**Instructions for Use**
Life Science Kits & Assays

# innuPREP cell-free microRNA Kit

Innuscreen GmbH

**Order No.:**

845-KS-8010010 10 reactions
845-KS-8010050 50 reactions
845-KS-8010250 250 reactions

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This documentation describes the state at the time of publishing.
It needs not necessarily agree with future versions. Subject to change!

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# Contents

# 1 Introduction

Mature cell-free microRNAs (miRNAs) are a class of naturally occurring, small, non-coding RNA molecules, about 18–25 nucleotides in length. They can circulate in blood inside of microvesicles (MVs) or as protein complexes. In the last few years, the importance of the cell-free miRNAs is constantly increasing.

## 1.1 Intended use

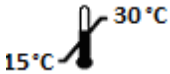
The innuPREP cell-free microRNA Kit has been designed as a tool for very fast and efficient isolation of cell-free microRNAs from exosomes and other MVs isolated. It is possible to combine this kit with the use of innuPREP PME Exosome Enrichment Kit, ultracentrifugation, filtration and similar methods. innuPREP cell-free microRNA Kit can also be successfully used for isolation of microRNA from unprocessed sample, exosome-depleted supernatant obtained after exosomes separation by above mentioned techniques or from samples sized by size exclusion chromatography. The extraction procedure is based on a new kind of chemistry, which combines a fast lysis step with a subsequent efficient binding of microRNA on a Spin Filter surface, followed by washing of the bound microRNA and finally elution of the microRNA. The recovery and the quality of microRNA are excellent. The whole protocol for the extraction of cell-free miRNAs requires approx. 60 minutes.

**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 uses the following warning and information symbols as well as the shown methodology:

| Symbol | Information |
|---|---|
|  | **REF**<br>Catalogue number. |
|  | **Content**<br>Contains sufficient reagents for <N> tests. |
|  | **Storage conditions**<br>Store at room temperature or shown conditions respectively. |
|  | **Consult instructions for use**<br>This information must be observed to avoid improper use of the kit and the kit components. |
|  | **Expiry date** |
|  | **Lot number**<br>The number of the kit charge. |
|  | **Manufactured by**<br>Contact information of manufacturer. |
|  | **For single use only**<br>**Do not use components for a second time.** |
| | **Note / Attention**<br>Observe the notes marked in this way to ensure correct function of the device 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 and the kit” p. 3).
- Work 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 that 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, immediately flush eyes or skin with a large amount of water.

**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 personnel in a laboratory environment!

If the buffer bottles are damaged or leaking, wear gloves and protective goggles when discarding the bottles to avoid any injuries. This kit could be used with potential infectious samples. 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

All kit components are shipped at ambient temperature.

Upon arrival, store lyophilized and dissolved Proteinase K at 2 °C to 8 °C.

The innuPREP cell-free microRNA 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. If there are any precipitates within the provided solutions solve these precipitates by careful warming. Before every use make sure that all components have room temperature.

For further information see chapter “Kit components” (→ p. 7).

# 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 product has been 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 innuPREP cell-free microRNA Extraction Kit, please do not hesitate to contact us. For technical support or further information in Germany please contact info.innu@ist-ag.com. 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 (→ “Intended use” p. 2) (→ “Product specifications” p. 8). Since the performance characteristics of IST Innuscreen GmbH kits have just 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 equivalent regulations required in other countries.

All products sold by 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 Components included in the kit

| | Σ 10 | Σ 50 | Σ 250 |
|---|---|---|---|
| REF | 845-KS-8010010 | 845-KS-8010050 | 845-KS-8010250 |
| **Lysis Solution CBVE** | 8 ml | 35 ml | 5 x 35 ml |
| **Proteinase K** | For 2 x 0.3 ml working solution | For 2 x 1.5 ml working solution | For 6 x 1.5 ml working solution |
| **Washing Solution HS (conc.)** | 3 ml | 15 ml | 70 ml |
| **Washing Solution LS (conc.)** | 2 ml | 8 ml | 36 ml |
| **RNase-free Water** | 2 ml | 6 ml | 30 ml |
| **Spin Filter** | 10 | 50 | 5 x 50 |
| **Receiver Tubes (2.0 ml)** | 10 | 50 | 5 x 50 |
| **Elution Tubes (1.5 ml)** | 10 | 50 | 5 x 50 |
| **Manual** | 1 | 1 | 1 |

## 6.2 Components not included in the kit

- 1.5 ml or 2.0 ml reaction tubes
- 96 %–99.8 % ethanol (molecular biology grade, undenaturated)
- dd$H_2O$ for dissolving Proteinase K
- DNase I (optional)

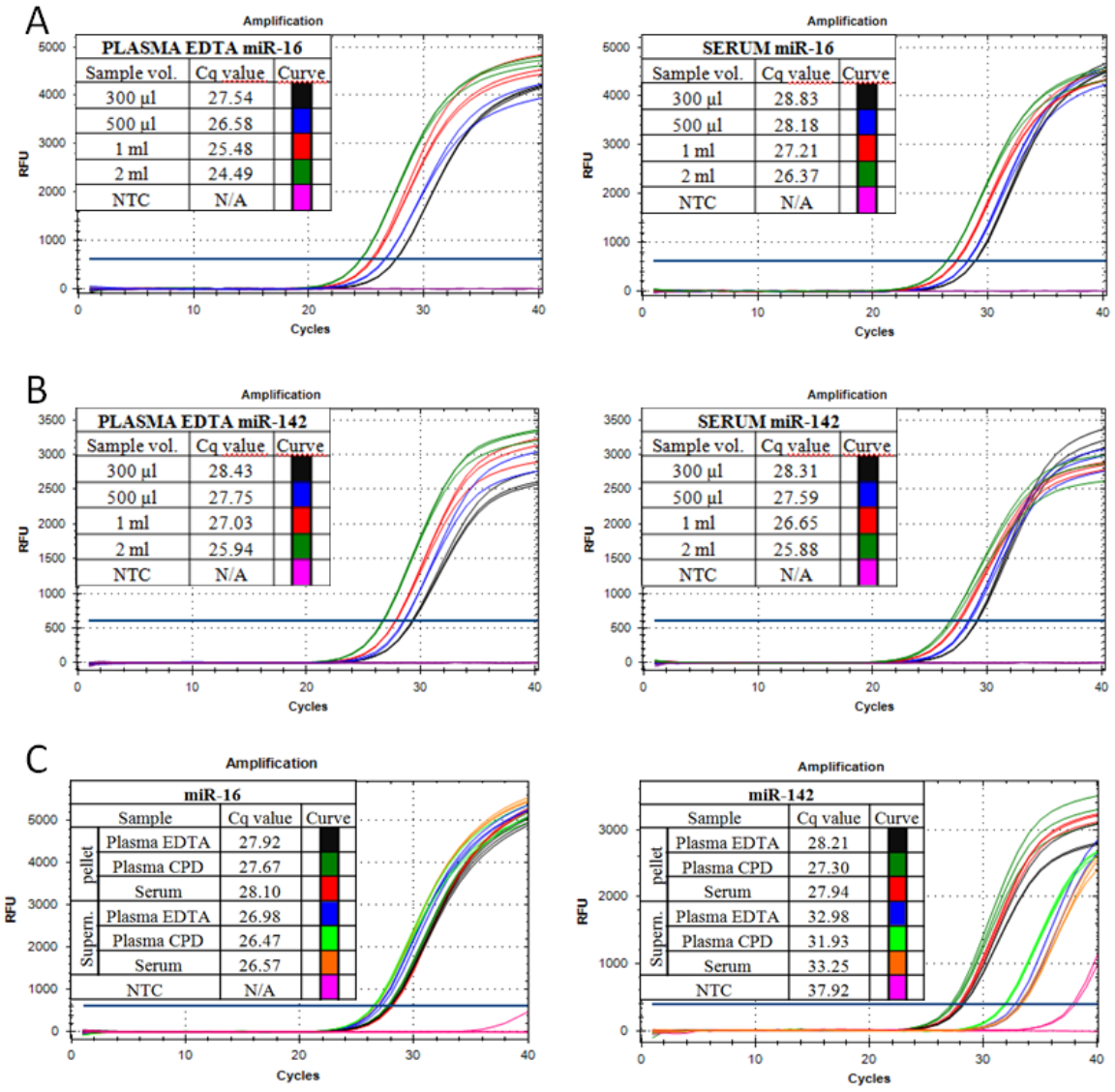
# 7 Product specifications

1. Starting material:
   - Exosomes and other MVs isolated with the use of innuPREP PME Exosome Enrichment Kit (845-IR-0010050, IST Innsucreen GmbH), ultracentrifugation, filtration, size exclusion chromatography and similar methods
   - Unprocessed sample (e.g. serum, plasma, urine, ascites, cell culture supernatant and other bio-fluids) or exosome-depleted supernatant obtained after exosomes separation by above mentioned techniques

2. Time for extraction:
   - Approximately 60 minutes

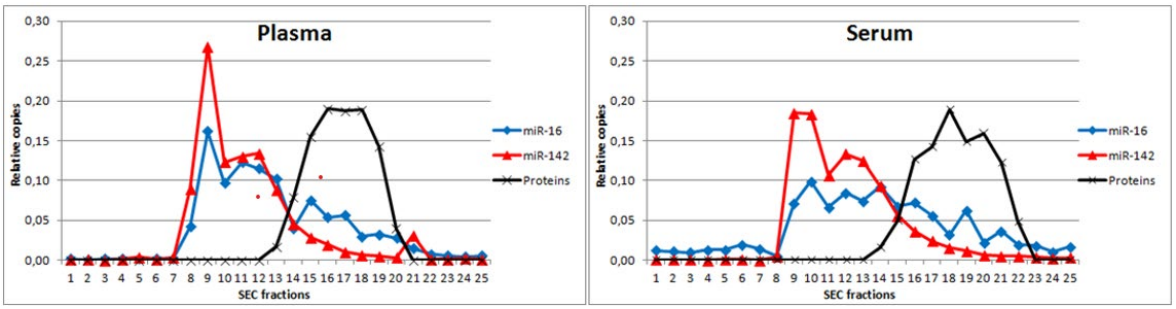
3. Application examples:
   - RT-qPCR

     MiRNAs were extracted from exosomes isolated by innuPREP PME Exosome Enrichment Kit of increasing plasma (EDTA) and serum volumes by innuPREP cell-free microRNA Extraction Kit and amplified by our in-house RT-qPCR. The Cq values derived from the real-time PCR are shown in the tables for miR-16 (A) and miR-142 (B). In addition, the Cq values of exosomal (PME-isolated pellet) and cell-free (supernatant) miR-16 and miR-142 derived from 500 µl of plasma (EDTA and CPD) and serum are compared (C).

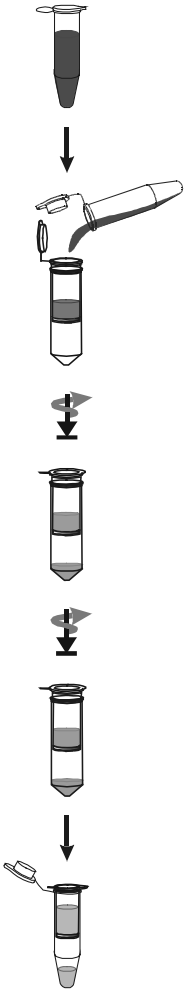



- Determination of the microRNA and protein content in size exclusion chromatography (SEC) fractions

  Using innuPREP PME Exosome Enrichment Kit and innuPREP cell-free microRNA Extraction Kit, microRNAs were enriched and isolated from twenty-five 1 ml SEC fractions derived from 500 µl od plasma or serum. In both, plasma and serum, miR-16 (blue) and miR-142 (red) were found in early fractions, whereas proteins (black) measured by the Bradford assay were detected in late fractions.




# 8 General procedure for cell-free microRNA extraction



Lysis of starting material

Binding of microRNA on Spin Filter

Washing of bound microRNA

Elution of microRNA

# 9 Initial steps before starting

- Add the indicated amount of dd$H_2O$ to each vial of **Proteinase K**, mix thoroughly and store as described above.

| | |
|---|---|
| 845-KS-8010010 | Add 0.3 ml dd$H_2O$ to lyophilized Proteinase K. |
| 845-KS-8010050<br>845-KS-8010250 | Add 1.5 ml dd$H_2O$ to lyophilized Proteinase K. |

- Add the indicated amount of absolute ethanol to **Washing Solution HS (conc.)**, mix thoroughly and store as described above. Always keep the bottle firmly closed.

| | |
|---|---|
| 845-KS-8010010 | Add 3 ml ethanol to 3 ml Washing Solution HS (conc.). |
| 845-KS-8010050 | Add 15 ml ethanol to 15 ml Washing Solution HS (conc.). |
| 845-KS-8010250 | Add 70 ml ethanol to 70 ml Washing Solution HS (conc.). |

- Add the indicated amount of absolute ethanol to **Washing Solution LS (conc.)**, mix thoroughly and store as described above. Always keep the bottle firmly closed.

| | |
|---|---|
| 845-KS-8010010 | Add 8 ml ethanol to 2 ml Washing Solution LS (conc.). |
| 845-KS-8010050 | Add 32 ml ethanol to 8 ml Washing Solution LS (conc.). |
| 845-KS-8010250 | Add 144 ml ethanol to 36 ml Washing Solution LS (conc.). |

- Heat thermal mixer or water bath to 55 °C.
- Centrifugation steps should be carried out at room temperature.
- Avoid freezing and thawing of starting material.

# 10 Protocol 1: Isolation of cell-free microRNA from pellets obtained by innuPREP PME Exosome Enrichment Kit, Protocol 1 (Isolation of exosomes from cell-free bio-fluids up to 1.5 ml)

**This protocol is applicable for isolation of microRNA from exosomes separated with techniques different than PME (e.g. ultracentrifugation) if the final volume of exosomes does not exceed 50 µl. If the final volume of exosomes exceeds 50 µl, proceed according to Protocol 3.**

**NOTE**
Before starting, prepare Washing Solution HS, Washing Solution LS and Proteinase K according to the instruction.

1. Add **400 µl Lysis Solution CBVE** to the reaction tube containing the pellet. Try to dissolve the pellet as much as possible by piercing it by the end of the tip and by pipetting up and down several times. Thereby avoid the formation of air bubbles!

**NOTE**
Complete dissolution of the pellet is not necessary for isolation of cell-free miRNA. Cell-free miRNA will be released from the polymer while incubated as in the following steps.

2. Add **20 µl Proteinase K** to the reaction tube and mix vigorously by pulsed vortexing for 10 seconds.
3. Incubate at **55 °C for 20 minutes** under continuous shaking at 1000 rpm.

**NOTE**
We recommend using a shaking platform (thermal mixer, water bath or another rocking platform) for a continuous shaking of the sample.

Alternatively, vortex the sample 3–4 times during the incubation. No shaking will reduce the lysis efficiency.

4. Centrifuge at **full speed** (>14,000 x g (~16.000 rpm)) for **3 minutes**.
5. Transfer the supernatant in the new 1.5 or 2.0 ml reaction tube (not provided with the kit). Do not disturb the pellet! In some cases, pellet might be transparent.
6. Add **800 µl absolute ethanol** into the reaction tube. Mix the sample by pipetting several times up and down.
7. Place a Spin Filter into a 2.0 ml Receiver Tube. Transfer 650 µl of the sample onto the Spin Filter. Centrifuge at 13,000 x g (~ 15,000 rpm) for 1 minute. Discard the filtrate and reuse the Receiver Tube. Place the Spin Filter back into the Receiver Tube.

**NOTE**

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

8. Transfer the residual sample onto the Spin Filter. Centrifuge at 13,000 x g (~ 15,000 rpm) for 1 minute. Discard the filtrate and reuse the Receiver Tube. Place the Spin Filter back into the Receiver Tube.

**NOTE**

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

**NOTE**

Optional DNA digestion on the spin column.

The innuPREP cell-free microRNA Extraction Kit may extract small amounts of cell-free DNA. If the cell-free DNA is interfering with the de-

tection procedure of miRNA, digest DNA on the Spin Column before starting step 9. Please note that the DNA digestion can lead to the partially loss of cell-free miRNA. DNase I is not included in this kit.

9. Open the Spin Filter and add **500 µl Washing Solution HS**, close the cap and centrifuge at 13,000 x g (~ 15,000 rpm) for 1 min. Discard the filtrate and reuse the Receiver Tube. Place the Spin Filter back into the Receiver Tube.
10. Open the Spin Filter and add **650 µl Washing Solution LS**, close the cap and centrifuge at 13,000 x g (~ 15,000 rpm) for 1 min. Discard the filtrate and reuse the Receiver Tube. Place the Spin Filter back into the Receiver Tube.
11. Centrifuge at full speed (>14,000 x g (~16.000 rpm)) for 3 minutes to remove all traces of ethanol. Discard the 2.0 ml Receiver Tube.
12. Place the Spin Filter into an Elution Tube. Carefully open the cap of the Spin Filter and add **100 µl RNase free $H_2O$**. Incubate at room temperature for 2 minutes. Centrifuge at 13,000 x g (~ 15,000 rpm) for 1 minute.

**NOTE**

Re-elution (loading the eluate on the same Spin Filter and repeating the procedure from point 12.) might increase the yield of extracted cell-free miRNA.

Increasing the incubation time up to 10 minutes might increase the yield of extracted cell-free miRNA.

The cell-free miRNA can be eluted with a lower (min. 30 µl) or a higher volume of RNase free $H_2O$ (depends on the expected yield of cell-free miRNA). Elution with lower volumes of RNase free $H_2O$ increases the final concentration of cell-free miRNA. Store the extracted miRNA at 4-8°C. For long time storage placing at -22°C to -18°C is recommended.

# 11 Protocol 2: Isolation of cell-free microRNA from pellets obtained by innuPREP PME Exosomes Enrichment Kit, Protocol 2 (Isolation of exosomes from cell-free bio-fluids from >1.5 ml up to 10 ml)

**NOTE**

Before starting, prepare Washing Solution HS, Washing Solution LS and Proteinase K according to the instruction.

1. Add **600 µl Lysis Solution CBVE** to the reaction tube containing the pellet. Try to dissolve the pellet as much as possible by piercing it by the end of the tip and by pipetting up and down several times. Thereby avoid the formation of air bubbles!

**NOTE**

Complete dissolution of the pellet is not necessary for isolation of cell-free miRNA. Cell-free miRNA will be released from the polymer while incubated as in the following steps.

If shaking platform for 15 ml reaction tubes is not available, sample after dissolving in Lysis Solution CBVE can be transferred into 1.5 or 2.0 ml reaction tube (not provided with the kit).

2. Add **30 µl Proteinase K** to the reaction tube and mix vigorously by pulsed vortexing for 10 seconds.
3. Incubate at **55 °C for 20 minutes** under continuous shaking at 1000 rpm.

**NOTE**

We recommend using a shaking platform (thermal mixer, water bath or another rocking platform) for a continuous shaking of the sample.

Alternatively, vortex the sample 3–4 times during the incubation. No shaking will reduce the lysis efficiency.

4. Centrifuge at **full speed** (>14,000 x g (~16.000 rpm)) for **3 minutes**.
5. Transfer the supernatant in the new 1.5 or 2.0 ml reaction tube (not provided with the kit). Do not disturb the pellet! In some cases, pellet might be transparent.
6. Add **1200 µl absolute ethanol** into the reaction tube. Mix the sample by pipetting several times up and down.
7. Place a Spin Filter into a 2.0 ml Receiver Tube. Transfer 650 µl of the sample onto the Spin Filter. Centrifuge at 13,000 x g (~ 15,000rpm) for 1 minute. Discard the filtrate and reuse the Receiver Tube. Place the Spin Filter back into the Receiver Tube.

**NOTE**

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

8. Repeat procedure from step 7 two times more, until whole sample pass through the Spin Filter. Discard the filtrate and reuse the Receiver Tube. Place the Spin Filter back into the Receiver Tube.

**NOTE**

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

**NOTE**

Optional DNA digestion on the spin column.

The innuPREP cell-free microRNA Extraction Kit may extract small amounts of cell-free DNA. If the cell-free DNA is interfering with the detection procedure of miRNA, digest DNA on the Spin Column before starting step 9. Please note that the DNA digestion can lead to the partially loss of cell-free miRNA. DNase I is not included in this kit.

9. Open the Spin Filter and add **500 µl Washing Solution HS**, close the cap and centrifuge at 13,000 x g (~ 15,000 rpm) for 1 min. Discard the filtrate and reuse the Receiver Tube. Place the Spin Filter back into the Receiver Tube.
10. Open the Spin Filter and add **650 µl Washing Solution LS**, close the cap and centrifuge at 13,000 x g (~ 15,000 rpm) for 1 min. Discard the filtrate and reuse the Receiver Tube. Place the Spin Filter back into the Receiver Tube.
11. Centrifuge at full speed (>14,000 x g (~16.000 rpm)) for 3 minutes to remove all traces of ethanol. Discard the 2.0 ml Receiver Tube.
12. Place the Spin Filter into an Elution Tube. Carefully open the cap of the Spin Filter and add **100 µl RNase free $H_2O$**. Incubate at room temperature for 2 minutes. Centrifuge at 13,000 x g (~ 15,000 rpm) for 1 minute.

**NOTE**

Re-elution (loading the eluate on the same Spin Filter and repeating the procedure from point 12.) might increase the yield of extracted cell-free miRNA.

Increasing the incubation time up to 10 minutes might increase the yield of extracted cell-free miRNA.

The cell-free miRNA can be eluted with a lower (min. 30 µl) or a higher volume of RNase free $H_2O$ (depends on the expected yield of cell-free miRNA). Elution with lower volumes of RNase free $H_2O$ increases the final concentration of cell-free miRNA. Store the extracted miRNA at 4-8°C. For long time storage placing at -22°C to -18°C is recommended.

# 12 Protocol 3: Isolation of cell-free microRNAs from unprocessed sample (starting material), exosome-depleted supernatant or exosomes separated with different techniques which final volume exceeds 50 µl

**The following protocol is foreseen for 200 µl of sample volume. If higher or lower volumes of sample are intended to be proceeded, keep volumes of Lysis Solution CBVE, Proteinase K and absolute ethanol proportional.**

**NOTE**

Before starting, prepare Washing Solution HS, Washing Solution LS and Proteinase K according to the instruction.

1. Add **200 µl sample**, **400 µl Lysis Solution CBVE** and **20 µl Proteinase K** to the reaction tube (not provided with the kit) and mix vigorously by pulsed vortexing for 10 seconds.

**NOTE**

Complete dissolution of the pellet is not necessary for isolation of cell-free miRNA. Cell-free miRNA will be released from the polymer while incubated as in the following steps.

2. Incubate at **55 °C for 20 minutes** under continuous shaking at 1000 rpm.

**NOTE**

We recommend using a shaking platform (thermal mixer, water bath or another rocking platform) for a continuous shaking of the sample.

Alternatively, vortex the sample 3–4 times during the incubation. No shaking will reduce the lysis efficiency.

3. Add **1200 µl absolute ethanol** into the reaction tube. Mix the sample by pipetting several times up and down.

4. Place a Spin Filter into a 2.0 ml Receiver Tube. Transfer 650 µl of the sample onto the Spin Filter. Centrifuge at 13,000 x g (~ 15,000 rpm) for 1 minute. Discard the filtrate and reuse the Receiver Tube. Place the Spin Filter back into the Receiver Tube.

**NOTE**

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

5. Repeat procedure from step 4 two times more or until whole sample pass through the Spin Filter. Discard the filtrate and reuse the Receiver Tube. Place the Spin Filter back into the Receiver Tube.

**NOTE**

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

**NOTE**

Optional DNA digestion on the spin column.

The innuPREP cell-free microRNA Extraction Kit may extract small amounts of cell-free DNA. If the cell-free DNA is interfering with the detection procedure of miRNA, digest DNA on the Spin Column before starting step 6. Please note that the DNA digestion can lead to the partially loss of cell-free miRNA. DNase I is not included in this kit.

6. Open the Spin Filter and add **500 µl Washing Solution HS**, close the cap and centrifuge at 13,000 x g (~ 15,000 rpm) for 1 min. Discard the filtrate and reuse the Receiver Tube. Place the Spin Filter back into the Receiver Tube.

7. Open the Spin Filter and add **650 µl Washing Solution LS**, close the cap and centrifuge at 13,000 x g (~ 15,000 rpm) for 1 min. Discard the filtrate and reuse the Receiver Tube. Place the Spin Filter back into the Receiver Tube.
8. Centrifuge at full speed (>14,000 x g (~16.000 rpm)) for 3 minutes to remove all traces of ethanol. Discard the 2.0 ml Receiver Tube.
9. Place the Spin Filter into an Elution Tube. Carefully open the cap of the Spin Filter and add **100 µl RNase free $H_2O$**. Incubate at room temperature for 2 minutes. Centrifuge at 13,000 x g (~ 15,000 rpm) for 1 minute.

**NOTE**

Re-elution (loading the eluate on the same Spin Filter and repeating the procedure from point 9.) might increase the yield of extracted cell-free miRNA.

Increasing the incubation time up to 10 minutes might increase the yield of extracted cell-free miRNA.

The cell-free miRNA can be eluted with a lower (min. 30 µl) or a higher volume of RNase free $H_2O$ (depends on the expected yield of cell-free miRNA). Elution with lower volumes of RNase free $H_2O$ increases the final concentration of cell-free miRNA. Store the extracted miRNA at 4-8°C. For long time storage placing at -22°C to -18°C is recommended.

# 13 Troubleshooting

| Problem / probable cause | Comments and suggestions |
|---|---|
| Pellet is difficult to dissolve | |
| Too much of VCR-1 or VCR-2 used (applicable for innuPREP PME Exosome Enrichment Kit). | Make sure that both VCR-1 and VCR-2 are added as described in protocol. |
| Not enough Lysis Solution CBVE added to the pellet. | Follow the adequate protocol. |
| Pipette tip is clogged while dissolving the pellet. | Cut the slide edge of pipette tip and try to transfer the pellet as much as possible. |
| Clogged Spin Filter | |
| Insufficient lysis and/or too much starting material. | Increase lysis time.<br>Increase centrifugation speed.<br>After lysis centrifuge the lysate to pellet unlysed material.<br>Reduce amount of starting material. |
| Low amount of extracted cell-free miRNA | |
| Loss of exosome pellet. | Ensure to not disturb or remove the pellet while processing sample. |
| Insufficient lysis. | Increase lysis time.<br>Reduce amount of starting material.<br>Overloading of Spin Filter reduces miRNA yield! |

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