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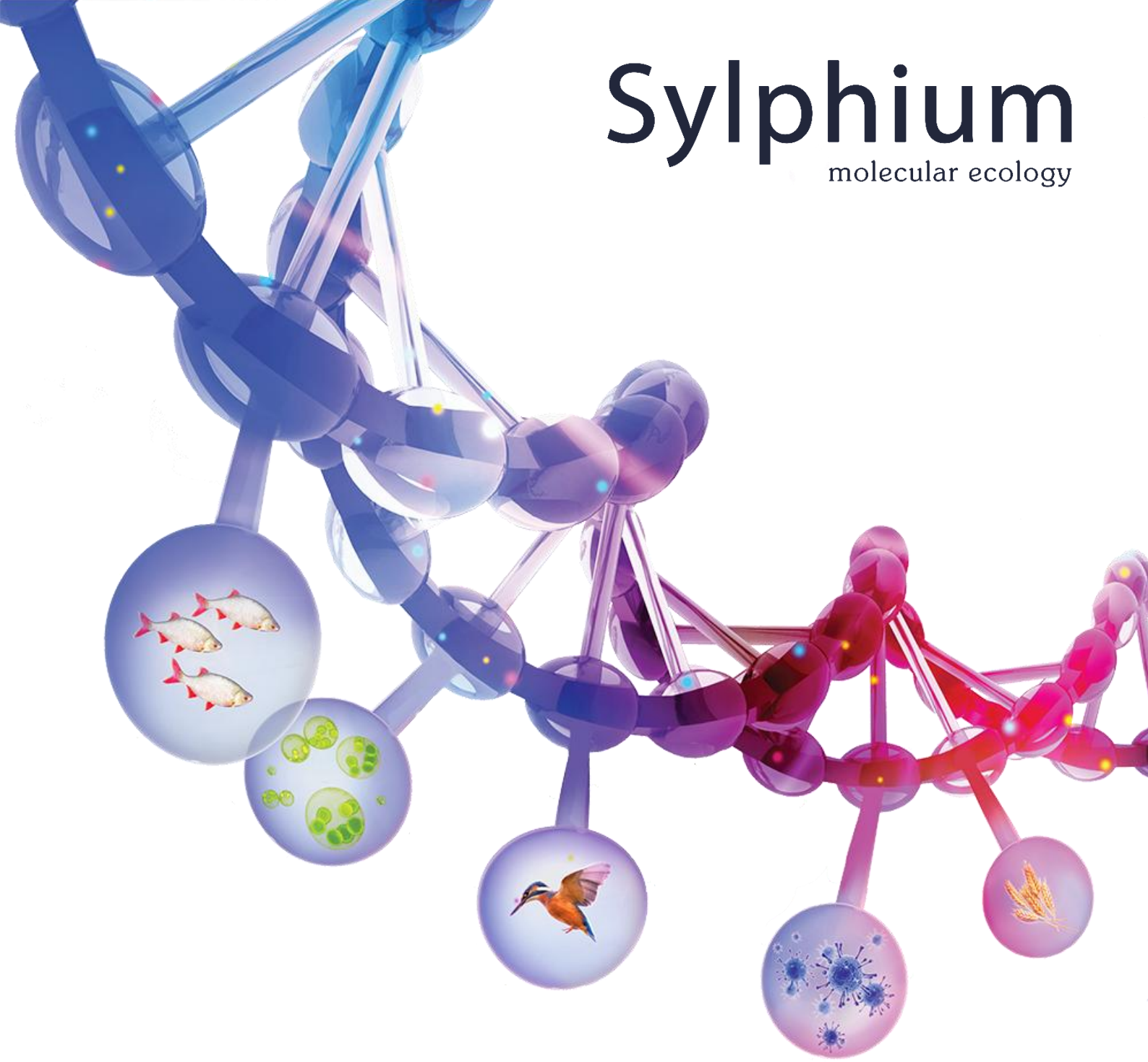

Environmental DNA Isolation Kit

with optional IPC quantification kit based on the eDNA master mix

#SYL002

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For general laboratory and research use only.

Quick Protocol for SYL002 eDNA Isolation Kit:

Before starting the eDNA isolation, please read through the entire protocol to understand all the steps. The full protocol can be found at: https://sylphium.com/webshop, or scan the QR code to go directly to the download page.

This protocol describes the eDNA isolation process from an eDNA Dual Filter. If you are using a different type of sample, begin at Step 4 after having prepared the 15 mL tubes in Step 2.

1. Incubate the eDNA dual filter capsule at 55 - 60 °C for 60 min or use pre-lysed samples (samples older than 1 day).
2. Prepare 15 mL tubes with 500 µL S2 and 2 mL tubes with 100 µL S3 + 900 µL S4 for each sample.
3. Connect a 60 mL syringe to the filter capsule outlet, remove the cap, and slowly draw in the liquid.
4. Add the syringe contents to the 15 mL tube containing S2, labelled with the sample number.
5. Centrifuge samples at ± 5000 x *g* for 5 minutes to form a bilayer.
6. Transfer up to 1 mL of the water layer to the mixture of S3 and S4, mix by shaking, and cool at -20 °C for 30 minutes.
7. Transfer the remaining water layer (± 1 mL) to a 2 mL tube as a backup and store at -20 °C.
8. Centrifuge samples in a cooled centrifuge (≤ 4 °C) at ≥ 13000 x *g* for 30 minutes (for eDNA). Remove liquid.
9. Add 500 µL S5, centrifuge at maximum speed for 5 minutes, and remove liquid. Repeat this step once.
10. Dissolve the pellet in 50 µL S6 for qPCR. Use 2 µL for analysis.

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1. Components

The Environmental DNA Isolation Kit enables fast and efficient isolation of environmental DNA from water samples. The procedure is suitable for both qualitative and quantitative PCR amplification. The complete procedure for isolation of total eDNA takes approximately 2 hours. The isolated DNA is dissolved in TE buffer and is ready for use in qPCR.

Optional: internal positive control (IPC) quantification kit

All Sylphium sample sets include an internal positive control (IPC) within the preservation solution (contained in the small syringe in the sampling set SYL009). The IPC consists of a small fragment of synthetic xenobiotic DNA. This DNA sequence is unknown to aquatic environments and does not interfere with the detection of target organisms. The IPC serves as an efficiency check for the DNA isolation procedure and as a quality and purity check of the isolated DNA. Optionally, the Internal Positive Control (IPC) Quantification Kit can be ordered alongside the eDNA Isolation Kit. Detection of the IPC is performed via the FAM channel.

1.1. Kit components DNA isolation

	Name	20 isolates	50 isolates	100 isolates
S1	Internal negative control[1]	1 x 15 mL	1 x 30 mL	2 x 30 mL
S2	Phase separation buffer	1 x 20 mL	1 x 40 mL	2 x 40 mL
S3	Precipitation buffer	1 x 4 mL	1 x 15 mL	2 x 15 mL
S4	Precipitation solution	1 x 30 mL	1 x 100 mL	2 x 100 mL
S5	Wash solution	1 x 30 mL	1 x 50 mL	2 x 50 mL
S6	eDNA conservation buffer	1 x 4 mL	1 x 20 mL	2 x 20 mL

1.2. Kit components internal positive control with eDNA master mix (optional)

	Name	20 isolates	50 isolates	100 isolates
S1	Positive control	1 x 500 µL	3 x 500 µL	5 x 500 µL
S2	eTaq qPCR mix (2x)	1 x 3 mL	3 x 3 mL	5 x 3 mL
S3	Primer-probe mix[2] (10x)	2 x 250 µL	3 x 250 µL	10 x 250 µL
S4	eTaq DNA polymerase	1 x 200 µL	3 x 200 µL	5 x 200 µL
S5	PCR water	1 x 2 mL	3 x 2 mL	5 x 2 mL

[1] Consists of the same substance as the conservation buffer in the environmental isolation kit
[2] For detection of the internal positive control (FAM dye)

1.3. Equipment and consumables to be supplied by user

Equipment	Consumables
• Centrifuge for 15 mL tubes capable of reaching approximately 5,000 x *g* (*optional*) • Centrifuge for microcentrifuge tubes (1.5 mL or 2 mL) capable of reaching 13,000 x *g* • Freezer -20°C • Incubator or heat block at 55°C • Pipettors • Personal protection equipment • PCR cooling rack (*optional*) • qPCR machine multiplex capable for detection of the FAM dye (*optional*)	• Collection tubes 15 mL with lid (*optional*) • Collection tubes 2 mL with lid • qPCR 96-wells reaction plates with seal cover • Disposable pipettor tips (filter pipette tips are recommended)

1.4. User manual information

We strongly recommend reading the detailed sections of this user manual before using the Enivornmental DNA Isolation Kit for the first time. Experienced users may refer to the "Quick Protocol for SYL002 Isolation Kit" as a quick-reference guide. Please note that this quick protocol is intended only as a supplementary tool for referencing support during the isolation procedure.

2. Product information

The Environmental DNA Isolation Kit enables fast and efficient isolation of environmental DNA from water samples. The procedure is suitable for both qualitative and quantitative PCR amplification. The complete procedure for isolation of total eDNA takes approximately 2 hours. The isolated DNA is dissolved in TE buffer and is ready for use in qPCR.

Optional: internal positive control (IPC) quantification kit

All Sylphium sample sets include an internal positive control (IPC) within the preservation solution (contained in the small syringe in the sampling set SYL009). The IPC consists of a small fragment of synthetic xenobiotic DNA. This DNA sequence is unknown to aquatic environments and does not interfere with the detection of target organisms. The IPC serves as an efficiency check for the DNA isolation procedure and as a quality and purity check of the isolated DNA. Optionally, the Internal Positive Control (IPC) Quantification Kit can be ordered alongside the eDNA Isolation Kit. Detection of the IPC is performed via the FAM channel.

2.1. qPCR

The provided primer and probe mix is a probe-based PCR method. During PCR amplification, one set of primers and a probe hybridize to synthetic DNA present in the preservation buffer (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 is 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 preservation buffer (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 then 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 in that case. The sampling and isolation procedure should then be repeated after cleaning the lab and equipment.

2.5. qPCR experimental plate setup

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

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. 96-wells plate setup for the analyses of 45 samples in duplicate (2-fold).

1.4. Kit Storage

Store reagents and kit components for DNA isolation at room temperature (15-30°C). Store all reagents and kit components of the IPC quantification kit in the dark and in a freezer (-15 to -20°C).

1.5. Notices and disclaimers

This product is developed, designed and sold for research purposes only. Sylphium Molecular Ecology (Trade name of Eelco Wallaart BV) 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).

3. Protocol

This kit can be used to isolate DNA from various source, with a specific protocol provided for each source.

Source	
eDNA Dual filter Capsule	For samples taken with the "Environmental sampling kit (SYL009)" using an eDNA Dual Filter Capsule. Used for detection of macro organisms like fish, amphibians, mammals, crustaceans, flatworms, etc.) **See section 3.2** for the protocol.
Sterivex unit samples	For samples taken with the "Environmental sampling kit (SYL001)" using a Sterivex unit. Used for detection of macro organisms like fish, amphibians, mammals, crustaceans, flatworms, etc.) **See section 3.3** for the protocol.
Conserved water samples	For samples taken with the "Microorganism sampling kit (SYL005)" using conserved water samples. Used for isolation of DNA from unicellular organisms like (cyano)bacteria and protozoa. **See section 3.4** for the protocol.
Droppings	For samples taken with the "Dropping sampling kit (SYL006)" using conserved dropping samples from mammals. **See section 3.5** for the protocol.

3.1. Precautions

- **Prevent contaminations**. Before starting your experiments, clean the work surface and all equipment with diluted thin bleach (1:10) or another DNA-removing agent to remove any DNA from previous experiments. Wear gloves to prevent DNA contamination between samples and clean your gloves with a paper towel soaked in diluted thin bleach between each handling step.
- **Minimize exposure to light**. It is recommended to limit light exposure during the preparation of the PCR plate and mixtures for the internal positive control. The PCR mix contains light-sensitive probes, and prolonged exposure to light may reduce the signal strength.
- **Keep samples cool for as long as possible**. After adding Taq DNA polymerase to the internal positive control mixtures, store them in a PCR cooling rack to prevent non-specific hybridization of primers and probe, as well as unintended extension by the DNA polymerase at room temperature. While Taq DNA polymerase is stable for at least a month at room temperature in its storage buffer, its stability in the PCR mixture is reduced to only a few hours at room temperature.

3.2. Procedure DNA isolation from water (eDNA dual filter capsule)

1. **Incubate the eDNA dual filter capsule containing the preservation liquid at 55 - 60°C for 60 minutes to release all DNA from cell material.** Samples older than one day have already lysed at room temperature and can therefore be used directly in step 4.

2. **Prepare 15 mL tubes with 500 µL S2 (phase separation solution).** Prepare one tube for each sample and one additional tube for the Internal Negative Control (INC). Preparing these tubes before handling the samples prevents contamination of the kit reagents.

   Note: *If a centrifuge for 15 mL tubes is not available, the phase separation step can also be performed using 2 x 2 mL tubes per sample. In that case, prepare two 2 mL tubes per sample, each containing 250 µL S2. After extracting the sample liquid from the filter capsule, divide the sample liquid over the two 2 mL tubes in step 7. Continue the protocol for both tubes. After DNA isolation and dissolution of the DNA pellet in 50 µL S6 (step 14), pool the two tubes by combining the dissolved DNA into one tube.*

3. **Prepare 2 mL tubes with 100 µL S3 (precipitation buffer) and 900 µL S4 (precipitation solution).** Prepare one tube for each sample and one additional tube for the Internal Negative Control (INC). Preparing these tubes before handling the samples prevents contamination of the kit reagents.

4. **Remove the cap from the outlet of the filter capsule and connect the 60 mL syringe (used during sampling) to the outlet.**

5. **Hold the filter capsule above the syringe, remove the second cap, and slowly pull the plunger to draw the liquid inside the syringe.** Take care not to spill any of the DNA-containing liquid, as this can lead to contamination of other samples. If a spill occurs, clean all contaminated surfaces and materials with a DNA decontamination reagent.

6. **To maximize liquid extraction from the filter capsule, slowly push and pull the a few times (allowing air into the capsule and liquid out).** Take care not to push the liquid back into the filter capsule.

7. **Transfer the liquid inside the syringe to the 15 mL tube containing 500 µL S2 (phase separation solution).** Label this tube with the corresponding sample number written on the sample bag.

8. **Take the additional 15 mL tube and add 1 mL S1 (internal negative control) to this tube. Mix by shaking and label this tube as INC.** This is the Internal Negative Control and should be processed in the following steps in the same way as the other samples.

9. **Centrifuge the samples at ± 5.000 x *g* for 5 minutes.** A bilayer will form: the upper layer is the aqueous phase containing the DNA, while a small solid layer of denatured proteins may form between the two liquid layers.

   Note: *If 2 mL tubes were used instead of 15 mL tubes (see step 2), centrifuge these tubes at maximum speed (≥13.000 x g) for approximately 30 minutes to achieve optimal phase separation. Proceed with care when transferring the upper water layer to avoid disturbing the protein/CIA interface.*

10. **Transfer a maximum of 1 mL of the aqueous layer to the prepared mixture of S3 and S4. Mix well by shaking and cool down to -20°C for at least 30 minutes.** During this step, DNA will precipitate while salts and other unwanted substances will remain in the solution. Avoid transferring the protein or the CIA (bottom) layer into the new tube.

11. **Transfer the remaining aqueous layer (± 1 mL) to an empty 2 mL tube and store it at -20°C as a backup.** If no backup is required, this sample can also be used for extraction to maximize eDNA recovery. In that case, prepare additional tubes as described in step 2 if these samples will be processed further.

12. **Centrifuge the samples for 30-45 minutes (for small eDNA fragments) or 5 minutes for genomic DNA (e.g. from bacteria) at maximum speed (≥ 13.000 x *g*). Carefully remove the supernatant by decanting and pipetting.** Be aware that the DNA pellet may not be firmly attached to the tube. It is important to remove all remaining liquid to improve the sensitivity of subsequent qPCR detection.

13. **Add 500 µL S5 (wash solution) to the pellet, mix, and centrifuge at maximum speed (≥ 13.000 x *g*) for 5 minutes. Completely remove the supernatant by pipetting. Repeat this wash step to eliminate PCR-inhibiting substances.** Be careful, as the DNA pellet may still not be firmly attached to the tube.

14. **Dissolve the pellet in 50 µL S6 (eDNA conservation buffer) and use 2 µL for qPCR.** If multiple tubes were processed for a single sample, pool the dissolved DNA from these tubes into one and use 2 µL of the pooled DNA extract for qPCR.

3.3. Procedure DNA isolation from water (Sterivex unit samples)

1. **Incubate the filter unit containing the preservation liquid at 55 - 60°C for 60 minutes to release all DNA from cell material.** Samples older than one day have already lysed at room temperature and can therefore be used directly in step 4.

2. **Prepare 15 mL tubes with 500 µL S2 (phase separation solution).** Prepare one tube for each sample and one additional tube for the Internal Negative Control (INC). Preparing these tubes before handling the samples prevents contamination of the kit reagents.

   Note: If a centrifuge for 15 mL tubes is not available, the phase separation step can also be performed using 2 x 2 mL tubes per sample. In that case, prepare two 2 mL tubes per sample, each containing 250 µL S2. After extracting the sample liquid from the filter unit, divide the sample liquid over the two 2 mL tubes in step 7. Continue the protocol for both tubes. After DNA isolation and dissolution of the DNA pellet in 50 µL S6 (step 11), pool the two tubes by combining the dissolved DNA into one tube.

3. **Prepare 2 mL tubes with 100 µL S3 (precipitation buffer) and 900 µL S4 (precipitation solution).** Prepare one tube for each sample and one additional tube for the Internal Negative Control (INC). Preparing these tubes before handling the samples prevents contamination of the kit reagents.

4. **Remove both caps and connect the small syringe to the luer-lock connection of the Sterivex filter. Slowly pull the plunger until all the liquid is inside the syringe.** Take care not to spill any of the DNA-containing liquid, as this can lead to contamination of other samples. If a spill occurs, clean all contaminated surfaces and materials with a DNA decontamination reagent.

5. **Transfer the liquid inside the syringe to the 15 mL tube containing 500 µL S2 (phase separation solution).** Label this tube with the corresponding sample number written on the sample bag.

6. **Take the additional 15 mL tube and add 1 mL S1 (internal negative control) to this tube. Mix by shaking and label this tube as INC.** This is the Internal Negative Control and should be processed in the following steps in the same way as the other samples.

7. **Centrifuge the samples at ± 5.000 x *g* for 5 minutes.** A bilayer will form: the upper layer is the aqueous phase containing the DNA, while a small solid layer of denatured proteins may form between the two liquid layers.

   Note: If 2 mL tubes were used instead of 15 mL tubes (see step 2), centrifuge these tubes at maximum speed (≥13.000 x g) for approximately 30 minutes to achieve optimal phase separation. Proceed with care when transferring the upper water layer to avoid disturbing the protein/CIA interface.

8. **Transfer a maximum of 1 mL of the aqueous layer to the prepared mixture of S3 and S4. Mix well by shaking and cool down to -20°C for at least 30 minutes.** During this step, DNA will precipitate while salts and other unwanted substances will remain in the solution. Avoid transferring the protein or the CIA (bottom) layer into the new tube.

9. **Centrifuge the samples for 30-45 minutes (for small eDNA fragments) or 5 minutes for genomic DNA (e.g. from bacteria) at maximum speed (≥ 13.000 x *g*). Carefully remove the supernatant by decanting and pipetting.** Be aware that the DNA pellet may not be firmly attached to the tube. It is important to remove all remaining liquid to improve the sensitivity of subsequent qPCR detection.

10. **Add 500 µL S5 (wash solution) to the pellet, mix, and centrifuge at maximum speed (≥ 13.000 x *g*) for 5 minutes. Completely remove the supernatant by pipetting. Repeat this wash step to eliminate PCR-inhibiting substances.** Be careful, as the DNA pellet may still not be firmly attached to the tube.

11. **Dissolve the pellet in 50 µL S6 (eDNA conservation buffer) and use 2 µL for qPCR.** If multiple tubes were processed for a single sample, pool the dissolved DNA from these tubes into one and use 2 µL of the pooled DNA extract for qPCR.

3.4. Procedure DNA isolation from water (conserved water samples)

1. **Prepare 15 mL tubes with 500 µL S2 (phase separation solution). Prepare one tube for each sample and one additional tube for the Internal Negative Control (INC).** Preparing these tubes before handling the samples prevents contamination of the kit reagents.

   Note: *If a centrifuge for 15 mL tubes is not available, the phase separation step can also be performed using 2 x 2 mL tubes per sample. In that case, prepare two 2 mL tubes per sample, each containing 250 µL S2. Divide the lysed and conserved water sample over the two 2 mL tubes in step 4. Continue the protocol for both tubes. After DNA isolation and dissolution of the DNA pellet in 50 µL S6 (step 10), pool the two tubes by combining the dissolved DNA into one tube.*

2. **Prepare 2 mL tubes with 100 µL S3 (precipitation buffer) and 900 µL S4 (precipitation solution). Prepare one tube for each sample and one additional tube for the Internal Negative Control (INC).** Preparing these tubes before handling the samples prevents contamination of the kit reagents.

3. **Incubate the tube containing the preservation liquid at 55 - 60°C for 60 minutes to release all DNA from cell material.** If the tube was incubated overnight at room temperature, this step is not necessary.

4. **Add 2 mL of the lysed and conserved water sample to the tube prepared in step 1. Close the tube with the cap and mix thoroughly by shaking.** This solution will remove PCR-inhibiting substances.

5. **Add 2 mL S1 (internal negative control) to the additional 15 mL tube and label this tube as INC. Close the tube with the cap and mix thoroughly by shaking.** This is the Internal Negative Control (INC) and should be handled in the same wat as the other samples starting from step 3.

6. **Centrifuge the samples and INC at ± 5.000 x *g* for 5 minutes**. A bilayer will form: the upper layer is the aqueous phase containing the DNA, while a small solid layer of denatured proteins may form between the two liquid layers.

   Note: *If 2 mL tubes were used instead of 15 mL tubes (see step 2), centrifuge these tubes at maximum speed (≥13.000 x g) for approximately 30 minutes to achieve optimal phase separation. Proceed with care when transferring the upper water layer to avoid disturbing the protein/CIA interface.*

7. **Transfer exactly 1 mL of the aqueous layer to the prepared mixture of S3 and S4. Mix well by shaking and cool down to -20°C for at least 30 minutes.** During this step, DNA will precipitate while salts and other unwanted substances will remain in the solution. Avoid transferring the protein or the CIA (bottom) layer into the new tube.

8. **Centrifuge the samples for 30-45 minutes (for small eDNA fragments) or 5 minutes for genomic DNA (e.g. from bacteria) at maximum speed (≥ 13.000 x g). Carefully remove the supernatant by decanting and pipetting.** Be aware that the DNA pellet may not be firmly attached to the tube. It is important to remove all remaining liquid to improve the sensitivity of subsequent qPCR detection.

9. **Add 500 µL S5 (wash solution) to the pellet, mix, and centrifuge at maximum speed (≥ 13.000 x g) for 5 minutes. Completely remove the supernatant by pipetting.** Be careful, as the DNA pellet may still not be firmly attached to the tube.

10. **Dissolve the pellet in 50 µL S6 (eDNA conservation buffer) and use 2 µL for qPCR.** If multiple tubes were processed for a single sample, pool the dissolved DNA from these tubes into one and use 2 µL of the pooled DNA extract for qPCR.

3.5. Procedure DNA isolation from dropping samples

1. **Prepare 2 mL tubes with 100 µL S3 (precipitation buffer) and 900 µL S4 (precipitation solution). Prepare one tube for each sample and one additional tube for the Internal Negative Control (INC).** Preparing these tubes before handling the samples prevents contamination of the kit reagents.
2. **Incubate the sampling tube containing the preservation liquid at 55 - 60°C for 60 minutes to release all DNA from cell material.** Samples older than one day have already lysed at room temperature and can therefore be used directly in step 4.
3. **Transfer 50 µL of the dissolved dropping sample to a new 2 mL tube containing 1 mL of water.** Due the high concentration of target DNA in dropping samples, it is recommended to perform this dilution step to prevent inhibition of the PCR reaction and to reduce the risk of contamination when handing high concentrations of target DNA.
4. **Add 500 µL S2 (phase separation solution) to the diluted dropping sample. Close the tube with the cap and mix thoroughly by shaking.** This solution will remove PCR-inhibiting substances.
5. **Take an additional 2 mL tube and add 1 mL S1 (Internal negative control) and 500 µL S2 (Phase separation solution) to this tube. Label this tube as INC, close it with the cap, and mix thoroughly by shaking.** This is the Internal Negative Control (INC) and it should be handled in the following steps in the same way as the other samples starting from step 3.
6. **Centrifuge the samples and INC at maximum speed (≥ 13.000 x g) for 5 minutes.** A bilayer will form: the upper layer is the aqueous phase containing the DNA, while a small solid layer of denatured proteins may form between the two liquid layers.
7. **Transfer a maximum of 1 mL of the aqueous layer to the prepared mixture of S3 and S4. Mix well by shaking and cool down to -20°C for at least 30 minutes.** During this step, DNA will precipitate while salts and other unwanted substances will remain in the solution. Avoid transferring the protein or the CIA (bottom) layer into the new tube.
8. **Centrifuge the samples for 30-45 minutes (for small eDNA fragments) or 5 minutes for genomic DNA (e.g. from bacteria) at maximum speed (≥ 13.000 x g). Carefully remove the supernatant by decanting and pipetting.** Be aware that the DNA pellet may not be firmly attached to the tube. It is important to remove all remaining liquid to improve the sensitivity of subsequent qPCR detection.
9. **Add 900 µL S5 (wash solution) to the pellet, mix, and centrifuge at maximum speed (≥ 13.000 x *g*) for 5 minutes. Completely remove the supernatant by pipetting.** Be careful, as the DNA pellet may still not be firmly attached to the tube.
10. **Dissolve the pellet in 50 µL S6 (eDNA conservation buffer) and use 2 µL for qPCR.**

3.6. Quality check isolated DNA

To prevent false negative results, a quality control is required on the isolated DNA samples. The kit is optionally provided with a qPCR quality control check (Internal positive control; IPC) based on a fragment of xenobiotic synthetic DNA present in the preservation solution. This quality control will provide information on isolation efficiency and the presence of inhibiting substances in the isolate.

3.6.1. 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.

x µL **sample +** x µL **S6**	2x diluted
x µL **2x diluted** + x µL **S6**	4x diluted
x µL **4x diluted** + x µL **S6**	8x diluted

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.

3.6.2. Precautions

- **Prevent contaminations**. Before starting your experiments, clean the table surface and all equipment with thin bleach (5 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.
- **Prevent breakdown of Taq DNA polymerase and unwanted products.** Put the PCR plate into the PCR machine within 5-10 minutes after preparation to prevent unwanted non-specific hybridization of primers and probe. This would also prevent breakdown of the Taq DNA polymerase.

3.6.3. Procedure

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	30°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.

3.7. 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).

3.7.1. 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.

3.7.2. Determination of isolation efficiency

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

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

	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:

- 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:

$$\frac{407.5 \cdot 100}{2 \cdot 2 \cdot 11000} * 100 = 92.6\% \; efficiency$$

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