preservation solution of Sylphium already contains the Internal Positive Control (IPC) at a concentration of 5000 copies per ml. The provided positive control tube can be used to add the IPC to your own sample preservation solution at the desired concentration. The concentration of the IPC is printed on the tube.

3.3. Experimental setup quality control

In most cases, the samples can be used undiluted in the quality control check. If samples are (slightly) brown of color, it is recommended to make a dilution series of 2x, 4x and 8x (**Figure 3**). This dilution series should be made in S6 (low TE). Make sure to use the same sample volume in the quality control check as in the target organism analysis. Due to pipetting errors with small volumes, quantitative analysis should be performed with a sample volume of 5 µL per reaction. Qualitative analysis can be performed with a sample volume of either 2 µL or 5 µL per reaction.

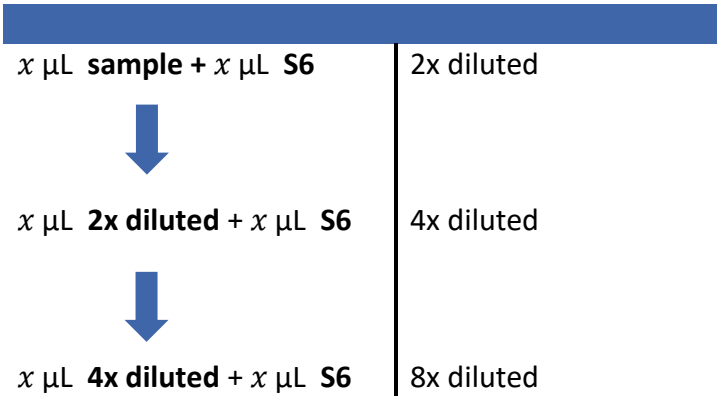

Figure 3: Pipetting scheme for preparing dilution series. x = qualitative analyses: 5 µL , quantitative analyses: 12 µL

An example experimental plate setup is shown in **Figure 4**, in which samples can be analyzed in duplicate. If dilution series are made, regard each dilution as a normal sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	5	9	13	17	21	25	29	33	37	41	10^4
B												
C	2	6	10	14	18	22	26	30	34	38	42	10^3
D												
E	3	7	11	15	19	23	27	31	35	39	43	10^2
F												
G	4	8	12	16	20	24	28	32	36	40	44	10
H												

Figure 4: Colum 12 contains the calibration dilutions in duplicate (2-fold) of the positive control.

3.4. Preparing PCR mixture and thermal cycling conditions

In addition to the specific primer/probe mix, the kit contains all components of the eDNA qPCR master mix. The protocol associated with the eDNA qPCR master mix (SYL1003) is also valid for the Internal positive control detection kit (SYL113). The complete protocol can be found under "Relevant documents" on the eDNA isolation kit page at sylphium.com/webshop.

Thermal cycling conditions of the Internal positive control detection kit are as follows:

Step	Temperature	Time*	Number of cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	15 s	
Annealing	55°C	15 s	50
Extension	72°C	30 s	

Table 1. **Thermal cycling conditions of the Internal positive control detection kit (SYL113).** Fluorogenic data should be collected during the extension step. Internal positive control detection DNA detection via FAM channel.

3.5. Interpretation of results

All samples isolated with the environmental DNA isolation kit should give a robust qPCR signal, before proceeding to the analysis of the target organism(s).

3.5.1 Inhibition check:

If lower dilutions result in a lower CT value, inhibiting substances are present in the isolated sample. Use the dilution with the lowest CT value and determine isolation efficiency.

3.5.2 Determination of isolation efficiency

The isolation efficiency (%) can be calculated via the following formula:

$$\frac{a.c}{b.d.e} * 100 = efficiency$$

	Volume
a	Measured concentration in well (total amount)
b	Volume of preservation solution (mL)
c	Total volume of isolate (µL)
d	Volume isolate added to reaction well (µL)
e	Given concentration in preservation solution (molecules per mL)*

* The concentration of the IPC is printed on the label of the preservation solution tube and on the box label of the sample sets (SYL001).

Example calculation:

Experimental setup

- In total, 2 mL preservation solution was extracted from the filter capsule and used in the isolation procedure.
- The isolated DNA was resuspended in 100 µL S6.
- Per reaction, 2 µL isolate was added to the qPCR mix in each well
- The given concentration of IPC in the preservation solution is 11000 molecules per mL
- The determined concentration was 410 and 405 for the duplicate analyses. This will give an average of 407,5

This will give the following formula:

$$\frac{\mathbf{407.5} * \mathbf{100}}{\mathbf{2} * \mathbf{2} * \mathbf{11000}} * \mathbf{100} = \mathbf{92.6\%}\ \boldsymbol{efficiency}$$

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