Instructions for Use
Life Science Kits & Assays

PME free-circulating DNA Extraction Kit

iST Innuscreen GmbH

Order No.:
845-IR-0003010 10 reactions
845-IR-0003050 50 reactions

Publication No.: HB_IR-0003_e_220404

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Contents

1. Introduction

1.1 Intended use

Free-circulating DNA in serum, plasma or urine is of high interest as a diagnostic target. The content of free-circulating DNA is usually very low and varies among different individuals. Further, the free-circulating DNA is present as short fragments, usually smaller than 1000 nt. Therefore, the extraction of cell-free-circulating DNA is difficult. Commercially available kits use standard nucleic acid extraction procedures based on sample lysis, binding of nucleic acids to solid material, washing and finally elution of nucleic acids. Due to commonly high sample volumes, the procedures are very time-consuming, labor-intensive and require various reagents.

The PME free-circulating DNA Extraction Kit is based on a new technology called: PME – Polymer Mediated Enrichment. The procedure doesn't start with sample lysis, like commonly used methods or kits. The first step consists of capturing free-circulating DNA with a special polymer. Subsequently the captured DNA is dissolved in a special buffer, followed by DNA extraction. The whole procedure for isolation of free-circulating DNA from 1 ml of sample volume takes approx. 30 minutes and from 2 - 5 ml serum or plasma or from 5 - 10 ml urine, it takes less than 1 hour.

The PME free-circulating DNA Extraction Kit contains two different Lysis/Binding Solution systems. Both systems are applicable for extraction of free-circulating DNA. Furthermore, the kit contains Carrier RNA. Addition of Carrier RNA is recommended, if very low amounts of free-circulating DNA are expected. In this case, the addition of Carrier RNA can increase the final yield. Real-time PCR as a downstream application has shown a decrease by 0.5 – 1 Ct-value when Carrier RNA was used. In all other cases the addition of Carrier RNA is not necessary.

The kit works with 1 - 5 ml serum or plasma or 5 - 10 ml urine sample. The extracted free-circulating DNA is suitable for downstream applications like PCR, Real-time PCR, bisulfite conversion or any kind of enzymatic reaction.

The detection limit for a specific free-circulating DNA depends on the individual procedures, e.g., in-house PCR or commercial detection assays.

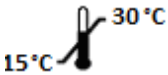
Please note that in case of using the Carrier RNA, the eluates contain free-circulating DNA and Carrier RNA. When using Carrier RNA, it is recommended to quantify the extracted DNA with Real-time PCR instead of photometric or fluorometric methods, as Carrier RNA is also detected by these methods. As Carrier RNA may inhibit PCR reactions, the amount of added Carrier RNA has to be carefully optimized depending on the individual PCR system used.

CONSULT INSTRUCTION FOR USE

This package insert must be read carefully before use. Package insert instructions must be followed accordingly. Reliability of results cannot be guaranteed if there are any deviations from the instructions in this package insert.

1.2 Notes on the use of this manual and the kit

For easy reference and orientation, the manual and labels use the following warning and information symbols as well as the shown methodology:

Symbol	Information
	**REF** Catalogue number.
	**Content** Contains sufficient reagents for <N> reactions.
	**Storage conditions** Store at room temperature or shown conditions respectively.
	**Consult instructions for use** This information must be observed to avoid improper use of the kit and the kit components.
	**Expiry date**
	**Lot number** The number of the kit charge.
	**Manufactured by** Contact information of manufacturer.
	**For single use only** Do not use components for a second time.
	NOTE / ATTENTION Observe the notes marked in this way to ensure correct function of the kit and to avoid operating errors for obtaining correct results.

The following systematic approach is introduced in the manual

- The chapters and figures are numbered consecutively.
- A cross reference is indicated with an arrow (e.g. → "Notes on the use of this manual" p. 5).
- Working steps are numbered.

2. Safety precautions

NOTE

Read through this chapter carefully before use to guarantee your own safety and a trouble-free operation.

Follow all the safety instructions explained in the manual, as well as all messages and information, which are shown.

All due care and attention should be exercised in handling the materials and reagents contained in the kit. Always wear gloves while handling these reagents and avoid any skin contact! In case of contact, flush eyes or skin with a large amount of water immediately.

FOR SINGLE USE ONLY!

This kit is made for single use only!

ATTENTION!

Don’t eat or drink components of the kit!

The kit shall only be handled by educated personal in a laboratory environment!

If the buffer bottles are damaged or leaking, wear gloves and protective goggles when discarding the bottles in order to avoid any injuries. IST Innuscreen GmbH has not tested the liquid waste generated during using the kit for potential residual infectious components. This case is highly unlikely but cannot be excluded completely. Therefore, all liquid waste must be considered as potentially infectious and must be handled and discarded according to local safety regulation.

Please observe the federal, state and local safety and environmental regulations. Follow the usual precautions for applications using extracted nucleic acids. All materials and reagents used for DNA or RNA isolation should be free of DNases or RNases.

ATTENTION!

Do not add bleach or acidic components to the waste after sample preparation!

NOTE
Emergency medical information in English and German can be obtained 24 hours a day from:
Poison Information Center, Freiburg / Germany
Phone: +49 (0)761 19 240.

For more information on GHS classification and the Safety Data Sheet (SDS) please contact sds.innu@ist-ag.com

3. Storage Conditions

The kit is shipped at ambient temperature.

Upon arrival, store lyophilized **Proteinase K** and **Enrichment Reagent VCR-1** at 4 °C to 8 °C.

Store lyophilized **Carrier RNA** at -22 °C to -18 °C.

It is recommended to aliquot dissolved Carrier RNA into several tubes for storage at -22 °C to -18 °C. Do not freeze and thaw Carrier RNA stock solution more than 3 times.

All other components of the PME free-circulating DNA Extraction Kit should be stored dry at room temperature (15 °C to 30 °C). When stored at room temperature, the kit is stable until the expiration date printed on the label on the kit box.

Before every use make sure that all components are at room temperature. If there are any precipitates within the provided solutions dissolve these precipitates by careful warming.

4. Functional testing and technical assistance

The IST Innuscreen GmbH guarantees the correct function of the kit for applications as described in the manual. This kit was produced and tested in an ISO 13485 certified facility.

We reserve the right to change or modify our products to enhance their performance and design. If you have any questions or problems regarding any aspects of the PME free-circulating DNA Extraction Kit or other IST Innuscreen GmbH products, please do not hesitate to contact us. For technical support or further information in Germany please dial +49 30 9489 3380. For other countries please contact your local distributor.

5. Product use and warranty

The kit is not designed for the usage of other starting materials or other amounts of starting materials than those, referred to in the manual (→ “Product specifications” p. 10). Since the performance characteristics of our kits have only been validated for the application described above, the user is responsible for the validation of the performance of IST Innuscreen GmbH kits using other protocols than those described below. IST Innuscreen GmbH kits may be used in clinical diagnostic laboratory systems after the laboratory has validated the complete diagnostic system as required by CLIA’ 88 regulations in the U.S. or equivalents in other countries.

All products sold by the IST Innuscreen GmbH are subjected to extensive quality control procedures and are warranted to perform as described when used correctly. Any problems should be reported immediately

NOTE

The kit is for research use only!

6. Kit components

6.1 Included kit components

	Σ 10	Σ 50
REF	845-IR-0003010	845-IR-0003050
Enrichment Reagent VCR-1	1.2 ml	5 x 1.2 ml
Enrichment Reagent VCR-2	10 ml	32 ml
Lysis Solution GS	8 ml	32 ml
Lysis Solution SE	6 ml	25 ml
Lysis Solution SEP	8 ml	32 ml
Carrier RNA	1x lyophilized powder	1x lyophilized powder
Proteinase K	for 2 x 0.3 ml working solution	for 2 x 1.5 ml working solution
Binding Solution VL	6 ml	20 ml
Binding Solution SBS	8 ml	32 ml
Washing Solution GS	15 ml	2 x 40 ml
Washing Solution BS (conc.)	1 ml	5 ml
RNase-free Water	2 ml	2 x 2 ml
Spin Filter	10	50
Receiver Tubes (2.0 ml)	60	6 x 50
Elution Tubes (1.5 ml)	10	50
Manual	1	1

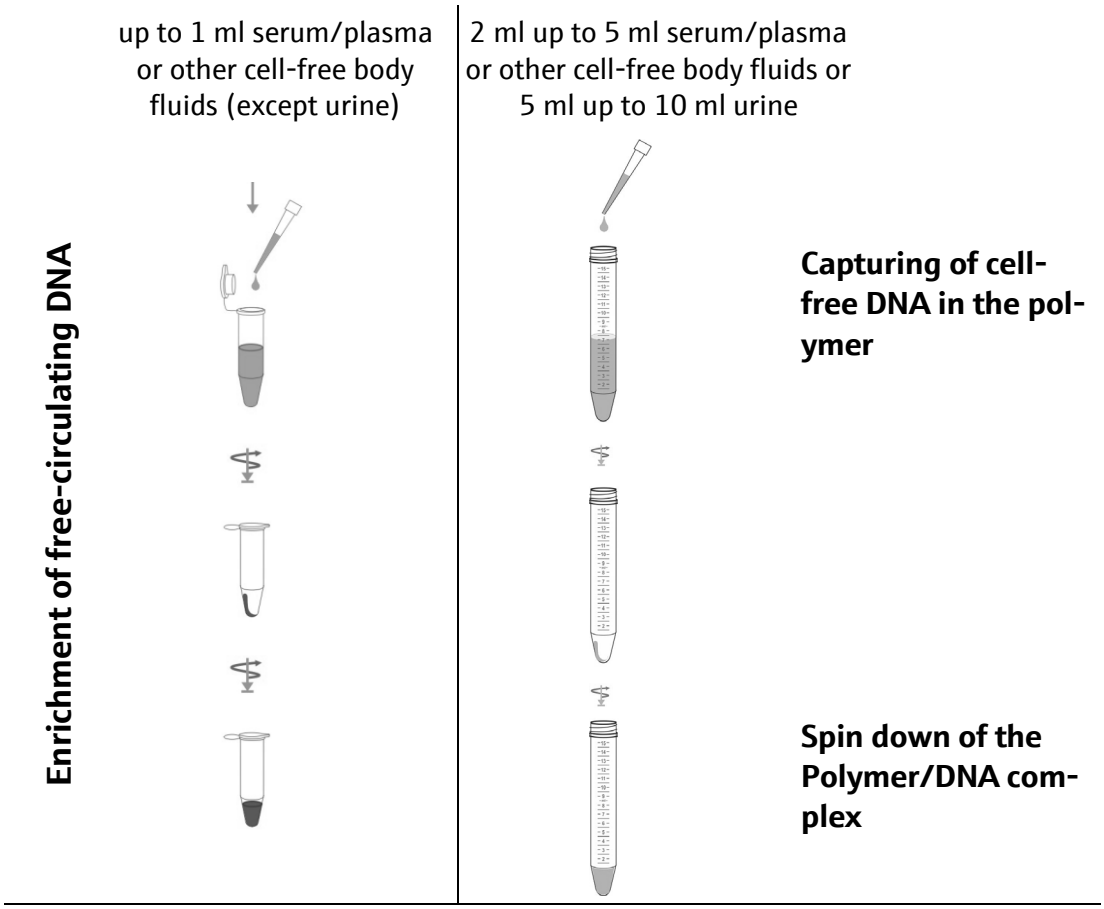
6.2 Components not included in the kit

- 1.5 ml reaction tubes
- 15 ml reaction tubes
- ddH_2O for dissolving Proteinase K
- 96–99.8 % ethanol (molecular biology grade, non-denatured)

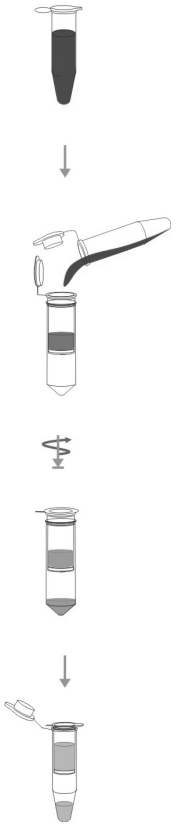
7. Product specifications

1. Starting material:
   - Serum, plasma, cell culture supernatants or medium and other cell-free body fluids from 1 - 5 ml
   - Urine from 5 - 10 ml
2. Time for isolation:
   - Approximately 30 – 60 minutes

8. General procedure of enrichment and isolation of free-circulating DNA



Sample preparation of free-circulating DNA



Lysis of the Polymer/DNA complex

Binding of cell-free DNA to Spin Filter

Washing of the bound cell-free DNA

Elution of cell-free DNA

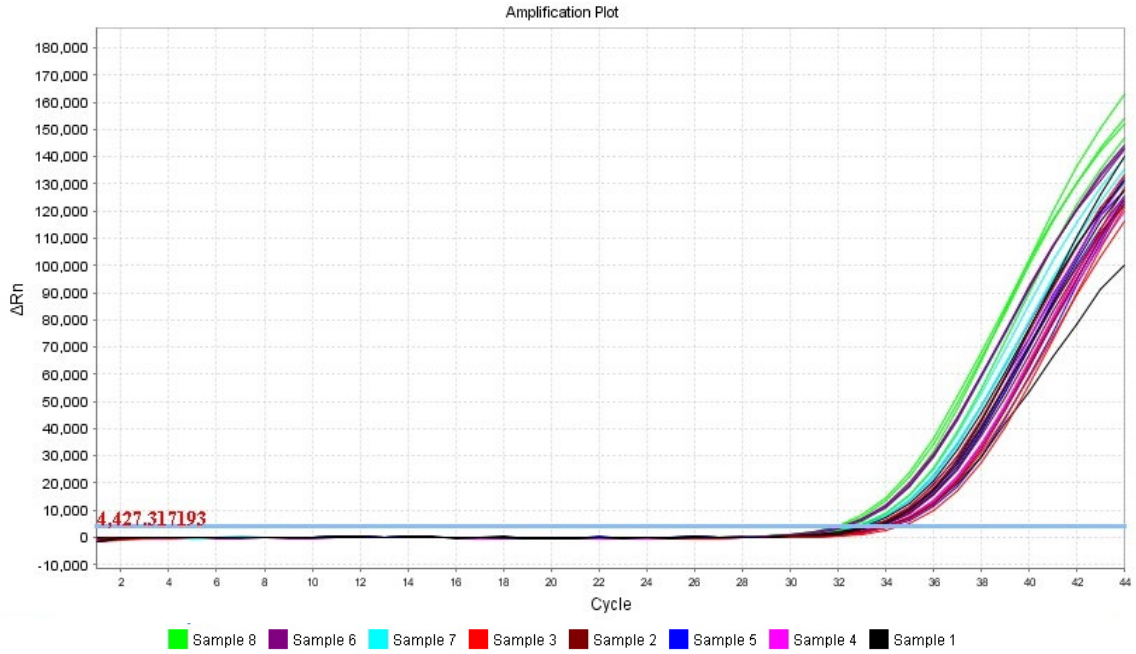
9. Validation results

1. Testing of different blood collecting systems for extraction of free-circulating DNA:

Apart from the varying amounts of free-circulating DNA from different individuals, the blood collection systems used also have influence on the recovery of free-circulating DNA. To date, we have tested the following blood collection systems:

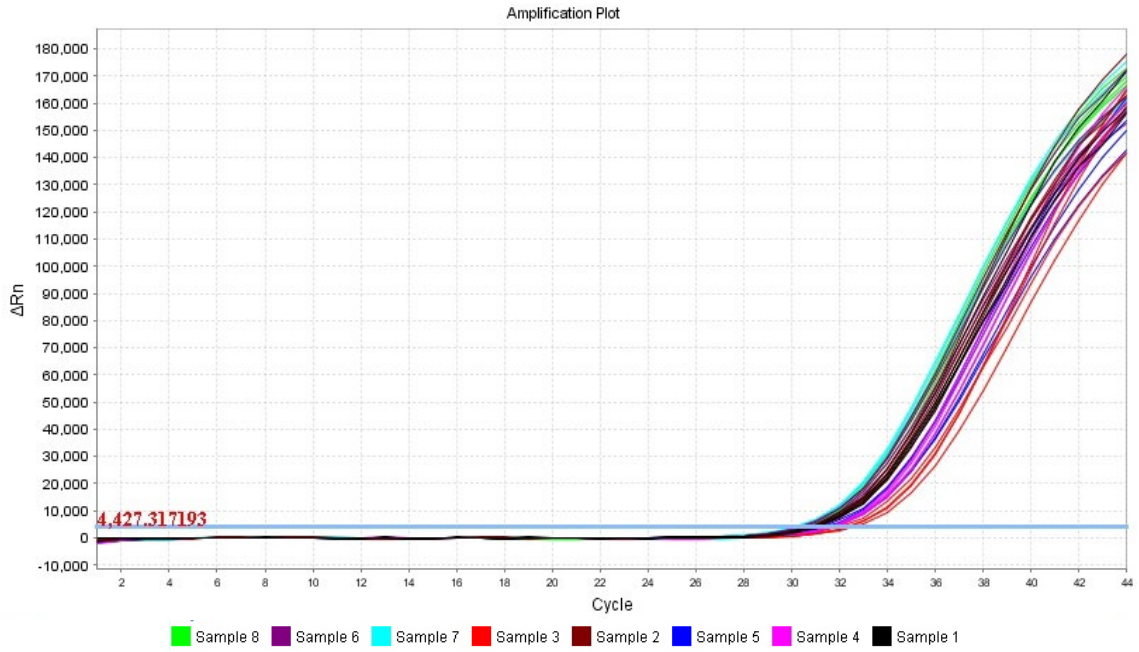
No.	Blood collecting system	Manufacturer
1	S-Monovette® 9 ml Silicat	Sarstedt
2	S-Monovette® 9 ml Polyacrylester Gel	Sarstedt
3	S-Monovette® 8.5 ml CPDA	Sarstedt
4	S-Monovette® 9 ml K3E (EDTA K3)	Sarstedt
5	S-Monovette® 10 ml 9NC (Trisodium Citrate Solution, Citrate Solution)	Sarstedt
6	S-Monovette® 7.5 ml NH (Natrium-Heparin)	Sarstedt
7	S-Monovette® 7.5 ml LH-Gel (Lithium-Heparin)	Sarstedt
8	S-Monovette® 9 ml LH (Lithium-Heparin)	Sarstedt

Extractions of free-circulating DNA from 1 ml of serum or plasma using the listed blood collection systems. Extracted free-circulating DNA was amplified with human-specific target primers:



▲ The amplification plots show differences depending on the kind of blood collection system used. Best results can be obtained using S-Monovette® 9 ml LH (Lithium-Heparin); (Sarstedt) or S-Monovette® 7.5 ml NH (Natrium-Heparin); (Sarstedt).

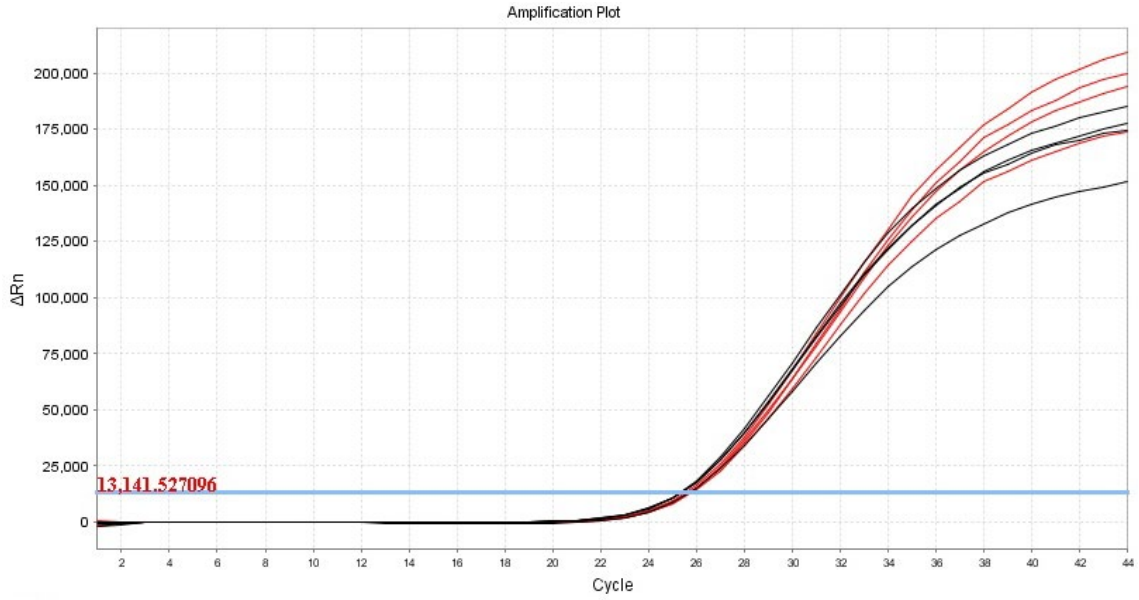
Extractions of free-circulating DNA from 5 ml of serum or plasma using the listed blood collection systems. Extracted free-circulating DNA was amplified with human-specific target primers:



▲ The amplification plots show differences depending on the kind of blood collection system used. Best results can be obtained using S-Monovette® 9 ml LH (Lithium-Heparin); (Sarstedt) or S-Monovette® 7.5 ml NH (Natrium-Heparin); (Sarstedt) and S-Monovette® 7.5 ml LH-Gel (Lithium-Heparin); (Sarstedt).

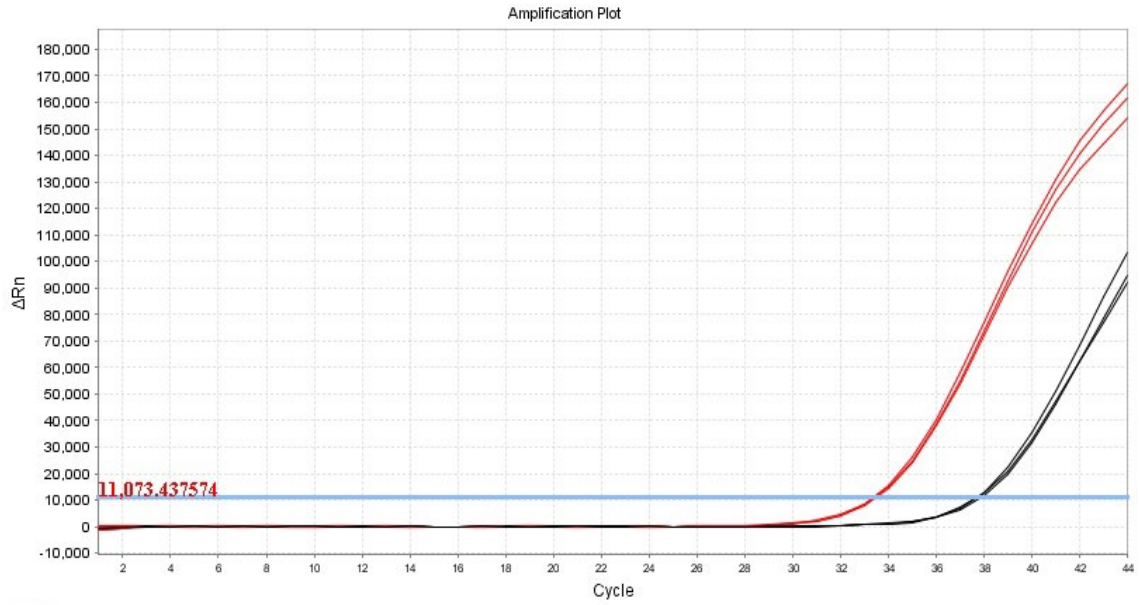
2. Comparison of Lysis Solution GS / Binding Solution VL and Lysis Solution SE / Binding Solution SBS system:

Extraction of free-circulating DNA from 5 ml human serum using Lysis Solution GS / Binding Solution VL and Lysis Solution SE / Binding Solution SBS system. The amount of free-circulating DNA was compared using human estrogen receptor 1-specific Real-time PCR.



▲ The black graphs correspond to the extraction of free-circulating DNA by using Lysis Solution GS / Binding Solution VL system and the red graphs to Lysis Solution SE / Binding Solution SBS system.

Extraction of free-circulating DNA from 5 ml human urine sample using Lysis Solution GS / Binding Solution VL and Lysis Solution SE / Binding Solution SBS system. Following extraction, the free-circulating DNA was amplified with human-specific target primers:



▲ The black graphs correspond to the extraction of free-circulating DNA by using Lysis Solution GS / Binding Solution VL system and the red graphs to Lysis Solution SE / Binding Solution SBS system.

10. Selecting the optimal lysis solution system

The PME free-circulating DNA Extraction Kit contains two different Lysis/Binding Solution systems. Both systems are applicable for extraction of free-circulating DNA from different kinds of starting materials.

Serum, plasma, cell culture supernatants or medium and other cell-free body fluids differ in their compositions. They also vary in content and size of free-circulating DNA, e.g.: short DNA fragments vs. long DNA fragments. Therefore, all Lysis/Binding Solution systems are provided for optimal processing, purification performance, high yields, and excellent quality of DNA.

In order to find the optimal Lysis/Binding Solution system, when using a specific serum or plasma sample for the first time, it is recommended to split the sample and try all Lysis/Binding Solution systems (see protocol 1, 2, 4 and 5) and compare the results to choose the best system for the sample.

For urine samples, in order to obtain optimal results regarding DNA yield and removal of PCR inhibitors, we recommend using the Lysis Solution SEP / Binding Solution SBS system (protocol 3). Nevertheless, the Lysis Solution GS / Binding Solution VL system (protocol 6) could also be tested and used for individual urine sample.

11. Initial steps before starting

- Heat thermal mixer or water bath to 70 °C.
- Add 1 ml RNase-free Water to each tube of Carrier RNA, mix thoroughly by pipetting up and down and store as described above.
- Pre-heat RNase-free Water to 70 °C.

NOTE

Do not use pre-heated RNase-free Water for dissolving Carrier RNA!

- Add the indicated amount of ddH_2O to **Proteinase K**, mix thoroughly and store as described above.

845-IR-0003010	Add 0.3 ml ddH_2O to lyophilized Proteinase K.
845-IR-0003050	Add 1.5 ml ddH_2O to lyophilized Proteinase K.

- Add the indicated amount of absolute ethanol to each bottle of **Washing Solution BS (conc.)**, mix thoroughly. Store as described above.

845-IR-0003010	Add 9 ml ethanol to 1 ml Washing Solution BS (conc.).
845-IR-0003050	Add 45 ml ethanol to 5 ml Washing Solution BS (conc.)

- Centrifugation steps should be carried out at room temperature.
- Avoid freezing and thawing of starting material.

12. Protocols using Lysis Solution SE / Binding Solution SBS system, Lysis Solution SEP / Binding Solution SBS system

12.1 Protocol 1: Isolation of free-circulating DNA from serum, plasma, or other cell-free body fluids (except urine) up to 1 ml

IMPORTANT

Pre-fill the needed amount of RNase-free Water into a 1.5 ml reaction tube and keep at 70 °C until the elution step.

1. Add **30 µl of Enrichment Reagent VCR-1** and the sample into a 1.5 ml reaction tube and vortex shortly. Add **150 µl of Enrichment Reagent VCR-2** to the tube, mix shortly by vortexing. Incubate at room temperature for 1 minute.

2. Centrifuge at maximum speed for 3 minutes, open the tube and carefully remove the supernatant as completely as possible.

3. Add 1 ml ddH2O, invert the reaction tube three times and centrifuge at maximum speed for 3 minutes, open the tube and carefully remove the supernatant as completely as possible.

NOTE

Do not remove the pellet; it will be processed in the following steps!

4. **Add 400 µl Lysis Solution SE** to the reaction tube containing the pellet. Dissolve the pellet by pipetting up and down several times. Try to avoid the formation of air bubbles!

NOTE

Optionally, add 10 µl Carrier RNA to the sample after adding Lysis Solution SE. See → "Intended use" if Carrier RNA is necessary to add or not!

5. Add **50 µl Proteinase K** to the reaction tube and mix vigorously by pulsed vortexing for 10 seconds. Incubate at 70 °C for 15 minutes under continuous shaking at 1,000 rpm.

NOTE

We recommend using a shaking platform (thermal mixer, water bath or any other rocking platform) for a continuous shaking of the sample. Alternatively, vortex the sample 3-4 times during incubation. No shaking will reduce the lysis efficiency. After lysis centrifuge the tube shortly to remove condensate from the lid of the tube.

6. Add **400 µl Binding Solution SBS** to the lysed sample, mix by pipetting up and down several times.

NOTE

It is important that the sample and the Binding Solution SBS are mixed thoroughly to get a homogeneous solution. Avoid the formation of air bubbles!

7. Apply the **whole sample** to the Spin Filter located in a 2.0 ml Receiver Tube. Close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute.

NOTE

If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

8. Open the Spin Filter and add **500 µl Washing Solution GS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

9. Open the Spin Filter and add **650 µl Washing Solution GS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

10. Open the Spin Filter and add **650 µl Washing Solution BS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

11. Centrifuge at maximum speed for 3 minutes to remove all traces of ethanol. Discard the 2.0 ml Receiver Tube.

12. Place the Spin Filter into a 1.5 ml Elution Tube. Open the cap of the Spin Filter and add **50 µl RNase-free Water** (pre-warmed to 70 °C). Incubate at room temperature for 2 minutes. Centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Two elution steps with equal volumes of RNase-free Water (e.g., 30 µl + 30 µl) might increase the yield of extracted free-circulating DNA.

NOTE

The free-circulating DNA can be eluted with a lower (minimum 30 µl) or higher volume of RNase-free Water (depending on the expected yield of free-circulating DNA). Elution with lower volumes of RNase-free Water increases the final concentration of free-circulating DNA. Store the extracted free-circulating DNA at 4–8 °C. For long-time storage placing at -22 to -18 °C is recommended.

12.2 Protocol 2: Isolation of free-circulating DNA from serum, plasma, or other cell-free body fluids (except urine) of 2 ml up to 5 ml

IMPORTANT

Pre-fill the needed amount of RNase-free Water into a 1.5 ml reaction tube and keep at 70 °C until the elution step.

1. Add **100 µl of Enrichment Reagent VCR-1** and the sample into a 15 ml reaction tube and vortex shortly. Add **600 µl of Enrichment Reagent VCR-2** to the tube, mix shortly by vortexing. Incubate at room temperature for 10 minutes.

2. Centrifuge the tubes at min. 4,200 x g (~5,000 rpm) for 10 minutes, open the tube and carefully remove the supernatant as completely as possible.

3. Add 5 ml ddH_2O to the tube, invert the tube three times and centrifuge at min. 4,200 x g (~5,000 rpm) for 5 minutes, open the tube and carefully remove the supernatant as completely as possible.

NOTE

Do not remove the pellet; it will be processed in the following steps!

4. Add **600 µl Lysis Solution SEP** to the 15 ml reaction tube containing the pellet. Dissolve the pellet by pipetting up and down several times and transfer the suspension into a 1.5 ml reaction tube. Try to avoid the formation of air bubbles!

NOTE

Optionally, add 10 µl Carrier RNA to the sample after adding Lysis Solution SE. See → "Intended use" if Carrier RNA is necessary to add or not!

5. Add **50 µl Proteinase K** to the reaction tube and mix vigorously by pulsed vortexing for 10 seconds. Incubate at 70 °C for 15 minutes under continuous shaking at 1,000 rpm.

NOTE

We recommend using a shaking platform (thermal mixer, water bath or any other rocking platform) for a continuous shaking of the sample. Alternatively, vortex the sample 3-4 times during incubation. No shaking will reduce the lysis efficiency.

6. Centrifuge at maximum speed for 2 minutes, open the tube and **transfer the supernatant** carefully into a new reaction tube.

NOTE

If there is a pellet, don't disturb it and transfer the supernatant as completely as possible!

7. Add **600 µl Binding Solution SBS** to the lysate, mix by pipetting up and down several times.

NOTE

It is important that the sample and the Binding Solution SBS are mixed vigorously to get a homogeneous solution. Avoid the formation of air bubbles!

8. Apply **600 µl of the sample** to the Spin Filter located in a 2.0 ml Receiver Tube. Close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute.

NOTE

If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

9. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube and load the residual sample onto the Spin Filter. Close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute.

NOTE

If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

10. Open the Spin Filter and add **500 µl Washing Solution GS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

11. Open the Spin Filter and add **650 µl Washing Solution GS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

12. Open the Spin Filter and add **650 µl Washing Solution BS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

13. Centrifuge at maximum speed for 3 minutes to remove all traces of ethanol. Discard the 2.0 ml Receiver Tube.

14. Place the Spin Filter into a 1.5 ml Elution Tube. Open the cap of the Spin Filter and add **50 µl RNase-free Water** (pre-warmed to 70 °C). Incubate at room temperature for 2 minutes. Centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Two elution steps with equal volumes of RNase-free Water (e.g., 30 µl + 30 µl) might increase the yield of extracted free-circulating DNA.

NOTE

The free-circulating DNA can be eluted with a lower (minimum 30 µl) or higher volume of RNase-free Water (depending on the expected yield of free-circulating DNA). Elution with lower volumes of RNase-free Water increases the final concentration of free-circulating DNA. Store the extracted free-circulating DNA at 4–8 °C. For long-time storage placing at -22 to -18 °C is recommended.

12.3 Protocol 3: Isolation of free-circulating DNA from urine sample of 5 ml up to 10 ml

IMPORTANT

Pre-fill the needed amount of RNase-free Water into a 1.5 ml reaction tube and keep at 70 °C until the elution step.
Urine samples contain cellular materials and cellular nucleic acids. In order to enrich only free-circulating DNA from the urine sample it is recommended to centrifuge the urine sample at maximum speed (e.g., 16,000 x g) and use only the supernatant

1. Add **100 µl of Enrichment Reagent VCR-1** and the sample into a 15 ml reaction tube and vortex shortly. Add **600 µl of Enrichment Reagent VCR-2** to the tube, mix shortly by vortexing. Incubate at room temperature for 10 minutes.

2. Centrifuge the tubes at min. 4,200 x g (~5,000 rpm) for 10 minutes, open the tube and carefully remove the supernatant as completely as possible.

3. Add 5 ml ddH2O to the tube, invert the tube three times and centrifuge at min. 4,200 x g (~5,000 rpm) for 5 minutes, open the tube and carefully remove the supernatant as completely as possible.

NOTE

Do not remove the pellet; it will be processed in the following steps!

4. Add **600 µl Lysis Solution SEP** to the 15 ml reaction tube containing the pellet. Dissolve the pellet by pipetting up and down several times and transfer the suspension into a 1.5 ml reaction tube. Try to avoid the formation of air bubbles!

NOTE

Optionally, add 10 µl Carrier RNA to the sample after adding Lysis Solution SE. See → "Intended use" if Carrier RNA is necessary to add or not!

5. Add **50 µl Proteinase K** to the reaction tube and mix vigorously by pulsed vortexing for 10 seconds. Incubate at 70 °C for 15 minutes under continuous shaking at 1,000 rpm.

NOTE

We recommend using a shaking platform (thermal mixer, water bath or any other rocking platform) for a continuous shaking of the sample. Alternatively, vortex the sample 3-4 times during incubation. No shaking will reduce the lysis efficiency. After lysis centrifuge the tube shortly to remove condensate from the lid of the tube.

6. Centrifuge at maximum speed for 2 minutes, open the tube and **transfer the supernatant** carefully into a new reaction tube.

NOTE

If there is a pellet, don't disturb it and transfer the supernatant as completely as possible!

7. Add **600 µl Binding Solution SBS** to the lysate, mix by pipetting up and down several times.

NOTE

It is important that the sample and the Binding Solution SBS are mixed vigorously to get a homogeneous solution. Avoid the formation of air bubbles!

8. Apply **600 µl of the sample** to the Spin Filter located in a 2.0 ml Receiver Tube. Close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute.

NOTE

If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

9. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube and load the residual sample onto the Spin Filter. Close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute.

NOTE

If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

10. Open the Spin Filter and add **500 µl Washing Solution GS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

11. Open the Spin Filter and add **650 µl Washing Solution GS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

12. Open the Spin Filter and add **650 µl Washing Solution BS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

13. Centrifuge at maximum speed for 3 minutes to remove all traces of ethanol. Discard the 2.0 ml Receiver Tube.

14. Place the Spin Filter into a 1.5 ml Elution Tube. Carefully open the cap of the Spin Filter and add **50 µl RNase-free Water** (pre-warmed to 70 °C). Incubate at room temperature for 2 minutes. Centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Two elution steps with equal volumes of RNase-free Water (e.g., 30 µl + 30 µl) might increase the yield of extracted free-circulating DNA.

NOTE

The free-circulating DNA can be eluted with a lower (minimum 30 µl) or higher volume of RNase-free Water (depending on the expected yield of free-circulating DNA). Elution with lower volumes of RNase-free Water increases the final concentration of free-circulating DNA. Store the extracted free-circulating DNA at 4–8 °C. For long-time storage placing at -22 to -18 °C is recommended.

13. Protocols using Lysis Solution GS / Binding Solution VL system

13.1 Protocol 4: Isolation of free-circulating DNA from serum, plasma, or other cell-free body fluids (except urine) up to 1 ml

IMPORTANT

Pre-fill the needed amount of RNase-free Water into a 1.5 ml reaction tube and keep at 70 ℃ until the elution step.

1. Add **30 µl of Enrichment Reagent VCR-1** and the sample into a 1.5 ml reaction tube and vortex shortly. Add **150 µl of Enrichment Reagent VCR-2** to the tube, mix shortly by vortexing. Incubate at room temperature for 1 minute.

2. Centrifuge at maximum speed for 3 minutes, open the tube and carefully remove the supernatant as completely as possible.

3. Add 1 ml ddH2O, invert the reaction tube three times and centrifuge at maximum speed for 3 minutes, open the tube and carefully remove the supernatant as completely as possible.

NOTE

Do not remove the pellet; it will be processed in the following steps!

4. Add **400 µl Lysis Solution GS** to the reaction tube containing the pellet. Dissolve the pellet by pipetting up and down several times. Try to avoid the formation of air bubbles!

NOTE

Optionally, add 10 µl Carrier RNA to the sample after adding Lysis Solution SE. See → "Intended use" if Carrier RNA is necessary to add or not!

5. Add **50 µl Proteinase K** to the reaction tube and mix vigorously by pulsed vortexing for 10 seconds. Incubate at 70 °C for 15 minutes under continuous shaking at 1,000 rpm.

NOTE

We recommend using a shaking platform (thermal mixer, water bath or any other rocking platform) for a continuous shaking of the sample. Alternatively, vortex the sample 3-4 times during incubation. No shaking will reduce the lysis efficiency. After lysis centrifuge the tube shortly to remove condensate from the lid of the tube.

6. Add **200 µl Binding Solution VL** to the lysed sample, mix by pipetting up and down several times.

NOTE

The Binding Solution VL is very viscous, please pipette carefully. It is important that the sample and the Binding Solution VL are mixed thoroughly to get a homogeneous solution. Avoid the formation of air bubbles!

7. Apply the **whole sample** to the Spin Filter located in a 2.0 ml Receiver Tube. Close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute.

NOTE

If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

8. Open the Spin Filter and add **500 µl Washing Solution GS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

9. Open the Spin Filter and add **650 µl Washing Solution GS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

10. Open the Spin Filter and add **650 µl Washing Solution BS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

11. Centrifuge at maximum speed for 3 minutes to remove all traces of ethanol. Discard the 2.0 ml Receiver Tube.

12. Place the Spin Filter into a 1.5 ml Elution Tube. Open the cap of the Spin Filter and add **50 µl RNase-free Water** (prewarmed to 70 °C). Incubate at room temperature for 2 minutes. Centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Two elution steps with equal volumes of RNase-free Water (e.g., 30 µl + 30 µl) might increase the yield of extracted free-circulating DNA.

NOTE

The free-circulating DNA can be eluted with a lower (minimum 30 µl) or higher volume of RNase-free Water (depending on the expected yield of free-circulating DNA). Elution with lower volumes of RNase-free Water increases the final concentration of free-circulating DNA. Store the extracted free-circulating DNA at 4–8 °C. For long-time storage placing at -22 to -18 °C is recommended.

13.2 Protocol 5: Isolation of free-circulating DNA from serum, plasma, or other cell-free body fluids (except urine) of 2 ml up to 5 ml

IMPORTANT

Pre-fill the needed amount of RNase-free Water into a 1.5 ml reaction tube and keep at 70 °C until the elution step.

1. Add **100 µl of Enrichment Reagent VCR-1** and the sample into a 15 ml reaction tube and vortex shortly. Add **600 µl of Enrichment Reagent VCR-2** to the tube, mix shortly by vortexing. Incubate at room temperature for 10 minutes.

2. Centrifuge the tube at min. 4,200 x g (~5,000 rpm) for 10 minutes, open the tube and carefully remove the supernatant as completely as possible.

3. Add 5 ml ddH_2O to the tube, invert the tube three times and centrifuge at min. 4,200 x g (~5,000 rpm) for 5 minutes, open the tube and carefully remove the supernatant as completely as possible.

NOTE

Do not remove the pellet; it will be processed in the following steps!

4. Add **600 µl Lysis Solution GS** to the 15 ml reaction tube containing the pellet. Dissolve the pellet by pipetting up and down several times and transfer the suspension into a 1.5 ml reaction tube. Try to avoid the formation of air bubbles!

NOTE

Optionally, add 10 µl Carrier RNA to the sample after adding Lysis Solution SE. See → "Intended use" if Carrier RNA is necessary to add or not!

5. Add **50 µl Proteinase K** to the reaction tube and mix vigorously by pulsed vortexing for 10 seconds. Incubate at 70 °C for 15 minutes under continuous shaking at 1,000 rpm.

NOTE

We recommend using a shaking platform (thermal mixer, water bath or any other rocking platform) for a continuous shaking of the sample. Alternatively, vortex the sample 3-4 times during incubation. No shaking will reduce the lysis efficiency.

6. Centrifuge at maximum speed for 2 minutes, open the tube and **transfer the supernatant** carefully into a new reaction tube.

NOTE

If there is a pellet, don't disturb it and transfer the supernatant as completely as possible!

7. Add **300 µl Binding Solution VL** to the lysate, mix by pipetting up and down several times.

NOTE

The Binding Solution VL is very viscous, please pipette carefully. It is important that the sample and the Binding Solution VL are mixed thoroughly to get a homogeneous solution. Avoid the formation of air bubbles!

8. Apply **600 µl of the sample** to the Spin Filter located in a 2.0 ml Receiver Tube. Close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute.

NOTE

If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

9. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube and load the residual sample onto the Spin Filter. Close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute.

NOTE

If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

10. Open the Spin Filter and add **500 µl Washing Solution GS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

11. Open the Spin Filter and add **650 µl Washing Solution GS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

12. Open the Spin Filter and add **650 µl Washing Solution BS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

13. Centrifuge at maximum speed for 3 minutes to remove all traces of ethanol. Discard the 2.0 ml Receiver Tube.

14. Place the Spin Filter into a 1.5 ml Elution Tube. Open the cap of the Spin Filter and add **50 µl RNase-free Water** (pre-warmed to 70 °C). Incubate at room temperature for 2 minutes. Centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Two elution steps with equal volumes of RNase-free Water (e.g., 30 µl + 30 µl) might increase the yield of extracted free-circulating DNA.

NOTE

The free-circulating DNA can be eluted with a lower (minimum 30 µl) or higher volume of RNase-free Water (depending on the expected yield of free-circulating DNA). Elution with lower volumes of RNase-free Water increases the final concentration of free-circulating DNA. Store the extracted free-circulating DNA at 4–8 °C. For long-time storage placing at -22 to -18 °C is recommended.

13.3 Protocol 6: Isolation of free-circulating DNA from urine sample of 5 ml up to 10 ml

IMPORTANT

Pre-fill the needed amount of RNase-free Water into a 1.5 ml reaction tube and keep at 70 °C until the elution step.

1. Add **100 µl of Enrichment Reagent VCR-1** and the sample into a 15 ml reaction tube and vortex shortly. Add **600 µl of Enrichment Reagent VCR-2** to the tube, mix shortly by vortexing. Incubate at room temperature for 10 minutes.

2. Centrifuge the tubes at min. 4,200 x g (~5,000 rpm) for 10 minutes, open the tube and carefully remove the supernatant as completely as possible.

3. Add 5 ml ddH2O to the tube, invert the tube three times and centrifuge at min. 4,200 x g (~5,000 rpm) for 5 minutes, open the tube and carefully remove the supernatant as completely as possible.

NOTE

Do not remove the pellet; it will be processed in the following steps!

4. Add **600 µl Lysis Solution GS** to the 15 ml reaction tube containing the pellet. Dissolve the pellet by pipetting up and down several times and transfer the suspension into a 1.5 ml reaction tube. Try to avoid the formation of air bubbles!

NOTE

Optionally, add 10 µl Carrier RNA to the sample after adding Lysis Solution SE. See → "Intended use" if Carrier RNA is necessary to add or not!

5. Add **50 µl Proteinase K** to the reaction tube and mix vigorously by pulsed vortexing for 10 seconds. Incubate at 70 °C for 15 minutes under continuous shaking at 1,000 rpm.

NOTE

We recommend using a shaking platform (thermal mixer, water bath or any other rocking platform) for a continuous shaking of the sample. Alternatively, vortex the sample 3-4 times during incubation. No shaking will reduce the lysis efficiency.

6. Centrifuge at maximum speed for 2 minutes, open the tube and transfer the supernatant carefully into a new reaction tube.

NOTE

If there is a pellet, don't disturb it and transfer the supernatant as completely as possible!

7. Add **300 µl Binding Solution VL** to the lysate, mix by pipetting up and down several times.

NOTE

The Binding Solution VL is very viscous, please pipette carefully. It is important that the sample and the Binding Solution VL are mixed thoroughly to get a homogeneous solution. Avoid the formation of air bubbles!

8. Apply **600 µl of the sample** to the Spin Filter located in a 2.0 ml Receiver Tube. Close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute.

NOTE

If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

9. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube and load the residual sample onto the Spin Filter. Close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute.

NOTE

If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

10. Open the Spin Filter and add **500 µl Washing Solution GS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

11. Open the Spin Filter and add **650 µl Washing Solution GS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

12. Open the Spin Filter and add **650 µl Washing Solution BS**, close the cap and centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter into a new 2.0 ml Receiver Tube.

13. Centrifuge at maximum speed for 3 minutes to remove all traces of ethanol. Discard the 2.0 ml Receiver Tube.

14. Place the Spin Filter into a 1.5 ml Elution Tube. Open the cap of the Spin Filter and add **50 µl RNase-free Water** (pre-warmed to 70 °C). Incubate at room temperature for 2 minutes. Centrifuge at 11,000 x g (~12,000 rpm) for 1 minute. Two elution steps with equal volumes of RNase-free Water (e.g., 30 µl + 30 µl) might increase the yield of extracted free-circulating DNA.

NOTE

The free-circulating DNA can be eluted with a lower (minimum 30 µl) or higher volume of RNase-free Water (depending on the expected yield of free-circulating DNA). Elution with lower volumes of RNase-free Water increases the final concentration of free-circulating DNA. Store the extracted free-circulating DNA at 4–8 °C. For long-time storage placing at -22 to -18 °C is recommended.

14. Troubleshooting

Problem / probable cause	Comments and suggestions
No pellet after first centrifugation step	
Insufficient addition of VCR-1 or VCR-2	Make sure that both VCR-1 and VCR-2 are added to the reaction tube. Make sure that the right volumes of VCR-1 and VCR-2 are added.
Insufficient centrifugation	Make sure that centrifugation steps are carried out as described in the manual. Otherwise repeat centrifugation.
Removal of pellet	Ensure that the pellet is not discarded during removal of supernatant. In some cases, the pellet cannot be seen until the supernatant is removed completely.
Pellet is difficult to dissolve	
Pipette tip is clogged while dissolving the pellet	Cut the pipette tip or use a wide-bore tip.
Clogged Spin Filter	
Insufficient lysis and/or too much starting material	Increase lysis time. Increase centrifugation speed. After lysis centrifuge the lysate to pellet unlysed material. Reduce amount of starting material.
Low amount of extracted free-circulating DNA	
Insufficient lysis	Increase lysis time. Reduce amount of starting material. Overloading of Spin Filter reduces yield!
Incomplete elution	Prolong the incubation time with RNase-free Water to 5 minutes or repeat elution step once again. Take a higher volume of RNase-free Water.

Insufficient mixing with Binding Solution	Mix sample with Binding Solution by pipetting or by vortexing prior to transfer of the sample onto the Spin Filter.
Low concentration of extracted free-circulating DNA	
Too much RNase-free Water	Elute the free-circulating DNA with lower volume of RNase-free Water.
No Carrier RNA added	Add Carrier RNA to the sample as described in the manual above.

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