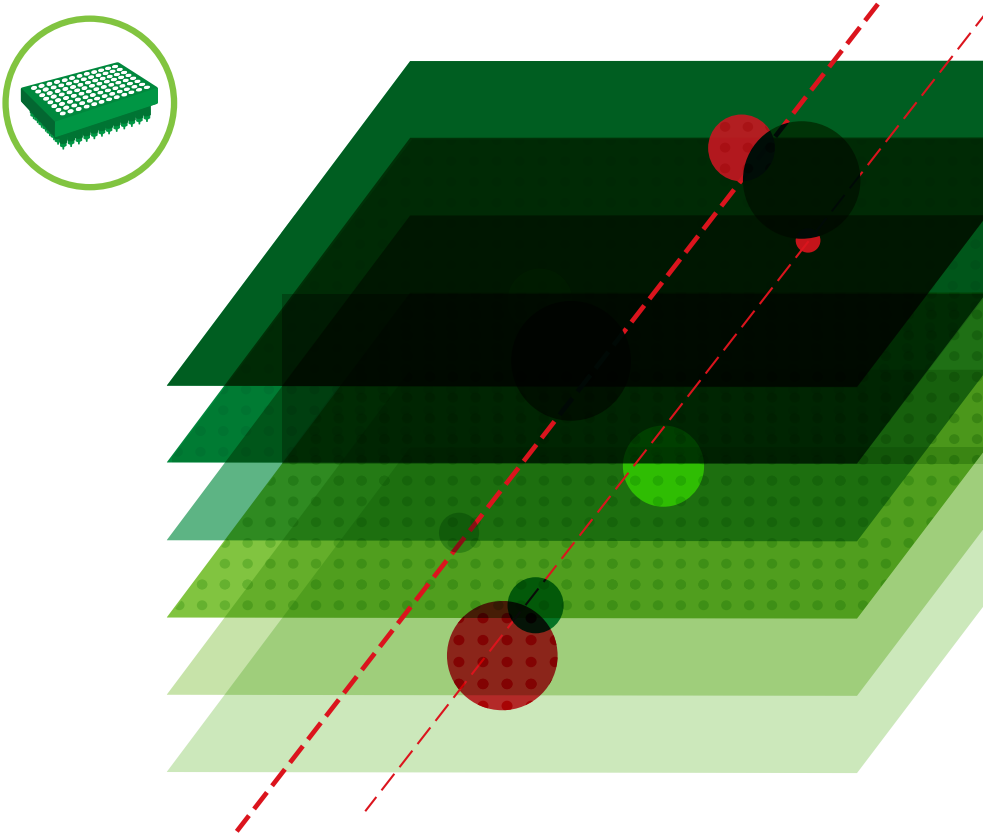
EXTRACTME®
GENOMIC DNA 96-WELL KIT

Cat. No. EM33
Version: 1.2018

Kit for genomic DNA isolation from a variety of sample sources in 96-well format



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I. INTENDED USE

The ***EXTRACTME*** **GENOMIC DNA 96-WELL KIT** is designed for a rapid and efficient purification of high quality genomic, mitochondrial, bacterial, parasite or viral DNA from solid tissues (fresh, frozen, formalin-preserved or paraffin-embedded), physiological fluids (urine, cerebrospinal fluid, peritoneal fluid, pleural fluid, sputum), fresh and frozen blood (human and mammalian), human and animal mucosa membrane swabs (including buccal, nasal, pharyngeal and vaginal swabs), semen, hair, rodent tails, insects, bacteria, yeast and cell cultures. The isolation protocol and buffer formulations have been optimized for high isolation efficiency and DNA purity. The product is intended for research use only.

II. COMPONENTS OF THE KIT AND STORAGE CONDITIONS

NUMBER OF ISOLATIONS	192 ISOLATIONS	960 ISOLATIONS	Storage Conditions[1]
Catalogue number	EM33-192	EM33-960	
GL Buffer (Lysis Buffer)	72 ml	360 ml	RT
⚠ **Proteinase K** ** (lyophilized)	1 tube	5 tubes	-20°C[2]
Proteinase Buffer	5.8 ml	29 ml	RT
⚠ **RNase A*** (lyophilized)	1 tube	5 tubes	-20°C[3]
RNase Buffer	920 µl	4.6 ml	RT
GB Buffer (conc.)*** (Binding Buffer)	38 ml	184 ml	RT
GW1 Buffer (conc.)*** (Wash Buffer 1)	69 ml	2x 173 ml	RT
GW2 Buffer (conc.)*** (Wash Buffer 2)	35 ml	2x 87 ml	RT
Elution Buffer	39 ml	5x 39 ml	RT
DNA Binding Plates	2 pcs	10 pcs	RT
Collection Plates	2 pcs	10 pcs	RT
DNA Elution Plates	2 pcs	10 pcs	RT
Elution Adhesive Seals	2 pcs	10 pcs	RT

[1] RT – room temperature (+15°C to +25°C)

[2] After reconstitution, **Proteinase K** should be kept **at -20°C**.

[3] After reconstitution, **RNase A** should be kept **at +4°C for short term storage (several days) or in aliquots at -20°C**.

* Prior to the first use, add 920 µl RNase Buffer to a tube containing RNase A lyophilizate.

** Prior to the first use, add 5.8 ml Proteinase Buffer to a tube containing Proteinase K lyophilizate.

*** Prior to the first use, add appropriate amount of **96–100% ethanol** to **GB, GW1 and GW2 Buffer**; for details, see the instructions on the bottle label and in the table below. Marking the bottle after adding the alcohol is recommended.

NUMBER OF ISOLATIONS	192 ISOLATIONS	960 ISOLATIONS
Catalogue number	EM33-192	EM33-960
GB Buffer	38 ml	184 ml
96–100% ethanol	57 ml	276 ml
Total volume	95 ml	460 ml
GW1 Buffer	69 ml	2 x 173 ml
96–100% ethanol	69 ml	2 x 173 ml
Total volume	138 ml	2 x 346 ml
GW2 Buffer	35 ml	2 x 87 ml
96–100% ethanol	82 ml	2 x 201 ml
Total volume	117 ml	2 x 288 ml

In order to avoid evaporation, ensure that the buffer bottles are tightly closed before storing.

Expiry date

Under proper storage conditions the kit will remain stable for at least 12 months from opening or until the expiry date.

III. ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED

- 96–100% PFA ethanol
- sterile microcentrifuge tubes (1.5–2 ml)
- multichannel pipettes and sterile DNase-free tips
- reagent reservoirs for multichannel pipets
- disposable gloves
- microcentrifuge with rotor for plates (≥ 3000 x g)
- thermal heating block or water bath (up to 70°C)
- vortex mixer
- 96 deep-well plates for sample preparation
- adhesive seals
- vacuum manifold and vacuum pump (producing pressure of -400 to -600 mbar) or automated liquid handling workstations

Might be also needed:

- xylene – paraffin blocks
- PBS buffer – cell cultures, formalin preserved tissues, physiological fluids
  Preparation: dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 800 ml H_2O. Set the pH to 7.4 with HCl. Fill up to 1000 ml and autoclave. Store at +4°C.
- 1M DTT – hair and semen
  Preparation: dissolve 1.54 g DTT in 10 ml H_2O. Aliquot and store at -20°C.
- sterile swab sticks
- RBC Lysis Buffer (Red Blood Cell Lysis Buffer) (EM05-RBC) – whole blood
- YS Buffer (Spheroplast Buffer) (EM10-YS) and lyticase – yeast
- lysostaphin – S*taphylococcus* species
- Lysozyme – Gram-positive bacteria
- scissors, scalpel
- bead-beating tubes with ceramic filling (HPLM100, HPLM100a)
- tissue homogenizer for 2 ml tubes
- mechanical homogenizer with knives
- thermomixer (shaking orbit of 2 mm minimum)
- 50–75 ml smooth-stroke mortar with fitted piston
- liquid nitrogen or dry ice
- centrifuge with a rotor for 10–15 ml tubes (physiological fluids, cell cultures)

IV. PRINCIPLE

DNA purification procedure consists of four steps and utilizes 96 minicolumns plates with membranes which efficiently and selectively bind nucleic acids. In the first step, biological material is lysed by Proteinase K in optimized GL Buffer. At this stage all the cellular membranes and proteins are degraded. When it is necessary to remove RNA the use of RNase A is recommended.
After the addition of chaotropic salts, the lysate is applied to 96 minicolumn plate's membrane and the DNA is bound. The two-step washing stage effectively removes impurities and enzyme inhibitors. Purified DNA is eluted with the use of either a low ionic strength buffer (Elution Buffer) or water (pH 7.0-9.0) and may be used directly in all downstream applications such as PCR, quantitive real-time PCR, pharmacogenic research, Southern blotting, single-nucleotide polymorphism (SNP), short tandem repeat (STR) genotyping, DNA sequencing, enzymatic restriction, ligation and so forth, or stored until ready to use.

V. QUALITY CONTROL

The quality of each production batch (LOT) of the ***E XTRACTME* GENOMIC DNA 96-WELL KIT** is tested with the use of standard QC procedures. Purified DNA concentration and quality are evaluated by gel electrophoresis and spectrophotometer.

VI. PRODUCT SPECIFICATIONS

SAMPLE MATERIAL

- fresh or frozen solid tissue: 1–20 mg depends on type of tissue (10 mg optimally)
- formalin-preserved tissue: 1–20 mg depends on type of tissue (10 mg optimally)
- paraffin-embedded tissue: 1–20 mg depends on type of tissue (10 mg optimally)
- physiological fluids (urine, peritoneal fluid, cerebrospinal fluid, pleureal fluid, sputum): up to 5 ml
- cell culture: 10^3–10^6 cells
- broth or plate bacterial (up to 10^9 cells) or yeast (up to 10^8 cells) culture, frozen cell pellet
- buccal, nasal, pharyngeal, vaginal, blood and saliva swabs or semen
- fresh or frozen blood: up to 1 ml
- hair: 10–30 mg
- insects: 1–10 mg

EFFICIENCY

up to 20 µg

BINDING CAPACITY

50 µg DNA per well

TIME REQUIRED

- approx. 35 minutes for purification using centrifuge (lysis time not included)
- approx. 25 minutes for purification using vacuum manifold (lysis time not included)

DNA PURITY

A_{260}/A_{280} ratio = 1.7–1.9

VII. SAFETY PRECAUTIONS

- Biological samples are treated as a biohazardous material of its potential pathogen content or health and life-threatening substances. While working with biological material it is essential to comply with all safety requirements for working with biohazardous material.
- It is advisable to conduct the entire isolation procedure in a Class II Biological Safety Cabinet or at laboratory burner as well as wearing disposable gloves and suitable lab coat at all times.
- Use of sterile pipette filter tips is recommended.
- Avoid the cross-contamination of DNA between minicolumns.
- Guanidine salts' residues may form highly reactive components when combined with oxidation compounds. In case of spillage, clean the surface with a detergent-water solution.
- In case of spillage of a liquid containing microorganisms, clean the contaminated surface with a detergent-water solution.

VIII. RECOMMENDATIONS AND IMPORTANT NOTES

Vacuum manifold use

Establish a reliable vacuum source for the ***EXTRACTME*** **GENOMIC DNA 96-WELL KIT** vacuum manifold protocol. The manifold may be used with a vacuum pump or water aspirator. Use a vacuum pressure of -400 to -600 mbar or reduce the vacuum pressure until a flow rate 1–2 drops per second is achieved. Using higher vacuum pressure than recommended may cause sample splattering, while using lower vacuum pressure will affect the elution resulting in lower recovery.

Keep all unused well in DNA Binding Plate sealed with an adhesive seal during purification to obtain a uniform vacuum and avoid contaminating unused wells.

Pressure conversions

	Recommended pressure (mbar)
	-400 to -600
Conversion from millibars	**Multiply by**
Millimeters of Mercury (mmHg)	0.75
Kilopascals (kPa)	0.1
Inches of Mercury (inchHg)	0.0295
Torrs (Torr)	0.75
Atmospheres (atmos)	0.000987
Pounds per Square Inch (psi)	0.0145

To check the vacuum pressure place an unused DNA Binding Plate on top of the vacuum manifold. Seal the plate with an adhesive seal. Apply vacuum and check the vacuum pressure on the vacuum regulator. Adjust the vacuum pressure on the regulator to obtain the recommended pressure.

DNA elution

An optimal volume of Elution Buffer used should be chosen in accordance with the quantity of sample material and final DNA concentration expected.

Tissue and cell culture: The use of 100–200 µl Elution Buffer is recommended when extracting from 2–10 mg of tissue or $<10^4$ cells. However, while extracting from 10-20 mg of tissue or 10^4–10^6 cells the volume of Elution Buffer should be increased to 200 µl.

Swab and Semen: The use of 50–100 µl Elution Buffer is recommended. The quantity of purified DNA depends on sample type and number of cells it contains; quality, however, on site's features the swab was taken from and interindividual diversity. Usually the isolation efficiency from a single buccal swab is equal to 1–3 µg of DNA whereas, from 150 µl of semen to 2–7 µg.

Bacteria and Yeast: The use of 50–100 µl Elution Buffer is recommended when extracting from no more than 10^9 cells (bacteria) or 10^8 cells (yeast).

Blood: The use of 50–100 µl of Elution Buffer while extracting from 100–500 µl of blood is recommended. However, while extracting from 500–1000 µl of blood the volume of Elution Buffer should be increased to 200 ul.

The quantity of purified DNA depends on the type of sample and the number of white blood cells it contains (patient's age, his health condition, sample transport conditions, as well as storage time and method). Usually the isolation efficiency from 200 µl of blood sample from a healthy person is equal to 3–10 µg of DNA. A greater amount of DNA may be acquired from clinical samples containing increased number of white blood cells ($3x\ 10^6$–$1x\ 10^7$ cells/ml).

If a high DNA concentration is desired, the elution volume may be reduced. However, it may reduce the efficiency of the DNA retrieval. It is essential to apply Elution Buffer precisely to the center of the membrane.

When isolating from a insignificant amount of sample material, use 200 µl Elution Buffer and precipitate the DNA according to standard procedures.

In order to maximize the DNA retrieval, heat Elution Buffer to 80°C and incubate it on the membrane for 10 minutes.

If full DNA retrieval is required, a second, 50–200 µl elution should be performed. For the second elution, repeat steps 10–13 of the Isolation Protocol while purification using centrifuge (see section XI.IIA) or steps 16–19 while purification using vacuum manifold (see seciotn XI.IIB), placing the DNA Binding Plate in a new Elution Plate (provided by the user).

Elution Buffer does not contain EDTA, which may interfere with some enzymatic reactions. If desired, DNA can alternatively be eluted from the minicolumn with nuclease-free water, pH 7.0–9.0, or 5–10 mM of TE buffer.

RNA contamination

Most fresh or frozen tissue contains more RNA than DNA, especially metabolically active tissues like glands, nerve tissue and epithelium. RNA may interfere with some enzymatic reactions, but does not inhibit PCR.If an RNA-free DNA sample is desired, add 4 µl of RNase A solution and incubate at 37°C for 5 minutes (step 4 of the Isolation Protocol, section XI.I).

Foam formation in GL Buffer

The non-ionic detergent content of lysis buffer may cause a foam appearance after homogenization, vortexing or intensive pipetting. In order to eliminate the foam, centrifuge the buffer at 11 000 x g for 60 s.

Recovery of elution volume

A reduction in recovered volume relative to the starting volume is normal when eluting plates. The table below presents the typical volume of elute recovered from the initial volume of Elution Buffer, when following the kit protocol.

Elution Buffer volume	Recovered elution buffer (±5 µl)	
	Centrifuge	Vacuum
50 µl	35 µl	25 µl
75 µl	65 µl	50 µl
100 µl	90 µl	80 µl
125 µl	115 µl	95 µl
150 µl	140 µl	120 µl
175 µl	155 µl	145 µl

Balance the centrifuge

The use of second DNA Binding Plate placed onto a Collection Plate avoids the need to balance the centrifuge.

IX. SAMPLE PREPARATION

A. FRESH OR FROZEN SOLID TISSUE

Quantity: 1–20 mg depends on type of tissue (10 mg optimally);
Sample material: animal or human tissues (muscle, liver, heart, brain, kidney, bone marrow and others).
(preparation for one minicolumn well on the 96 well plate)

General procedure, applies to all homogenization methods

Divide a tissue into small fragments with tweezers and scissors or a scalpel. Follow one of the homogenization methods described below or go to step 1 of the Isolation Protocol (section XI.I).

Liquid nitrogen, dry ice (LN_2, CO_2)

1. Place tissue frozen in LN_2 or CO_2 in a previously chilled, sterile mortar. Using a chilled piston, carefully, but firmly crush the tissue into smaller pieces and then, into a pulp.
2. Transfer the powder obtained this way into a well of a 96 deep well plate (provided by the user) containing **375 µl GL Buffer** and go to step 2 of the Isolation Protocol (section XI.I).

   ⚠ *After pulping, a thin, sticky layer may be formed, rather than a powder. If this occurs, add 375 µl of GL Buffer to a mortar and reconstitute the tissue by pipetting afterwards transfer the lysate into a sterile, 96 deep well plate. Remember to retrieve the tissue remains from the piston.*

Homogenization using a mechanical homogenizer equipped with knives

1. Place the tissue in a 2 ml tube, add **100 µl GL Buffer** and carefully homogenize with a sterile homogenizer tip.
2. After homogenization, retrieve the tissue remains from the knife tip by washing it with **275 µl GL Buffer**. Combine the fractions obtained this way and transfer the entire volume to a well of a 96 deep well plate (provided by the user).
3. Continue the isolation from step 2 of the Isolation Protocol (section XI.I).

Homogenization using a bead-beating tube

We recommend the use of homogenization **bead-beating tubes** (HPLM100, HPLM100a) pre-filled with ceramic beads.

1. Add **150 µl GL Buffer** to a 2 ml ceramic bead-beating tube and suspend the sliced tissue in the buffer. Place the tube in a tissue homogenizer and homogenize at 3000–4000 x g for 30 s. If necessary, repeat the procedure.
   - ⚠ *If evaluation of the degree of tissue fragmentation is compromised by foam formation, centrifuge the tube at 11 000 x g.*
   - ⚠ *If a tissue homogenizer is not available, the tissue may be homogenized by vortexing, using the appropriate 2 ml tube adaptor, for at least 5 min at maximum speed.*
2. Add **225 µl GL Buffer** and mix by pipetting.
3. Add **25 µl Proteinase K** and **4 µl RNase A**. Mix by vortexing for 20 s. Incubate at 37°C for 5 min.
4. Add **400 µl GB Buffer** and mix thoroughly for 10 s.
5. Centrifuge for 120 s at 11 000–21 000 x g.
6. Transfer the supernatant to a well of a 96 deep well plate (provided by the user).
   - ⚠ *Carefully pipette the supernatant by placing a 200 µl pipette tip (N.B.: a 1000 µl tip may become clogged by the beads) into the filling. Tissue remains should either lie on one side of the tube or at the bottom.*
7. Continue the isolation from step 1 of the Protocol for centrifugation or vacuum manifold (section XI.IIA or XI.IIB).

B. FORMALIN-PRESERVED TISSUE

Quantity: 1-20 mg depends on type of tissue (10 mg optimally);
Sample material: animal tissues preserved in 4% formalin under cooling conditions.

1. Remove the formalin by washing it from the tissue two or three times, using PBS buffer or H_2O.
   - ⚠ *Formalin is an irritating agent. Do not monitor its removal by inhaling the fumes from a tube.*
2. Continue the isolation following the procedure described for fresh or frozen solid tissue in section IXA.

C. PARAFFIN-EMBEDDED TISSUE

Quantity: 1–20 mg depends on type of tissue (10 mg optimally);
Sample material: animal tissue embedded in paraffin block by standard histological procedure.
(preparation for one minicolumn well on the 96 well plate)

1. Prepare a fragment no larger than 30 mg by cutting it out of a paraffin block and place it in a 2 ml tube.
2. Add **1 ml xylene**. Mix by vortexing for 30 s.
   ⚠ *Xylene is toxic, irritating and very inflammable. Conduct the procedure in a working fume cupboard.*
3. Centrifuge at 15 000 x g for 5 min. Remove the supernatant by pipetting.
4. Repeat steps 2–3.
5. Add **1 ml 96–100% ethanol**. Mix by pipetting or vortexing for 15 s.
6. Centrifuge at 15 000 x g for 120 s. Remove the supernatant by pipetting.
7. Repeat steps 5–6.
8. In order to remove the remains of the ethanol, dry the pellet in the open tube at **50°C** for 5–20 min.
9. Add **375 µl GL Buffer** and mix by vortexing for 20 s.
10. Transfer the entire volume to a well of a 96 deep well plate (provided by the user) and continue the isolation from step 2 of the Isolation Protocol (section XI.I).

D. CELL CULTURES

Quantity: 10^3-10^6 cells;
Sample material: cell suspension or adherent cells, fresh or frozen at -80°C or -196°C.
(preparation for one minicolumn well on the 96 well plate)

1. Thaw frozen cells at **37°C** or remove growth medium from the culture plate and harvest adherent cells by trypsinization or a method of choice or centrifuge the cell suspension at 500 x g for 5 min. Remove the supernatant. If a compact cell pellet is not formed, wash the cells twice with **1 ml cold PBS buffer**.
   Add **375 µl GL Buffer**. Mix thoroughly by vortexing for 30 s followed by pipetting.
   ⚠ *In some cases, where the cells tend to form either syncytia (myoblasts) or tight connections (epithelial cells) or they are high in number (approx. 10^6), it may be difficult to resuspend them in a GL Buffer. Should this happen pipette carefully, using a ≥1000 µl pipette tip or a sterile syringe.*
2. Transfer suspension obtained this way to a well of a 96 deep well plate (provided by the user) and continue the isolation from step 2 of the Isolation Protocol (section XI.I).

E. BACTERIA

Quantity: up to 10^9 cells (~1 ml of overnight *E.coli* culture);
Sample material: broth and plate bacterial culture, frozen cell pellet.
(preparation for one minicolumn well on the 96 well plate)

Isolation from broth culture of Gram-negative bacteria

Harvest up to 10^9 Gram-negative bacteria in a 96 deep well plate by centrifugation at ≥ 3000 x g for 5–10 min. Resuspend the pellet in **375 µl of GL Buffer**. Continue the isolation following the Isolation Protocol from step 2 (section XI.I).

Isolation from frozen bacterial cells

Immediately after retrieving the frozen cell pellet from the freezer, resuspend it in **300 µl of GL Buffer**. Do not allow the cell pellet to thaw. Continue the isolation following the Isolation Protocol from step 2 (section XI.I).

Isolation from plate culture

Transfer **300 µl of GL Buffer** to a well of 96 deep well plate (provided by the user). Using a loop, take a sufficient amount of culture from the plate and suspend it in GL Buffer. Continue the isolation following the Isolation Protocol from step 2 (section XI.I).

Isolation from Gram-positive bacteria

Gram-positive bacteria must be treated with an appropriate enzyme before commencing the isolation. For DNA isolation from *Staphylococci* use lysostaphin and from *Enterococci*, lysozyme.

Staphylococcus:

1. Harvest up to 10^9 cells in a 96 deep well plate by centrifugation at ≥3000 x g for 5–10 min.
2. Discard the supernatant and resuspend the cell pellet in **200 µl TE*** (provided by the user).
3. Add **30 µl lysostaphin 400 U/ml** solution and **4 µl RNase A**. Seal the plate with the adhesive seal (provided by the user).
4. Mix well by vortexing. Briefly centrifuge the plate to collect any lysate from the adhesive seal.
   ⚠ *Extensive, high speed centrifugation is not recommended since it may cause the cells pelleting.*
5. Incubate at **37°C** for 20–60 min** (until sample is lysed).
6. Remove the adhesive seal and add **300 µl GL Buffer** and **17 µl Proteinase K**. Re-seal the plate and mix well by vortexing. Briefly centrifuge the plate to collect any lysate from the adhesive seal.
   ⚠ *Extensive, high speed centrifugation is not recommended.*
7. Incubate the plate at **55°C** for 10 min.
8. Continue the isolation following the Isolation Protocol from step 5 (section XI.I).

* TE Buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

** When isolating from coagulase-negative strains, use 50 µl of lysostaphin and incubate 1h at 37°C.

Enterococcus:

1. Harvest up to 10^9 cells in a 96 deep well plate by centrifugation at ≥ 3000 x g for 5–10 min.
2. Discard the supernatant and resuspend the cell pellet in **200 µl TE** (provided by the user), mix thoroughly.
3. Add **40 µl lysozyme 100 mg/ml** solution and **4 µl RNase A**. Seal the plate with the adhesive seal (provided by the user).
4. Mix well by vortexing. Briefly centrifuge the plate to collect any lysate from the adhesive seal.
   ⚠ *Extensive, high speed centrifugation is not recommended since it may cause the cells pelleting.*
5. Incubate at **37°C** for 40–60 min (until sample is lysed).
6. Remove the adhesive seal and add **300 µl GL Buffer** and **17 µl Proteinase K**. Re-seal the plate and mix well by vortexing. Briefly centrifuge the plate to collect any lysate from the adhesive seal.
   ⚠ *Extensive, high speed centrifugation is not recommended.*
7. Incubate the plate at **55°C** for 10 min.
8. Continue the isolation following the Isolation Protocol from step 5 (section XI.I).

F. YEAST

Quantity: up to 10^8 cells;
Sample material: broth and plate yeast culture, frozen cell pellet.
(preparation for one minicolumn well on the 96 well plate)

1. Harvest up to 10^8 cells in a 96 deep well plate by centrifugation at 3000 x g for 5–10 min.
2. Discard the supernatant, and resuspend the cell pellet thoroughly in **200 µl YS Buffer** (Spheroplast Buffer, not included in the kit, EM10-YS).
3. Add **50–200 U lyticase**. Seal the plate with the adhesive seal (provided by the user) and mix by vortexing. Briefly centrifuge the plate to collect any lysate from the adhesive seal.
   ⚠ *Extensive, high speed centrifugation is not recommended since it may cause the cells pelleting.*
4. Incubate at **30°C** for at least 30 min, mixing occasionally by inverting.
5. After incubation, centrifuge for 10 min at 3000 x g.
6. Remove the adhesive seal and carefully remove the supernatant with a pipette tip.
   ⚠ *Do not disturb the spheroplasts pellet!*
7. Resuspend the spheroplast pellet thoroughly in **375 µl GL Buffer**.
8. Continue the isolation from step 2 of the Isolation Protocol (section XI.I).

G. PHYSIOLOGICAL FLUIDS

Quantity: up to 5 ml of fluid;

Sample material: urine, cerebrospinal fluid, peritoneal fluid, pleural fluids, sputum.

⚠ Physiological fluids are a valuable diagnostic material, but also represent a great biological hazard due to the potential pathogen and/or cancer cell content. While working with biological material it is essential to comply with all safety requirements for working with biohazardous material.

(preparation for one minicolumn well on the 96 well plate)

1. **Urine and other fluids:** depending on the volume, centrifuge in an appropriate tube at approx. 500 x g for 5 min. Discard the supernatant.
   Sputum: prior to centrifugation, add the appropriate quantity of a mucolytic agent (bromhexine, acetylcysteine). Centrifuge at 3000 x g for 5 min. Discard the supernatant.
2. Wash the cell pellet with **1 ml PBS buffer** or **saline**. Centrifuge at 3000 x g for 60 s.
3. Add **375 µl GL Buffer**. Mix thoroughly by vortexing for 30 s.
4. Transfer the entire volume to a well of a 96 deep well plate (provided by the user) and continue the isolation from step 2 of the Isolation Protocol (section XI.I).

H. BLOOD

Quantity: up to 1000 µl;

Sample material: fresh or frozen blood.

⚠ Blood represent a great biological hazard due to the potential pathogen. While working with blood, compliance with all the safety requirements for working with biohazardous material is essential.

(preparation for one minicolumn well on the 96 well plate)

1. Transfer 50–1000 µl of blood to a well of a 96 deep well plate (provided by the user) and add the same volume of the **RBC Lysis Buffer** (not included in the kit, EM05-RBC).
   ⚠ *For example, add 200 µl RBC Buffer to 200 µl blood. When isolating from less than 200 µl of blood, add Elution Buffer or PBS buffer in order to obtain a volume of 200 µl and then add 200 µl of RBC Lysis Buffer.*
2. Seal the plate with the adhesive seal (provided by the user) and mix well by inverting the tube until a clear red solution is obtained.
3. Centrifuge for 10 min at 3000 x g. Higher speeds are not recommended as they may hinder the subsequent suspension of the white blood cell pellet in lysis buffer.

4. Remove the adhesive seal and carefully discard the supernatant from over the white blood cell pellet.
5. Resuspend the cell pellet completely in **375 µl GL Buffer** and add **10 µl Proteinase K**.
6. Re-seal the plate and mix by inverting the plate several times or vortexing. Briefly centrifuge the plate to collect any lysate from the adhesive seal.
   ⚠ *Extensive, high speed centrifugation is not recommended.*
7. Incubate at **55°C** for 10 min.
8. Continue the isolation from step 5 of the Isolation Protocol (section XI.I).

I. SEMEN

Quantity: up to 150 µl;
Sample material: semen.
(preparation for one minicolumn well on the 96 well plate)

1. Transfer 150 µl semen to a well of a 96 deep well plate (provided by the user).
   ⚠ *If the sample volume is less than 150 µl, add Elution Buffer or PBS buffer (not included in the kit) up to obtain a capacity of 150 µl. It should be noted that the efficiency of the DNA isolation will be lower.*
2. Add **375 µl GL Buffer**, **10 µl Proteinase K and 20 µl 1M DTT**.
3. Seal the plate with the adhesive seal (provided by the user) and vortex for 3 s. Briefly centrifuge the plate to collect any lysate from the adhesive seal.
   ⚠ *Extensive, high speed centrifugation is not recommended since it may cause the cells pelleting.*
4. Incubate at **55°C** for 30 min. Mix by inverting the plate at several-minute intervals during the incubation. After the incubation period, spin the plate briefly.
5. Continue the isolation from step 5 of the Isolation Protocol (section XI.I).

J. SWABS

Quantity: –
Sample material: swabs (buccal, eye, nasal pharyngeal, vaginal and others).
(preparation for one minicolumn well on the 96 well plate)

1. Place the swab holding material in a 1.5 ml Eppendorf tube and cut off the excess from the end of the shaft so that the tube lid can be closed without difficulty.
2. Add **375 µl GL Buffer and 10 µl Proteinase K**, vortex for 3 s.
3. Incubate at **55°C** for 30 min. Mix by inverting the tube at several-minute intervals during the incubation.
4. Press the swab firmly against one side of the tube in order to retrieve the maximum possible volume of the lysate.
5. Discard the swab holding material. Transfer the lysate to a well of a 96 deep well plate (provided by the user).
6. Continue the isolation from step 5 of the Isolation Protocol (section XI.I).

K. HAIR

Quantity: 1–20 mg hair, approx. 10 mg of hair roots;
Sample material: hair, preferably with roots, or hair roots alone.

⚠ Hair roots contain living cells, while the rest of the hair bears only traces of degraded gDNA and mtDNA. Downstream applications such as PCR or qPCR should therefore involve small products ≤200 bp.

(preparation for one minicolumn well on the 96 well plate)

1. Cut off the hair roots and transfer them to a well of a 96 deep well plate (provided by the user). If the sample material does not contain hair roots, cut the hair into 3 mm fragments.
2. Add **375 µl GL Buffer**, **40 µl 1M DTT** and **25 µl Proteinase K**.
   ⚠ *The DTT supplement is optional. Most hair should be lysed without it; however, some hair types, such as curly, for instance, contain too many disulphide bridges for Proteinase K to handle.*
3. Seal the plate with the adhesive seal (provided by the user) and vortex for 3 s. Briefly centrifuge the plate to collect any lysate from the adhesive seal.
   ⚠ *Extensive, high speed centrifugation is not recommended.*
4. Incubate at **55°C** for at least 6 h, or overnight. Vortex for 60–120 s from time to time. A thermomixer may be used. After the incubation period, spin the plate briefly.
5. Continue the isolation from step 5 of the Isolation Protocol (section XI.I).

L. RODENT TAILS

Quantity: up to 20 mg (10 mg optimally);
Sample material: rat or mouse tail.
(preparation for one minicolumn well on the 96 well plate)

1. Cut the tail into smaller fragments and transfer them to a well of a 96 deep well plate (provided by the user).
   ⚠ *For fragmentation instructions refer to the homogenization methods for fresh or frozen solid tissue (section IXA).*
2. Add **375 µl GL Buffer**, **4 µl RNase A** and **25 µl Proteinase K**.
3. Seal the plate with the adhesive seal (provided by the user) and vortex for 20 s. Briefly centrifuge the plate to collect any lysate from the adhesive seal.
   ⚠ *Extensive, high speed centrifugation is not recommended.*
4. Incubate at **37°C** for 5 min and then at **55°C**, depending on the fragmentation' degree; 2–3 hours for well-homogenized samples or 5–16 hours for small fragments. Vortex vigorously for 20 s at least every 1–2 h. A thermomixer may be used. After the incubation period, spin the plate briefly.
5. Continue the isolation from step 5 of the Isolation Protocol (section XI.I).

M. INSECTS

Quantity: 1–20 mg (10 mg optimally);
Sample material: insects at various stages of life, fresh, frozen or preserved in formalin or ethanol.

1. Wash insects preserved in formalin or ethanol twice with the **PBS buffer** or **distilled water**. Centrifuge for 60 s at 500 x g. Depending on the fragmentation' degree go to step 3 or proceed with homogenization (step 2).
2. **Homogenization:** cut the insects into smaller fragments. Pound in a mortar with liquid nitrogen until a powder is obtained. Transfer the powder to a 2 ml tube. Homogenization may also be carried out using a tube with bead-beating filling (for instructions refer to section IXA). For fragmentation instructions refer to the homogenization methods for fresh or frozen solid tissue (section IXA).
3. Add **375 µl GL Buffer** and vortex vigorously for 60 s.
4. Transfer the entire volume to a well of a 96 deep well plate (provided by the user).
5. Add **4 µl RNase A** and **25 µl Proteinase K**.
6. Seal the plate with the adhesive seal (provided by the user) and vortex for 20 s. Briefly centrifuge the plate to collect any lysate from the adhesive seal.
   ⚠ *Extensive, high speed centrifugation is not recommended.*

7. Incubate at **37°C** for 5 min and then at **55°C**, depending on the fragmentation's degree; 2–3 h for well-homogenized samples, or 5–16 h for small fragments. Vortex vigorously for 20 s at least every 1–2 h. A thermomixer may be used. After the incubation period, spin the plate briefly.
   ⚠ *Extensive, high speed centrifugation is not recommended.*
8. Continue the isolation from step 5 of the Isolation Protocol (section XI.I).

X. PRIOR TO ISOLATION

1. Mix well each buffer supplied with the kit.
2. Prepare **Proteinase K** solution by reconstituting the lyophilizate in an appropriate quantity of Proteinase Buffer.
3. If necessary, reconstitute **RNase A** lyophilizate in an appropriate quantity of RNase Buffer.
4. Ensure that ethanol has been added to **GB**, **GW1** and **GW2 Buffers**. If not, add the appropriate quantity of **96–100% ethanol** (the volumes can be found on the bottle labels or in the table given in section II).
5. Examine the buffers. If a sediment has occurred in any of them, incubate it at **37°C** (**GB**, **GW1** and **GW2 Buffers**) or at **50–60°C** (other buffers) mixing occasionally until the sediment has dissolved. Cool to room temperature.
6. Set a dry block heater or water bath to **70°C**.
7. Unless otherwise stated, conduct all the isolation steps at room temperature.

XI. ISOLATION PROTOCOL

I. Preparation of lysates:

1. Place the properly prepared biological material* into each well of a 96 deep well plate (provided by the user). Add **375 µl GL Buffer**.
   ⚠ See section IX. Sample preparation.
2. Add **25 µl Proteinase K** to each well and seal the plate with the adhesive seal (provided by the user). Mix well by vortexing for 20 s. Briefly centrifuge the plate to collect any lysate from the adhesive seal.
   ⚠ Extensive, high speed centrifugation is not recommended.
3. Incubate at **55°C** until the material has been completely digested. Vortex vigorously for 20 s every 30–60 min.
   ⚠ For larger tissue pieces you may perform overnight digestion.
4. If the **RNase A** is to be used, remove the adhesive seal, add **4 µl RNase A** to each well and re-seal the plate. Mix well by vortexing and briefly centrifuge the plate. Incubate at **37°C** for 5 min.
   ⚠ Extensive, high speed centrifugation is not recommended.
5. Remove the adhesive seal and add **400 µl GB Buffer**. Seal the plate and mix thoroughly for 10 s to obtain homogenous solution.
6. Centrifuge for 5–10 min at ≥ 3000 x g.
7. Proceed immediately to purification protocol for centrifugation (section XI.IIA) or vacuum manifold (section XI.IIB).

II.A Purification using centrifuge:

1. Place DNA Binding Plate onto a Collection Plate (supplied with the kit).
2. Remove the adhesive seal and transfer the **half of the each lysate (~350 µl)** to a well of the DNA Binding Plate using multichannel pipettor. Ensure no remains are transferred along with it.
   ⚠ Unused wells should be covered with the adhesive seal (provided by the user).
3. Centrifuge the stacked plates for 5–10 min at minimum 3000 x g. Discard the filtrate and reuse Collection Plate.
   ⚠ If not all of the supernatant passes through the membrane, repeat the centrifugation for 2–5 min at ≥ 3000 x g. Should the problem persist, it means that the material was insufficiently homogenized or the digestion time was too short or too much sample material was used for the isolation.

4. Transfer the remaining mixture into the same well on DNA Binding Plate.
5. Centrifuge for 5–10 min at minimum 3000 x g. Discard the filtrate and reuse Collection Plate.
6. Add **600 µl GW1 Buffer** into each well of DNA Binding Plate and centrifuge for 120 s at minimum 3000 x g. Discard the filtrate and reuse Collection Plate.
7. Add **500 µl GW2 Buffer** into each well of DNA Binding Plate and centrifuge for 120 s at minimum 3000 x g. Discard the filtrate and reuse Collection Plate.
8. Centrifuge for 20 min at minimum 3000 x g or place DNA Binding Plate in an incubator for 10 min at **70°C** to evaporate residual alcohol.
   - ⚠ Wash buffer contains alcohol, which may interfere with some enzymatic reactions and also decrease the elution efficiency. It is therefore vital to remove the alcohol completely from DNA Binding Plate before elution.
   - ⚠ To ensure the completely drying of the membrane – do not seal the plate.
   - ⚠ Removal of alcohol by evaporation at 70°C is more efficient than prolonged centrifugation.
9. Discard Collection Plate and the filtrate and carefully transfer DNA Binding Plate to a sterile DNA Elution Plate.
10. Add **50–200 µl Elution Buffer**, directly onto the center of the membrane in each well.
    - ⚠ Other buffer volumes in the 20–200 µl range may be used. For instructions, see section VIII. Recommendations and important notes.
11. Incubate DNA Binding Plate at room temperature for 120 s.
12. Centrifuge the stacked plates at 3000 x g for 120 s.
13. Remove DNA Binding Plate then seal tightly DNA Elution Plate with Elution Adhesive Seal. Isolated DNA should be stored at +4°C or -20°C depending on further applications.

XI. ISOLATION PROTOCOL

II.B Purification using vacuum manifold:

1. Prepare the vacuum manifold according to manufacturer's instructions.
   ⚠ If using an automated liquid handling workstation, prepare the workstation deck as recommended by the manufacturer.
2. Place DNA Binding Plate on top of the manifold.
3. Transfer the **half of the each lysate (~350 µl)** to a well of the DNA Binding Plate using multichannel pipettor. Ensure no remains are transferred along with it.
   ⚠ Unused wells should be covered with the adhesive seal (provided by the user).
4. Apply vacuum for 60–120 s until lysate passes through DNA Binding Plate. Release the vacuum.
5. Transfer the remaining mixture into the same well on DNA Binding Plate.
6. Apply vacuum for 60–120 s until lysate passes through DNA Binding Plate. Release the vacuum.
7. Add **600 µl GW1 Buffer** into each well of DNA Binding Plate.
8. Apply vacuum for 120 s. Release the vacuum.
9. Add **500 µl GW2 Buffer** into each well of DNA Binding Plate.
10. Apply vacuum for 120 s. Release the vacuum.
11. Place DNA Binding Plate on a stack of paper towels and gently tap to remove residual liquid from the nozzles. Place DNA Binding Plate again on the manifold.

12. Apply vacuum for 10 min or place DNA Binding Plate in an incubator for 10 min at **70°C** to evaporate residual alcohol.
    - ⚠ Removal of alcohol by evaporation at 70°C is more efficient than prolonged vacuum membrane drying.
    - ⚠ To ensure the completely drying of the membrane – do not seal the plate.
    - ⚠ Wash buffer contains alcohol, which may interfere with some enzymatic reactions and also decrease the elution efficiency. It is therefore vital to remove the alcohol completely from DNA Binding Plate before elution.
13. Disassemble the manifold to remove the waste tray. Discard the filtrate.
14. Assemble the vacuum manifold with DNA Elution Plate.
15. Place DNA Binding Plate onto the vacuum manifold.
16. Add **50–200 µl Elution Buffer**, directly onto the center of the membrane in each well.
    - ⚠ Other buffer volumes in the 20–200 µl range may be used. For instructions, see section VIII. Recommendations and important notes.
17. Incubate DNA Binding Plate at room temperature for 120 s.
18. Apply vacuum for 120 s. Release the vacuum.
19. Disassemble the vacuum manifold to remove DNA Elution Plate, then seal tightly DNA Elution Plate with Elution Adhesive Seal. Isolated DNA should be stored at +4°C or -20°C depending on further applications.

XII. TROUBLESHOOTING

Problem	Possible cause	Solution
TISSUE: Column becomes clogged during purification	Inappropriate tissue homogenization.	Select the appropriate homogenization conditions (see section IXA).
	Incomplete protein degradation.	Prepare a fresh Proteinase K solution. Ensure that the Proteinase K solution is stored at -20°C. Extend the vortexing time, incubate the tissue with Proteinase K in GL Buffer for 16 h until the lysate is clear.
	Tissue remains were transferred onto the membrane.	Pipette the supernatant carefully, without disturbing the tissue pellet.
	The purification column is overloaded.	Do not exceed the recommended tissue amount or number of cell taken for DNA isolation.
SWAB: Column becomes clogged during purification	The buccal swab sample contained food remains.	Repeat the isolation, ensuring that the person providing the sample does not consumed any food or drink for 30 minutes prior to the sample collection.
BACTERIA: Column becomes clogged during purification	Incomplete cell lysis.	Reduce the amount of starting material or follow the instructions for the isolation from more than recommended cells. Extend the incubation time at 55°C to 20 min or until the cells are completely lysed.
	A thick slime of DNA was formed.	Repeat the centrifugation for 60 s at 15 000 x g or at the maximum speed.
BLOOD: Column becomes clogged during purification	Improper storage of the sample material.	Storing blood samples at -80°C is highly recommended. Avoid subjecting the samples to repeated freeze/thaw cycles. Store blood samples at +4°C no longer than 24h.
	Incomplete white blood cell lysis.	Resuspend the white blood cell pellet thoroughly and/or the extend incubation time at 70°C, vortexing the lysate at several-minute intervals.
Purified DNA is degraded	Old or damaged material was used.	Performing an isolation from fresh or properly preserved sample material is recommended.
	Inappropriate sample storage conditions.	Store samples at -80°C. Avoid subjecting the sample material to repeated freeze/thaw cycles.
	TISSUE: The DNA degraded as a result of over-intensive homogenization.	The recommended homogenization conditions should be applied (see section IXA).

Low yield of purified DNA	The purified DNA contains residual alcohol.	Repeat the isolation, paying a particular attention to whether any residual GW2 Buffer is left after step 7 (Purification using centrifuge XI.IIA) or step 12 (Purification using vacuum manifold XI.IIB).
	The purification minicolumn has become clogged.	See "Column becomes clogged during purification".
	Incomplete DNA elution from the membrane.	Before applying Elution Buffer directly to the centre of the membrane, heat it to 80°C. Extend the incubation time with Elution Buffer to 10 min. Perform second elution. Increase volume of Elution Buffer to 200 µl.
	The pH of the water used for elution is lower than 7.0.	Use Elution Buffer for DNA elution.
TISSUE: Low yield of purified DNA	The tissue was incorrectly stored or preserved; DNA degradation.	Store tissue at -80°C. Avoid subjecting the sample material to repeated freeze/thaw cycles. Ensure that a good quality fixative is used and that the storage conditions are adequate.
	Insufficient fragmentation of the sample material.	Ensure proper tissue homogenization in GL Buffer. The tissue must first be fragmented into the smallest possible pieces and homogenized by an appropriate method.
	Incomplete tissue lysis.	The tissue should be as well-fragmented as possible, increase the vortexing time, incubate the tissue with Proteinase K in GL Buffer for 16 h.
	Reduced Proteinase K activity.	Inappropriate storage conditions for Proteinase K. When applicable, ensure that the sample material is thoroughly washed with PBS buffer.
SWAB: Low yield of purified DNA	Improper sample collection method. The buccal swab contains too few peeling cells.	When collecting a sample, ensure that the swab stick is scraped firmly against the inside of the cheek.
	Incomplete cell lysis.	Extend the incubation time at 55°C. Mix by inverting at several-minute intervals.
BACTERIA: Low yield of purified DNA.	Starting material contained few bacterial cells.	Increase the amount of starting material or decrease the volume of Elution Buffer to 20 µl.
BLOOD: Low yield of purified DNA	Material contains few white blood cells.	Increase sample volume and/or decrease Elution Buffer volume to 20 µl.
	Incomplete white blood cell lysis.	Resuspend the white blood cell pellet thoroughly and/or the extend incubation time at 65°C, vortexing the lysate at several-minute intervals.

Isolated DNA is of low purity	One of the washing steps was omitted.	Repeat the isolation, performing both washing steps.
	The purified DNA contains residual alcohol.	Repeat the isolation, paying a particular attention to whether any residual GW2 Buffer is left after step 7 (Purification using centrifuge XI.IIA) or step 12 (Purification using vacuum manifold XI.IIB).
TISSUE: Isolated DNA is of low purity.	Incomplete protein degradation.	Prepare a fresh Proteinase K solution. Ensure that the solution is stored at -20°C. Extend the vortexing time, incubate the tissue with Proteinase K in GL Buffer for 16 h until the lysate is clear.
	Reduced Proteinase K activity.	Inappropriate storage conditions for Proteinase K. When applicable, ensure that the sample material is thoroughly washed with PBS buffer (see section IX).
SWAB: Isolated DNA is of low purity	The swab sample is of very low purity.	Use 600 µl of GW2 Buffer during second washing step (step 7 while purification using centrifugation or step 9 while purification using vacuum manifold) of Isolation Protocol. In case of buccal swab ensure that the person providing the sample does not consume any food or drink during 30 minutes prior to the sample collection.
BLOOD: Some red blood cells are present in the white blood cell pellet	The red blood cells have not been completely lysed.	Discard the supernatant from over the white blood cell pellet and repeat RBC lysis step. However, if a small quantity of red blood cells remain, this will not interfere with the subsequent DNA isolation steps.
RNA contamination present	Silica membrane binds total nucleic acid present in the sample	Perform digestion with RNase A (step 4 of the Isolation Protocol).
Inhibition of downstream enzymatic reactions	Purified DNA contains residual alcohol.	Repeat the isolation, paying a particular attention to whether any residual GW2 Buffer is left in the purification column after drying of the membrane.
Low elution volume or sample cross-contamination	Incorrect vacuum pressure.	Make sure the vacuum manifold is sealed tightly and there is no leakage. A vacuum pressure of -400 to -600 mbar is required to obtain the best results.

XIII. EXAMPLES OF AVERAGE FRESH BIOLOGICAL MATERIAL PROCESSING TIMES

SAMPLE MATERIAL	Digestion in a block heater, no homogenization, periodical mixing	Digestion in a thermomixer	Liquid nitrogen homogenization	Homogenization with Bead-Beating Tubes	Approximate lysis time
Rat liver	3–6 h	2–4 h	1–2 h	≤ 1h	min 1 h, max 6 h
Rat skeletal muscle	2–3 h	2 h	≤ 1h	0.5–1 h	min 0.5 h, max 3 h
Rat heart	2–3 h	1–1.5 h	≤ 1h	0.5–1 h	min 0.5 h, max 3 h
Yellow adipose tissue	1–1.5 h	1 h	0.5 h	0.5 h	min 0.5h, max 1.5h
Brown adipose tissue	1–1.5 h	1 h	0.5 h	0.5 h	min 0.5h, max 1.5h
Rat kidney	1.5–3 h	0.5–2 h	0.5–1.5 h	0.5–1.5 h	min 0.5 h, max 3 h
HT29 cell culture	0.5–1 h	5–30 min	No data	app. 5 min	min 5 min, max 1 h
HCT116 cell culture	0.5–1 h	5–30 min	No data	app. 5 min	min 5 min, max 1 h
Rat brain	0.5–1 h	0.5–1 h	No data	No data	min 0.5 h, max 1 h
Insects	No data	1–2 h	0.5–2 h	0.5–2 h	min 0.5 h, max 2 h

XIV. SAFETY INFORMATION

Proteinase K (lyophilized)

Danger
H315, H319, H334, H335
P261, P271, P304+P340, P342+P311, EUH208

GL Buffer

Warning
H319
P264, P305+P351+P338

GB Buffer

Danger
H318, H302, H332, H315, H412
P280, P261, P305+P351+P338, P301+P312 P330, P304+P340 P312

GW1 Buffer

Danger
H318, H315, H412
P280, P305+P351+P338 P310

EUH208 Contains Proteinase. May produce an allergic reaction. **H302** Harmful if swallowed. **H315** Causes skin irritation. **H318** Causes serious eye damage. **H319** Causes serious eye irritation. **H332** Harmful if inhaled. **H334** May cause allergy or asthma symptoms or breathing difficulties if inhaled. **H335** May cause respiratory irritation. **H412** Harmful to aquatic life with long-lasting effects. **P261** Avoid breathing dust/fumes/gas/mist/vapours/spray. **P264** Wash hands thoroughly after handling. **P271** Use only outdoors or in a well-ventilated area. **P280** Wear protective gloves/protective clothing/eye protection/face protection. **P305+P351+P338** IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. **P342+P311** If experiencing respiratory symptoms: call a POISON CENTER or doctor/physician. **P304+P340** IF INHALED: Remove person to fresh air and keep comfortable for breathing. **P312** Call a POISON CENTER/doctor if you feel unwell. **P301+P312 P330** IF SWALLOWED: Call a POISON CENTER/doctor if you feel unwell. Rinse mouth. **P310** Immediately call a POISON CENTER/doctor.