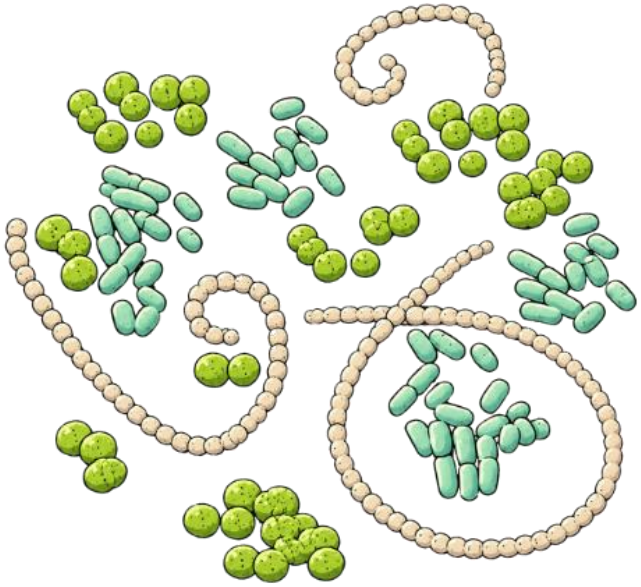
MANUAL

Microcystin Cyanotox qPCR detection kit

SYL102

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molecular ecology

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Table of Contents

1. Introduction 4

1.1. Kit contents, storage, and required equipment 5

1.2. Ordering information 5

1.3. Notices and disclaimers 5

1.4. Risk assessment microcystin/nodularin concentration 6

2. Precautions 8

3. Protocol 10

3.1. Reaction setup 10

3.2. Controls 11

3.3. Thermal cycling 13

4. Interpretation of results 14

Appendix A: Lab room layout and one-way workflow 15

Appendix B: Steps to overcome inhibition 16

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1. Introduction

The SYL102 *Microcystin Cyanotox* qPCR detection kit, combined with the eTaq kit buffer, provides excellent performance for sensitive, robust and accurate qPCR detection. The eTaq kit buffer is highly resistant to inhibitory substances commonly found in environmental samples, such as humic acids.

In addition to the eTaq kit buffer, this kit contains a primers and probe mix designed to detect a highly specific mitochondrial DNA sequence of *microcystin cyanotox*. The primers and probe have been specifically designed and validated for use with eDNA samples. The full validation report is available on the Sylphium website[1].

When this kit is used to support conclusions regarding the absence of the target species, the use of an internal positive control (IPC) is essential. Without an IPC, a negative target result cannot be reliably distinguished from a false-negative result caused by PCR inhibition, DNA degradation, or insufficient DNA recovery during sample processing. For further information on the use and interpretation of controls, refer to **Section 3.2**.

The Microcystin Cyanotox detection kit has:

- **High resistance to inhibiting factors**. This detection kit uses the **eTaq kit buffer**, which is highly resistant to inhibitory substances commonly found in environmental DNA (eDNA) extracts. Standard qPCR mixes may be sensitive to these inhibitors.
- **Strong fluorescence signal with low background noise**. Environmental DNA extracts may contain naturally occurring, auto-fluorescent compounds that can interfere with fluorescence measurements. A strong assay signal is therefore important to minimize the impact of background fluorescence.
- **High analytical sensitivity**. Environmental water samples may contain only very small amounts of target DNA, making high analytical sensitivity essential. The assay has demonstrated detection down to 10 target DNA copies per reaction under the validated test conditions.
- **High specificity**. Environmental DNA extracts contain billions of DNA fragments originating from bacteria, protozoa, plants, animals, and other organisms. During primer and probe design, not only closely related species must be considered; all available DNA sequences should be screened for potential nonspecific binding of the primers and probe.

[1] https://sylphium.com/available-downloads/

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1.1. Kit contents, storage, and required equipment

Kit contents:	Required equipment:
• Positive control (cloned target DNA) • Primers & probe mix (10x) (FAM dye) • eTaq DNA polymerase • eTaq kit buffer	• Freezer -20°C • Pipettors • Microcentrifuge (13,000 x *g*) • qPCR machine with FAM detection • PCR cooling rack (optional)

This kit contains sufficient materials for 200 reactions with a final reaction volume of 25 µL. Upon arrival, store all reagents and kit components protected from light at -20°C.

All kits are compatible with major qPCR platforms and are suitable for FAM dye detection on most modern qPCR instruments. They are optimized for use on eDNA isolates purified using **SYL002 Environmental DNA isolation kit**, but also work on isolates purified with other eDNA extraction methods or kits.

1.2. Ordering information

This qPCR detection kit (SYL102), a wide variety of other eDNA qPCR detection kits, eDNA sampling materials and equipment, eTaq flex kit, and eDNA isolation kits can be found and ordered at www.sylphium.com.

1.3. Notices and disclaimers

This product is developed, designed and sold for research purposes only. Sylphium Molecular Ecology B.V. does not take any responsibility and is not liable for any damage caused through use of this product, be it indirect, special, incidental or consequential damages (including but not limited to damages for loss of business, loss of profits, interruption or the like).

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1.4. Risk assessment microcystin/nodularin concentration

This kit is designed to provide a reliable risk assessment for microcystin/nodularin in recreational waters. According to The World Health Organization (WHO), a microcystin concentration of 20 µg is considered unsafe for recreational use. This concentration is also used as a threshold value in regulations and guidelines for recreational water quality in many countries. Therefore, a maximum concentration of 20 µg/L is used for the risk assessment performed with this kit.

Microcystins and nodularins are structurally similar cyanotoxins with closely related biosynthetic pathways and are considered equivalent for the purpose of this risk assessment[2]. The Microcystin Cyanotox qPCR detection kit is designed to detect the genetic potential for the production of both toxin groups simultaneously. For simplicity, the term microcystin is used throughout this manual to refer to both microcystins and nodularins.

The qPCR primers and probe used in this kit target the *mcyJ* gene. The *mcyJ* gene is part of the biosynthetic pathway responsible for microcystin production and occurs as a single copy within the microcystin biosynthesis gene cluster. It is therefore a suitable target for determining the number of microcystin biosynthesis gene copies present in a water sample.

1.4.1 Correlation between *mcyJ* copy number and microcystin concentration

To investigate the relationship between *mcyJ* gene copy number and the actual microcystin concentration, water samples were collected from several lakes in the northern Netherlands. The microcystin concentration in each sample was determined using an ELISA assay. Samples with microcystin concentrations between 2 and 35 µg/L, corresponding to the detection range of the ELISA assay, were subsequently analyzed using the Microcystin Cyanotox qPCR detection kit to determine the *mcyJ* gene copy number.

Figure 1 shows the relationship between the *mcyJ* gene copy number and the microcystin concentration per liter of water.

A correlation correlation between *mcyJ* gene copy number and microcystin concentration was found ($R^2 = 0.9713$). Based on these findings, the toxic gene potential (TGP) for microcystin was determined to be 1×10^{-9} µg microcystin per gene copy. The TGP is defined as the amount of toxin that can be produced per copy of the microcystin biosynthesis pathway. Based on this TGP, 2×10^{10} gene copies per liter of water correspond to a microcystin concentration of 20 µg/L, the maximum permissible concentration used in the WHO guideline.

[2] Boopathi, T., Ki, J. (2014) Impact of environmental factors on the regulation of cyanotoxin production. Toxins, 6 1951-1978

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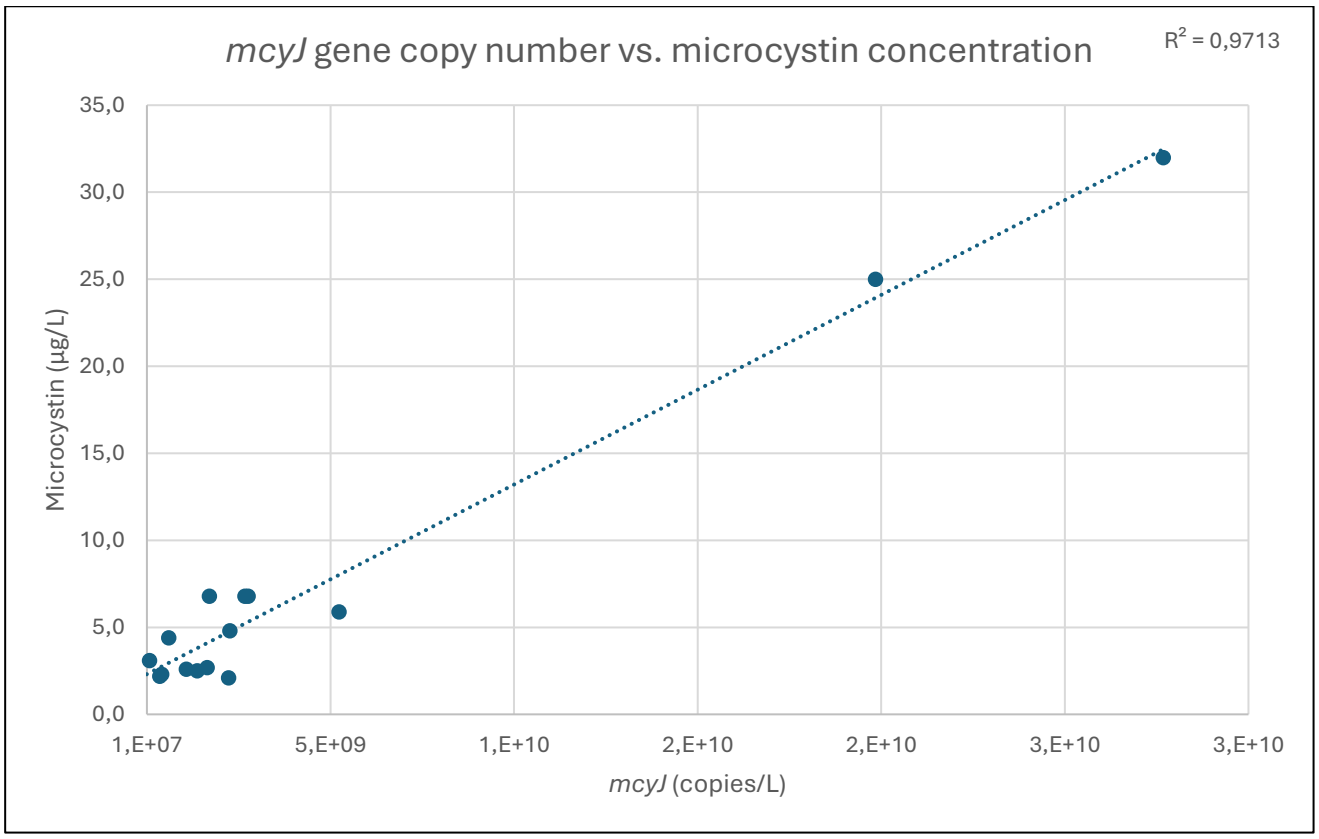


Figure 1. Correlation between *mcyJ* gene copy number and microcystin concentration in samples collected from 17 different Dutch lakes in 2015.

Similar results have been reported in the literature. Singh et al.[3] also used general primers targeting genes (*mcyA* and *mcyB*) involved in the microcystin biosynthesis pathway. Although they did not calculate the TGP, the data presented in their publication were sufficient to calculate it (**Table 1**). The calculated TGP values were within the same range as those determined from our data.

Table 1. *mcyA* and *mcyB* gene copy numbers and microcystin concentrations reported by Singh et al. (2015). Toxic gene potential (TGP) was calculated from the reported values.

Location and year	*mcyA* copies/L	*mcyB* copies/L	Microcystin (µg/L)	TGP *mcyA*	TGP
Lakshmikund, 2010	7.5×10^{10}	6.5×10^{10}	126	1.7×10^{-9}	1.9×10^{-9}
Lakshmikundr, 2011	3.3×10^{11}	3.0×10^{11}	128	3.9×10^{-10}	4.3×10^{-10}
Sankuldhara, 2011	1.4×10^{10}	7.2×10^{9}	39.6	2.9×10^{-9}	5.5×10^{-9}

[3] Singh, S., Rai, P.K., Chau, R., Ravi, A.K., Neilan, B.A., Asthana R.K. (2015) Temporal variations in microcystin-producing cells and microcystin concentrations in two freshwater ponds Water Res., 69 131-142

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It is important to use general primers for the detection of genes involved in the microcystin biosynthesis pathway. In many publications (e.g. Savela et al.[4]), species-specific assays targeting genes within the microcystin biosynthesis pathway were used. This may result in an underestimation of the total gene copy number and, consequently, an overestimation of the calculated toxic gene potential.

1.4.2 Toxic gene potential as a reliable risk assessment

Although a strong correlation was found between gene copy number and microcystin concentration, microcystin production is not constitutive. Instead, toxin production is regulated by environmental factors such as light intensity and iron availability[4567]. Therefore, this kit cannot be used to determine the exact microcystin concentration in a water sample. Instead, it provides an estimate of the toxic potential of the sample. Based on the measured gene copy number and the determined TGP, a predicted microcystin concentration can be calculated and used for risk assessment.

2. Precautions

[4] Sevilla, E., Martin-Luna, B.,Vela, L., Bes, M. T., Fillat, M. F., and Peleato, M. L. (2008). Iron availability affects mcyD expression and microcystinLR synthesis in Microcystis aeruginosa PCC7806. Environ. Microbiol. 10, 2476–2483.
[5] Kaebernick, M., Neilan, B. A., Boerner, T., and Dittmann, E. (2000). Light and the transcriptional response of the microcystin biosynthetic gene cluster. Appl. Environ. Microbiol. 66, 3387–3392.
[6] Kaebernick, M., Dittmann, E., Boerner, T., and Neilan, B. A. (2002). Multiple alternate transcripts direct the biosynthesis of microcystin, a cyanobacterial nonribosomal peptide. Appl. Environ. Microbiol. 68, 449–455.
[7] Tonk, L., Visser, P. M., Christiansen, G., Dittmann, E., Snelder, E. O., Wiedner, C., Mur, L. R., and Huisman, J. (2005). The microcystin composition of the cyanobacterium Planktothrix agardhii changes toward a more toxic variant with increasing light intensity. Appl. Environ. Microbiol. 71, 5177–5181

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- **Use separate rooms or PCR cabinets for different workflow steps**. DNA extraction, PCR mix preparation, and amplification should be performed in physically separated areas or dedicated PCR cabinets. In particular, high-concentration positive controls and PCR products should be handled separately to minimize the risk of target DNA contamination. Follow a one-way workflow from clean to potentially contaminated areas. Refer to Appendix B for more details.

- **Prevent DNA contamination**. Before starting an experiment, clean work surfaces and equipment with bleach containing 1% sodium hypochlorite or another suitable DNA-removing agent to remove residual DNA from previous experiments. Wear gloves at all times and clean or replace them between handling different samples. If gloves are cleaned, use a paper towel soaked with diluted bleach.

- **Minimize exposure to light**. Limit light exposure during preparation of PCR mixtures and PCR plates. Primers & probe mixes contain light-sensitive fluorescent probes, and prolonged exposure to light may reduce fluorescence signal intensity.

- **Prevent formation of non-specific products**. Keep the prepared PCR reaction mixture at 0-4°C until amplification to reduce non-specific primer and probe hybridization. Keeping the reaction mixture cooled during preparation may improve assay sensitivity.

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3. Protocol

3.1. Reaction setup

1. **Completely thaw** the eTaq PCR mix, primers & probe mix (10x), positive control, and your sample(s) to ensure that any precipitates are fully dissolved.
2. **Mix all tubes thoroughly** by gentle shaking, then briefly centrifuge them before opening. This ensures that the contents collect at the bottom of the tubes and reduces the risk of spillage.
3. **Prepare the reaction mixture**. Prepare only the amount of reaction mixture required for the analysis, including a few extra reactions to compensate for pipetting losses. For the required volumes, refer to **Table 1**.

Table 1. qPCR reaction setup for a final volume of 25 µL

Component	Volume per reaction	Volume per 96-wells plate*
eTaq kit buffer	19.5 µL	Whole tube
eTaq DNA polymerase	1 µL	100 µL
Primers & probe mix (10x)	2.5 µL	Whole tube
Sample	2-5 µL	2-5 µL per well

*Volumes already include an allowance for pipetting loss.

4. **Mix the prepared reaction mixture** thoroughly but gently to minimize the formation of air bubbles.
5. **Dispense the prepared reaction mixture** into the wells of the PCR plate, using the total reaction volume, minus the template volume per well. To minimize pipetting errors. Use a template volume of at least 2 µL for qualitative assays and at least 5 µL for quantitative assays. Cover the 96-well PCR plate with a seal to protect the reactions from airborne contamination.
6. **Add the template DNA, the positive control, and the negative control according to the desired plate setup**. To maximize assay sensitivity, minimize the time the reaction mixture is kept at room temperature after addition of the template. The use of a cooling block (approximately 4°C) is recommended.
7. **Briefly centrifuge the plate**, if necessary, to remove air bubbles. These can interfere with fluorescence detection.

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3.2. Controls

PCR positive control (PPC)

The PCR positive control (PPC) consists of PCR reagents to which target species DNA of a known concentration has been added. This control verifies that the qPCR reagents, primers, probe, and amplification conditions function correctly.

At least two positive control reactions should be included each time the kit is used. The PPC must show amplification with the expected Cq value. If the positive control does not show the expected amplification, the results of the qPCR run are considered invalid and the analysis must be repeated.

The positive control contains a relatively high concentration of target DNA and therefore represents a potential source of contamination. To minimize the risk of cross-contamination, add the positive control only after all samples and negative controls have been pipetted and sealed whenever possible. Avoid contact between the positive control and other kit components, samples, equipment, or work surfaces.

PCR negative control (PNC)

The PCR negative control (PNC) consists of PCR reagents without target species DNA. PCR-grade water is added instead of sample DNA. This control is used to detect DNA contamination introduced during preparation of the qPCR reactions.

At least one PCR negative control should be included each time the kit is used. The PNC should not show amplification of target species DNA. Detection of a positive amplification signal indicates possible DNA contamination during preparation of the qPCR reactions. In this case, the results of the qPCR run are considered unreliable, and the analysis should be repeated after identifying and eliminating the potential source of contamination.

Internal positive control (IPC) – not included in this kit

An internal positive control (IPC) can be used as an additional control to assess DNA-recovery and possible inhibition of the qPCR reaction. The IPC is analyzed separately from the target-species assay and is therefore not a component of the target qPCR reaction itself.

Sylphium preservation buffers contain an IPC by default. The same control is included as the IPC used with the **SYL002 Environmental DNA isolation Kit** and can be detected and quantified using the **SYL113 IPC quantification kit**.

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The IPC should produce amplification within the expected Cq range. The absence of amplification or an increased Cq value compared with the expected value may indicate reduced DNA recovery, PCR inhibition, and/or DNA degradation.

When inhibition is suspected, the sample may be diluted or subjected to a suitable PCR cleanup procedure before reanalysis. Refer to **Appendix B** for possible approaches to reduce PCR inhibition.

An IPC is not included as a standard component of this qPCR detection kit itself unless explicitly stated otherwise.

Blank procedure

When this qPCR detection kit is used as part of an eDNA workflow, inclusion of a blank procedure control during DNA extraction is recommended. The blank procedure is used to detect DNA contamination introduced during DNA extraction and subsequent analysis. The blank consists of preservation solution without an environmental sample and is processed through all DNA extraction and qPCR analysis steps in the same manner as a regular sample.

The blank procedure should not show amplification of target species DNA. Detection of target species DNA in the blank indicates that contamination may have occurred during sample processing, DNA extraction, or analysis. In this case, the affected results are considered unreliable and the source of contamination should be investigated before repeating the procedure.

Laboratory work surfaces and equipment should be thoroughly decontaminated before repeating the extraction and analysis.

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3.3. Thermal cycling

Table 2. Thermal cycling conditions of the *Microcystin Cyanotox* qPCR detection kit

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	95	5 min	1
Denaturation	95	15 sec	
Annealing	55	15 sec	35
Extension*	72	30 sec	

*Fluorogenic data should be collected during the extension step. *Microcystin Cyanotox* DNA is detected in the FAM channel.

To make a risk assessment, the toxic gene potential ($1*10^{-9}$ µg microcystin/gene) can be multiplied with the obtained *mcyJ* copy number to calculate the predicted microcystin concentration in your sample(s). For more information about the toxic gene potential see the section about risk assessment in this document.

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4. Interpretation of results

A sample is considered positive when at least one replicate within an analytical series shows positive amplification. Control results must meet the acceptance criteria specified in **Table 4**.

Table 4. Interpretation of assay controls and results

Sample	PPC	PNC	IPC*	Blank§	Result	Action
+	+	-	N/A or +	N/A or -	Positive detection	-
-	+	-	N/A or +	N/A or -	Negative detection	-
-	-	-	N/A or +	N/A	PCR procedure failed	Repeat the PCR procedure
-	+	-	-*	N/A	PCR inhibited, insufficient DNA recovery, or DNA degradation	See **Appendix B**
+	+	-	N/A or +	+§	DNA contamination during DNA extraction	See **Appendix A**
+	+	+	N/A or +	N/A or -	DNA contamination during PCR preparation	See **Appendix A**

PPC: PCR positive control; **PNC**: PCR negative control; "**+**" = positive amplification DNA; "**-**" = no amplification. **IPC**: Internal positive control; **Blank**: blank procedure control; N/A = not applicable or not included. ***** IPC-based interpretation is only applicable when an IPC has been included in the sample workflow and quantified using an appropriate IPC assay. **§** Blank-based interpretation is only applicable when the samples have undergone a DNA extraction workflow in which a blank procedure control was processed alongside the samples. For further explanatory notes see, **Section 3.2**.

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Appendix A: Lab room layout and one-way workflow

To prevent the unwanted spread of target DNA, use separate rooms or dedicated PCR cabinets for DNA extraction, eTaq kit buffer preparation, and PCR amplification (**Figure 1**).

Maintain a one-way workflow to minimize the risk of contamination. Do not transfer materials (e.g. pens and markers), consumables, or laboratory equipment (e.g. pipettes) from the DNA extraction area to the eTaq kit buffer preparation area. Similarly, do not transfer any materials from the PCR-room to the DNA extraction or eTaq kit buffer preparation areas. Color coding of laboratory equipment, lab coats, consumables, and other materials can help maintain this one-way workflow and reduce the risk of cross-contamination. Work instructions and notebooks should also remain within their designated areas. When these rooms are used for activities other than PCR-analysis, personnel should continue to follow the workflow applicable to the PCR areas (**Figure 1**).

In exceptional cases where the transfer of materials, consumables, or laboratory equipment between rooms is unavoidable, thoroughly clean and decontaminate the items using a suitable DNA-removing agent before transfer. Movement should always follow the direction of the workflow, from cleaner areas to more contamination-prone areas.

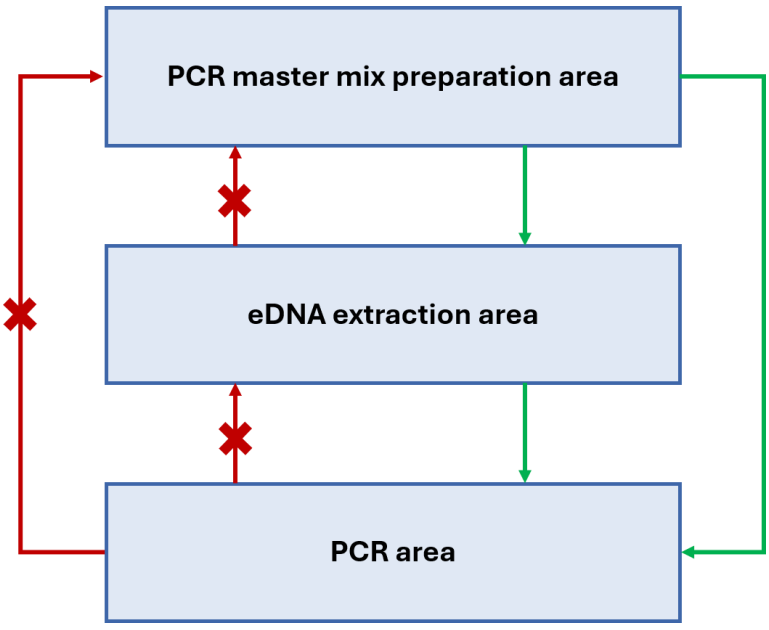


Figure 1. Laboratory room layout and one-way workflow. Transport is permitted only in the direction of the green arrows. Transport in the direction of the red arrows should be avoided, as it increases the risk of target DNA contamination. A dedicated PCR cabinet may function as a separate work area, but the use of physically separated rooms is preferred.

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Appendix B: Steps to overcome inhibition

Environmental samples often contain inhibitory substances, such as humic acids. The eTaq kit buffer is highly resistant to these compounds; however, inhibition may still occur in some samples. The absence of a fluorescent signal or an increased Cq value (ΔCq > 2) may indicate PCR inhibition.

There are two main approaches to reduce or overcome inhibition:

1. Dilute the sample

Diluting the DNA-extract reduces the concentration of PCR-inhibitory compounds and may restore reliable amplification. However, dilution also reduces the concentration of target DNA and may therefore decrease the probability of detecting low target concentrations.

2. Use a DNA cleanup kit

Commercial PCR cleanup kits can be used to remove or reduce PCR-inhibitory compounds from the sample. However, the cleanup procedure may result in partial loss of target DNA and/or an increase in the final sample volume. Both effects may reduce the probability of detecting low concentrations of target DNA. If sample cleanup is required, Sylphium recommends the EchoClean DNA Cleanup Kit[8].

[8] https://www.bioecho.com/products/nucleic-acid-cleanup

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